Avaliação *In Vitro* dos Potenciais Efeitos Terapêuticos e Toxicológicos Associados ao Consumo de Ayahuasca

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Tese para obtenção do grau de Doutor em
Biomedicina
(3º ciclo de estudos)

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11 de junho de 2024

Declaração de Integridade

Eu, Joana Domingos Gonçalves, que abaixo assino, estudante com o número de inscrição D2438 do 3º ciclo de estudos em Biomedicina da Faculdade de Ciências da Saúde, declaro ter desenvolvido o presente trabalho e elaborado o presente texto em total consonância com o **Código de Integridades da Universidade da Beira Interior**.

Mais concretamente afirmo não ter incorrido em qualquer das variedades de Fraude Académica, e que aqui declaro conhecer, que em particular atendi à exigida referenciação de frases, extratos, imagens e outras formas de trabalho intelectual, e assumindo assim na íntegra as responsabilidades da autoria.

Universidade da Beira Interior, Covilhã 20 / 11 / 2023

Joana Domingos Gonçalves

(assinatura conforme Cartão de Cidadão ou preferencialmente
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Preface

Para ser grande, sê inteiro: nada
    Teu exagera ou exclui.
Sê todo em cada coisa. Põe quanto és
    No mínimo que fazes.
Assim em cada lago a lua toda
    Brilha, porque alta vive.

-Ricardo Reis

List of publications

Scientific articles & book chapters related to this doctoral thesis:

i. **Joana Gonçalves**, Ângelo Luís, Ana Gradillas, Antonia García, José Restolho, Nicolás Fernández, Fernanda Domingues, Eugenia Gallardo and Ana Paula Duarte. "Ayahuasca Beverages: Phytochemical Analysis and Biological Properties" Antibiotics 2020, 9, 731

ii. **Joana Gonçalves**, Ângelo Luís, Eugenia Gallardo and Ana Paula Duarte. "Psychoactive Substances of Natural Origin: Toxicological Aspects, Therapeutic Properties and Analysis in Biological Samples" Molecules 2021, 26, 1397

iii. **Joana Gonçalves**, Miguel Castilho, Tiago Rosado, Ângelo Luís, José Restolho, Nicolás Fernández, Eugenia Gallardo and Ana Paula Duarte. "*In Vitro* Study of the Bioavailability and Bioaccessibility of the Main Compounds Present in Ayahuasca Beverages" Molecules 2021, 26, 5555

iv. **Joana Gonçalves**, Ângelo Luís, Eugenia Gallardo and Ana Paula Duarte. "Evaluation of the *In Vitro* Wound-Healing Potential of Ayahuasca" Molecules 2022, 27, 5760

v. **Joana Gonçalves**, Ângelo Luís, Eugenia Gallardo and Ana Paula Duarte. "A Systematic Review on the Therapeutic Effects of Ayahuasca" Plants 2023, 12, 2873

vi. **Joana Gonçalves,** Tiago Rosado, Mário Barroso, José Restolho, Nicolás Fernández, Ângelo Luís, Eugenia Gallardo and Ana Paula Duarte**. "**Comparative study of sample preparation procedures to determine the main compounds in ayahuasca beverages by QuEChERS and high-performance liquid chromatography analysis" Phytochemical Analysis 2024, 1-12

vii. **Joana Gonçalves**, Mariana Feijó, Sílvia Socorro, Ângelo Luís, Eugenia Gallardo and Ana Paula Duarte. "The Role of Ayahuasca in Colorectal Adenocarcinoma Cell Survival, Proliferation and Oxidative Stress" Pharmaceuticals 2024, 17, 719

viii. **Joana Gonçalves**, José Francisco Cascalheira, Patrícia Valentão, Ângelo Luís, Eugenia Gallardo and Ana Paula Duarte. "The role of ayahuasca in cell viability and oxidative stress in gastric adenocarcinoma cell line" Natural Product Research 2024

Other publications unrelated to this doctoral thesis:

i. Tiago Rosado*, Sofia Soares*, Sara Malaca*, **Joana Gonçalves***, Mário Barroso, Eugenia Gallardo. "The Role of Liquid Chromatography in Toxicological Analysis" In book: High-Performance Liquid Chromatography: Types, Parameters and Applications, Chapter: chapter 1 Publisher: Nova Science Publishers, Inc., Editors: Ivan Lucero, March 2018 [ISBN: 978-1-53613-543-5].
***These authors are contributed equally.**

ii. Tiago Rosado, **Joana Gonçalves**, Ângelo Luís, Sofia Soares, Sara Malaca, Mário Barroso, Eugenia Gallardo. "Synthetic Cannabinoids in Biological Specimens: A Review of Current Analytical Methods and Sample Preparation Techniques" Bionalysis 2018; 10(19):1609-1623

iii. **Joana Gonçalves**, Rodrigo Ramos, Ângelo Luís, Sandra Rocha, Tiago Rosado, Eugenia Gallardo and Ana Paula Duarte. "Assessment of the Bioaccessibility and Bioavailability of the Phenolic Compounds of *Prunus avium* L. by *in Vitro* Digestion and Cell Model" ACS Omega 2019; 4: 7605–7613

iv. **Joana Gonçalves***, Tiago Rosado*, Sofia Soares *, Ana Y. Simão *, Débora Caramelo*, Ângelo Luís *, Nicolás Fernández *, Mário Barroso, Eugenia Gallardo and Ana Paula Duarte. "Cannabis and Its Secondary Metabolites: Their Use as Therapeutic Drugs, Toxicological Aspects, and Analytical Determination" Medicines 2019, 6(1): 31
*** These authors contributed equally to this work.**

v. Vânia Meireles, Tiago Rosado, Mário Barroso, Sofia Soares, **Joana Gonçalves**, Ângelo Luís, Débora Caramelo, Ana Simão, Nicolas Fernández, Ana Paula Duarte, Eugenia Gallardo. "*Mitragyna speciosa*: Clinical, Toxicological Aspects and Analysis in Biological and Non-Biological Samples" Medicines 2019, 6 (1): 35

vi. **Joana Gonçalves**, Rodrigo Ramos, Tiago Rosado, Eugenia Gallardo and Ana Paula Duarte. "Development and validation of a HPLC–DAD method for quantification of phenolic compounds in different sweet cherry cultivars" SN Appl. Sci. 2019 1, 954

vii. Inês Tabarra, Sofia Soares, Tiago Rosado, **Joana Gonçalves**, Ângelo Luís, Sara Malaca, Mário Barroso, Thomas Keller, José Restolho and Eugenia Gallardo. "Novel synthetic opioids – toxicological aspects and analysis" Forensic Sciences Research 2019, 4 (2): 111-140

viii. Ana Y. Simão *, **Joana Gonçalves** *, Ana Paula Duarte, Mário Barroso, Ana Clara Cristóvão and Eugenia Gallardo. "Toxicological Aspects and Determination of the Main Components of Ayahuasca: A Critical Review" Medicines 2019, 6, 106
*** These authors contributed equally to this work.**

ix. Ana Y. Simão*, Mónica Antunes*, **Joana Gonçalves***, Sofia Soares*, Teresa Castro*, Tiago Rosado*, Débora Caramelo*, Mário Barroso*, André R. T. S. Araújo* Jesus Rodilla and Eugenia Gallardo. "The role of gas chromatography in clinical and forensic toxicology" In book: Gas Chromatography: History, Methods and Applications Chapter: 1 Publisher: Nova Science Publishers, Inc. Editors: Percy Henrichon, February 2020 [ISBN: 978-1-53617-350-5]
***These authors are contributed equally.**

x. Ana Sofia Vargas*, Débora Caramelo*, Ana Y. Simão*, Sofia Soares*, **Joana Gonçalves***, Tiago Rosado*, Mónica Antunes*, Mário Barroso*, Eugenia Gallardo. "Ketamine and Other Phencyclidine Analogues: A Review of Their Use As Drugs of Abuse, Toxicological Aspects and Bioanalytical Approaches" In book: Ketamine History, Uses and

Health Effects Chapter: 4 Publisher: Nova Science Publishers, Inc. Editors: Leland A. McBride, February 2020 [ISBN: 978-1-53616-731-3]
***These authors are contributed equally.**

xi. Adriana Silva*, Ana Y. Simão*, **Joana Gonçalves***, Débora Caramelo*, Sofia Soares*, Ângelo Luís*, Tiago Rosado*, Mário Barroso*, Ana Paula Duarte and Eugenia Gallardo. "*Salvia Divinorum*: Beyond Its Therapeutic Uses" In book: Salvia Chemistry and Effects Chapter: 5 Publisher: Nova Science Publishers, Inc. Editors: J. Rodolfo Rendón Villalobos, April 2020 [ISBN: 978-1-53617-819-7]
***These authors are contributed equally.**

xii. **Joana Gonçalves**, Ana Y. Simão*, Sofia Soares*, Carina Gameiro*, Ema Almeida*, Tiago Rosado*, Ângelo Luís*, Eugenia Gallardo and Ana Paula Duarte. "*Prunus avium* L.: Composition, Analysis and Health Benefits" In book: Prunus Classification, Cultivation and Toxicity Chapter: 2 Publisher: Nova Science Publishers, Inc. Editors: William Schneider, April 2020 [ISBN: 978-1-53617-755-8]
***These authors are contributed equally.**

xiii. Sofia Soares, **Joana Gonçalves**, Ana Y. Simão, Tiago Rosado, Débora Caramelo, Nicolás Fernández, André R.T.S. Araújo, M. Barroso and E. Gallardo. "Paroxetine in Human Biological Specimens: A Review of Current Sample Pre-treatment and Bioanalytical Approaches" In book: The Pharmacological Guide to Paroxetine Chapter: 4 Publisher: Nova Science Publishers, Inc. Editors: Charles M. Lepkowsky, July 2020 [ISBN: 978-1-53618-049-7]

xiv. Mónica Antunes*, Ana Y. Simão*, Suzana Fonseca*, **Joana Gonçalves***, Sofia Soares*, Ema Almeida*, Carina Gameiro*, Tiago Rosado1*, Ana Paula Duarte, Mário Barroso*, André R. T. S. Araújo*, Jesus Rodilla and Eugenia Gallardo. "The Role of CYP2D6 In Opiates And Opioids Consumers and Its Implications in Clinical and Forensic Toxicology" In book: Advances in Medicine and Biology Chapter: 1 Publisher: Nova Science Publishers, Inc. Editors: Leon V. Berhardt, October 2020 [ISBN: 978-1-53618-454-9]
***These authors are contributed equally.**

xv. Mónica Antunes*, Tiago Rosado*, Ana Y. Simão*, **Joana Gonçalves***, Sofia Soares*, Mário Barroso* and Eugenia Gallardo. "Liquid Chromatography Mass Spectrometry as a Tool to Identify Adulteration in Different Food Industries" In book: Food Toxicology and Forensics, Chapter: 5 Publisher: Elsevier Inc. Editors: Charis M. Galanakis, November 2020 [ISBN: 978-0-12-822360-4]
***These authors are contributed equally.**

xvi. Eugénia Solange Santos, Ângelo Luís, **Joana Gonçalves**, Tiago Rosado, Luísa Pereira, Eugenia Gallardo and Ana Paula Duarte. "*Julbernardia paniculata* and *Pterocarpus angolensis*: From Ethnobotanical Surveys to Phytochemical Characterization and Bioactivities Evaluation" Molecules 2020, 25(8), 1828

xvii. Alexandra M. Afonso, **Joana Gonçalves**, Ângelo Luís, Eugenia Gallardo and Ana Paula Duarte. "Evaluation of the *In Vitro* Wound-Healing Activity and Phytochemical Characterization of Propolis and Honey" Appl. Sci. 2020, 10, 1845
xviii. Ana Y Simão*, Mónica Antunes*, Hernâni Marques*, Tiago Rosado*, Sofia Soares*, **Joana Gonçalves***, Mário Barroso, Maristela Andraus and Eugenia Gallardo. "Recent bionalytical methods for the determination of new psychoactive substances in biological specimens" Bioanalysis 2020, 12(21):1557-1595
*** These authors contributed equally to this work**.

xix. Ana Y. Simão, **Joana Gonçalves**, Débora Caramelo, Tiago Rosado, Mário Barroso, José Restolho, Nicolás Fernández, Jesus Rodilla, Ana P. Duarte, Ana C. Cristóvão and Eugenia Gallardo. "Determination of *N,N*-dimethyltryptamine and beta-carbolines in plants used to prepare ayahuasca beverages by means of solid-phase extraction and gas-chromatography–mass spectrometry" SN Appl. Sci. 2020, 2, 463

xx. Ana Y. Simão, **Joana Gonçalves**, Ana Gradillas, Antonia García, José Restolho, Nicolás Fernández, Jesus M. Rodilla, Mário Barroso, Ana Paula Duarte, Ana C. Cristóvão and Eugenia Gallardo. "Evaluation of the Cytotoxicity of Ayahuasca Beverages" Molecules 2020, 25, 5594

xxi. Ema Almeida, Sofia Soares, **Joana Gonçalves**, Tiago Rosado, Nicolás Fernández, Jesus M. Rodilla, Luís A. Passarinha, Mário Barroso and Eugenia Gallardo. "Stability of Cocaine, Opiates, and Metabolites in Dried Saliva Spots" Molecules 2022, 27, 641

xxii. Ana Y. Simão, Mónica Antunes, Hernâni Marques, Tiago Rosado, Sofia Soares, **Joana Gonçalves**, Mário Barroso, and Eugenia Gallardo. "Amphetamine in Biological Specimens: Impact and Implications for Public Health" In book: Handbook of Substance Misuse and Addictions, Chapter: 95 Publisher: Springer, Cham. Editors: Patel, V.B., Preedy, V.R., October 2022 [ISBN: 978-3-030-92391-4]

xxiii. Carina Gameiro, **Joana Gonçalves**, Sofia Soares, Tiago Rosado, André Araújo, Luís Passarinha, Mário Barroso and Eugenia Gallardo. "Evaluation of Antipsychotic Drugs' Stability in Oral Fluid Samples" Molecules 2023, 28, 2030

xxiv. Joana Domingues, Manuela Goulão, Fernanda Delgado, José Carlos Gonçalves, **Joana Gonçalves** and Cristina Santos Pintado. "Essential Oils of Two Portuguese Endemic Species of *Lavandula* as a Source of Antifungal and Antibacterial Agents" Processes 2023, 11, 1165

xxv. Soraia Pedro, Carlos Antunes, Carmo Horta, Inês Pitacas, **Joana Gonçalves**, Jorge Gominho, Eugenia Gallardo and Ofélia Anjos. "Characterization of Mineral Composition and Nutritional Value of *Acacia* Green Pods" Plants 2023, 12 (9), 1853

List of Scientific Communications

Oral and Poster communications related to this doctoral thesis:

i. **Joana Gonçalves**, Ângelo Luís, Ana Gradillas, Antonia García, José Restolho, Nicolás Fernández, Fernanda Domingues, Eugenia Gallardo and Ana Paula Duarte. "Evaluation of antioxidant, anti-inflammatory and antimicrobial properties, and phytochemical characterization of Ayahuasca beverages" Online Meeting on Phytomedicine and Phytochemistry (Phytomedicine-2021), February 2021

ii. **Joana Gonçalves**, Miguel Castilho, Ângelo Luís, José Restolho, Eugenia Gallardo, Ana Paula Duarte. "Validação De Um Método Analítico Para Avaliar A Biodisponibilidade E Bioacessibilidade Dos Principais Constituintes Da Ayahuasca" III Jornadas Ibéricas de Toxicologia, online, junho 2021

iii. **Joana Gonçalves**, Ângelo Luís, Eugenia Gallardo, Ana Paula Duarte. "Ayahuasca Beverage: *In Vitro* Wound-Healing Activity" XVII International CICS-UBI Symposium, Covilhã, julho 2022

iv. **Joana Gonçalves**, Tiago Rosado, Mário Barroso, José Restolho, Ângelo Luís, Ana Paula Duarte e Eugenia Gallardo. "Deteção e quantificação dos principais constituintes de Ayahuasca com recurso a QuEChERS e cromatografia líquida de alta eficiência acoplada a um detetor de *diode array*" 21º Congresso Nacional de Medicina Legal e Ciências Forenses, Lamego, outubro 2023

v. **Joana Gonçalves**, Mariana Feijó, Sílvia Socorro, Ângelo Luís, Eugenia Gallardo e Ana Paula Duarte. "Avaliação *in vitro* do potencial anticancerígeno de decocções de Ayahuasca: proliferação celular, apoptose e stresse oxidativo" X Congresso Iberoamericano, Coimbra, outubro 2023

vi. **Joana Gonçalves**, Ângelo Luís, Ana Gradillas, Antonia García, José Restolho, Nicolás Fernández, Fernanda Domingues, Eugenia Gallardo and Ana Paula Duarte. "Phytochemical characterization and evaluation of the bioactive properties of ayahuasca beverages" Microbiotec 2023, Covilhã, dezembro 2023

Oral and Poster communications unrelated to this doctoral thesis:

i. Tiago Rosado, Hernâni Marques, **Joana Gonçalves**, Sofia Soares, Duarte Nuno Vieira, Eugenia Gallardo, Mário Barroso. "Desenvolvimento E Validação De Um Método Para A Determinação De Opiáceos Em Amostras De Cabelo Com Recurso À Microextracção Em Seringa Empacotada E GC/MS-MS" 17.º Congresso Nacional de Medicina Legal e Ciências Forenses, Coimbra, novembro 2018

ii. Andreia Ribeiro, Margarida Prata, Cristiana Vaz, Tiago Rosado, Sofia Soares, **Joana Gonçalves**, José Restolho, Mário Barroso, André RTS Araújo, Eugenia Gallardo. "Determinação De Metadona E Eddp Em Amostras De Fluido Oral Com Amostragem Por Saliva Seca" 17.º Congresso Nacional de Medicina Legal e Ciências Forenses, Coimbra, novembro 2018

iii. Margarida Prata, Andreia Ribeiro, David Figueirinha, Tiago Rosado, David Oppolzer, **Joana Gonçalves**, Sofia Soares, José Restolho, André RTS Araújo, Suzel Costa, Mário Barroso, Eugenia Gallardo. "Aplicação Da Microextracção Em Seringa Empacotada Para A Determinação De Opiáceos Em Amostras De Sangue Total Por Cromatografia Gasosa

Acoplada À Espetrometria De Massa Triplo Quadrupolo" 17.º Congresso Nacional de Medicina Legal e Ciências Forenses, Coimbra, novembro 2018

iv. Tiago Rosado, Sofia Soares, Débora Caramelo, **Joana Gonçalves**, Mário Barroso, Eugenia Gallardo. "Monitorização De Fármacos E Tóxicos: Uma Ferramenta Útil No Diagnóstico E Seguimento Clínico" 1ªs Jornadas de Investigação Clínica do Centro Académico Clínico das Beiras, Covilhã, novembro 2018

v. **Joana Gonçalves**, Rodrigo Ramos, Ângelo Luís, Eugénia Solange Santos, Sandra Rocha, Tiago Rosado, Eugenia Gallardo, Ana Paula Duarte. "Sweet cherries (*Prunus avium* L.): bioaccessibility and bioavailability of their phenolic compounds" Natural Products in Drug Discovery and Human Health (NatProdDDH), Lisboa, July 2019

vi. **Joana Gonçalves**, Rodrigo Ramos, Sandra Rocha, Tiago Rosado, Eugenia Gallardo and Ana Paula Duarte. "Study of Bioaccessibility and Bioavailability of Phenolic Content of Sweet Cherry (*Prunus avium* L.) by *In Vitro* Digestion and Cellular Model" XII Iberoamerican Congress of Food Engineering-CIBIA 2019, Faro, julho 2019

vii. Ana Y. Simão, **Joana Gonçalves**, Débora Caramelo, Tiago Rosado, Mário Barroso, José Restolho, Nicolás Fernández, Ana Paula Duarte, Ana Clara Cristóvão, Eugenia Gallardo. "Desenvolvimento E Validação De Um Método Para A Determinação De Compostos Em Extratos Comerciais Destinados À Preparação De Ayahuasca" 18.º Congresso Nacional de Medicina Legal e Ciências Forenses, Coimbra, novembro 2019

viii. Ana Y. Simão, **Joana Gonçalves**, Débora Caramelo, Tiago Rosado, Mário Barroso, José Restolho, Nicolás Fernández, Ana Paula Duarte, Ana Clara Cristóvão, Eugenia Gallardo. "Analysis of The Main Components of Ayahuasca And Their Cytotoxicity In Dopaminergic Cells" 57th Annual Meeting of The International Association of Forensic Toxicologists (TIAFT), Birmingham, UK, 2019

ix. Bruno Pires, Ângelo Luís, **Joana Gonçalves**, Tiago Rosado, Lígia Salgueiro, Jesus Rodilla, Ana Y. Simão, Sofia Soares, Gonçalo Catarro, Amélia Santos, Délfina Menezes, Fausta Parracho, Nuno Belino, Luís Passarinha e Eugenia Gallardo. "Essential Oils Potential as anti fungal agents: *in vitro* studies and proof of concept" X Congresso Iberoamericano, Coimbra, outubro 2023

x. Bruno Pires, Jesus Rodilla, **Joana Gonçalves**, Tiago Rosado, Amélia Santos, Lúcia Silva, Delfina Menezes, Fausta Parracho, Bruno Peliteiro, Rita Valério, Nelson Durães, Daniela Rodrigues, António Falcão, Sandra Cunha, Ana Afonso, Madalena Freitas, Mário Brito, Nuno Belino, Eugenia Gallardo "Essential Oils for Anti-Cellulite Applications: *In Vitro* Testing Of The Viability, Cytotoxicity, And Cell Permeability" 11º Simpósio Brasileiro de Óleos Essenciais Campinas (Brasil) novembro 2023

xi. E. Nunes, Ana T. Brinca, Sofia Soares, **Joana Gonçalves**, Ana Y. Simão, Hernâni Marques, Tiago Rosado, Eugenia Gallardo "Steroid Hormones Analysis in Blood Samples of Postmenopausal Women by Solid Phase Extraction and Gas Chromatography-Mass Spectrometry" 13th National Chromatography Meeting, Lisboa, Dezembro 2023

Resumo Alargado

A ayahuasca é uma bebida psicoativa consumida desde há séculos, sendo originalmente utilizada por tribos indígenas no noroeste da Amazónia. Esta bebida consiste numa decocção, espessa, oleosa e de cor acastanhada, que é originalmente preparada a partir das folhas de *Psychotria viridis* Ruiz & Pav. e de raspas do caule de *Banisteriopsis caapi* (Spruce ex Griseb.) C.V.Morton, embora ao longo dos anos tenham sido desenvolvidas diversas variações desta decocção. Atualmente, são conhecidos alguns adulterantes naturais ou sintéticos que conseguem mimetizar os efeitos das plantas utilizadas originalmente. Esta decocção contém o composto alucinogénico *N,N*-dimetiltriptamina (DMT) proveniente da *Psychotria viridis*e alcaloides β-carbolínicos como a harmina, tetrahidroharmina (THH) e harmalina, que provêm da *Banisteriopsis caapi*. O DMT é o principal componente psicoativo da ayahuasca, uma vez que atua como agonista dos recetores de serotonina (5-HT1A/2A/2C) e, quando ingerido sozinho, é metabolizado pela monoamina oxidase A (MAO-A) periférica, tornando-se inofensivo. Porém, em conjunto com os alcaloides β-carbolínicos consegue atingir a circulação e o sistema nervoso central, por inibição temporária da MAO-A. Adicionalmente, a THH também inibe a recaptação de serotonina aumentando os efeitos do DMT.

Esta sinergia entre compostos é conhecida pelos povos indígenas há cerca de 3000 anos. Assim, originalmente, as tribos recorriam a esta bebida para fins terapêuticos e religiosos. Era também utilizada pelos curandeiros nativos no tratamento de distúrbios psicológicos, estimulação da criatividade visual e pensamento criativo. Mais recentemente, entidades religiosas não indígenas de vários países, sobretudo da América Latina, também recorreram a esta bebida para as suas cerimónias, nomeadamente a Barquinha, a União do Vegetal e o Santo Daime. Estes dois últimos, atualmente encontram-se disseminados pelos restantes continentes. Nas últimas décadas, tem crescido significativamente em todo o mundo, sendo reconhecida como um fármaco de origem natural usado desde há milénios para a cura de várias doenças. Assim, atualmente esta bebida, apesar de continuar a ser usada de forma tradicional, é também consumida de forma recreativa um pouco por todo o mundo, bem como usada na medicina moderna.

Aquando do consumo de ayahuasca surgem frequentemente vómitos, diarreia e náuseas. Além destes sintomas físicos, foram verificadas alterações na temperatura corporal, tamanho da pupila e alterações imunológicas, endócrinas e cardiovasculares. Sintomas psicológicos como alterações na perceção (tempo, espaço e dos sentidos) e ao nível cognitivo também têm sido descritas. Adicionalmente, são também descritas conexões com entidades divinas e míticas, sendo este o aspeto que motiva o seu consumo em contexto religioso. Não obstante todos os efeitos descritos, ao longo dos anos têm sido

apontadas à ayahuasca diversas propriedades benéficas, acreditando-se mesmo no seu potencial terapêutico em doenças do foro psicológico, como a depressão, ansiedade, dependência e transtornos psicológicos. Além disso, também lhe são apontadas propriedades benéficas em tratamentos fisiológicos.

Nas últimas décadas o consumo de substâncias psicoativas tem vindo a aumentar, nomeadamente substâncias de origem natural, como é o caso da ayahuasca. Muitas vezes este consumo é incitado por motivações culturais ou por crenças espirituais e religiosas, tornando difícil avaliar o seu uso mundial. Segundo a literatura, o consumo desta mistura vegetal em ambientes controlados não apresenta perigo, não estando associado a episódios psicóticos. Porém, a expansão do seu consumo tem levantado algumas preocupações. Apesar do consumo de plantas fonte de DMT não ser controlado, este passou a ser uma substância controlada em alguns países, devido ao aumento da sua procura. Contudo, a legislação que regulamenta substâncias psicoativas é muito variável, podendo até ser ambígua.

Tendo em conta as potencialidades da ayahuasca, o primeiro objetivo desta tese foi desenvolver um método analítico para determinar e quantificar os principais compostos presentes em decocções de ayahuasca, bem como compreender o seu comportamento ao longo do processo digestivo. O segundo objetivo foi efetuar uma caracterização fitoquímica de decocções de ayahuasca e avaliar os seus potenciais efeitos antioxidante, anti-inflamatório, antimicrobiano e as suas propriedades terapêuticas na cicatrização de feridas e no tratamento do cancro.

Assim, inicialmente foram preparadas decocções, em quantidade suficiente para a realização de todo o trabalho, de quatro plantas individuais habitualmente utilizadas nas preparações de ayahuasca (*P. viridis* (folhas), *Mimosa hostilis* Benth. (raíz), *B. caapi* (caule) e *Peganum harmala* L. (sementes)), uma decocção de uma mistura comercial e quatro decocções de misturas de duas das plantas anteriores (*P. viridis* e *B. caapi*, *P. viridis* e *Peganum harmala*, *Mimosa hostilis* e *B. caapi* e *M. hostilis* e *P. harmala*). As amostras foram submetidas a três procedimentos de microextração e posteriormente, foi desenvolvido e validado um método analítico para a determinação e quantificação dos principais constituintes destas amostras. A técnica de *Quick*, *Easy*, *Cheap*, *Effective*, *Rugged* and *Safe* (QuEChERS) mostrou ser a mais promissora e, portanto, foi a eleita para aplicação nas amostras. O método analítico mostrou ser linear entre 0,16 e 10 μg/mL para as β-carbolinas e entre 0,016 e 1 μg/mL para o DMT, com coeficientes de determinação (R^2) entre 0,9968 e 0,9993. O limite de deteção (LOD) e o limite inferior de quantificação (LLOQ) foram de 0,16 μg/mL (0,016 μg/mL para o DMT) e as eficiências de extração variaram entre 60 e 88 %.

A biodisponibilidade e bioacessibilidade dos mesmos compostos também foi avaliada *in vitro*. Para tal, a bioacessibilidade das amostras foi avaliada por um processo de digestão *in vitro*, e posteriormente a sua biodisponibilidade foi avaliada com recurso à linha celular do adenocarcinoma colorretal (Caco-2). Após a quantificação, verificou-se que os compostos (DMT, harmina, harmalina, harmol, harmalol e THH) foram libertados da matriz durante o processo de digestão *in vitro*, tornando-se bioacessíveis. Do mesmo modo, alguns destes compostos, após serem incubados com a monocamada de células, foram absorvidos, tornando-se biodisponíveis, sem apresentarem risco para a integridade da mesma.

O perfil fitoquímico das amostras foi avaliado, tendo-se demonstrado que de forma geral, as amostras apresentam um elevado teor de compostos fenólicos e flavonoides. Estes resultados refletem as atividades antioxidante e anti-inflamatória também determinadas. Adicionalmente, as amostras também apresentaram propriedades antimicrobianas, sendo de destacar o efeito da *B. caapi* e *P. harmala* na estirpe *A. baumannii*, que resultou na inibição da formação de biofilmes e da inibição do *quorum sensing*.

As amostras de ayahuasca também demonstraram o seu potencial terapêutico, nomeadamente ao nível da cicatrização de feridas da pele. Recorrendo a fibroblastos dérmicos normais humanos (NHDF) realizou-se um ensaio de arranhão, tendo-se verificado que apenas uma amostra apresentou citotoxicidade e todas as outras promoveram a migração dos fibroblastos da pele, sem absorção cutânea de DMT e alcaloides β-carbolínicos. Do mesmo modo, as amostras de ayahuasca apresentaram um grande potencial anticancerígeno em células Caco-2, tendo-se verificado que após incubação, as amostras induziram significativamente a apoptose enquanto a proliferação celular diminuiu muito significativamente. Verificou-se ainda uma redução significativa do stresse oxidativo com algumas amostras, com um aumento significativo da atividade de enzimas antioxidantes. Do mesmo modo, em células do adenocarcinoma gástrico (AGS) foi verificado um aumento da indução da apoptose e uma redução do stresse oxidativo após incubação com as amostras de ayahuasca.

Em conclusão, os principais resultados desta tese demonstraram que a ayahuasca possui de facto um grande potencial terapêutico, sendo de destacar as suas ações antimicrobiana, antioxidante, anti-inflamatória, cicatrizante, mas sobretudo a sua atividade anticancerígena estudada em duas linhas celulares diferentes. É ainda de realçar o desenvolvimento e validação do método analítico onde foram comparadas três técnicas de extração miniaturizadas e onde a técnica de QuEChERS foi aplicada a amostras de ayahuasca pela primeira vez.

Palavras-chave

Ayahuasca; DMT; β-carbolinas; caracterização fitoquímica; caracterização analítica; propriedades terapêuticas

Abstract

Ayahuasca is a psychoactive beverage that has been consumed for centuries and was originally used by indigenous tribes in the northwest of the Amazon. This beverage consists of a decoction, thick, oily, and brownish in color, which is originally prepared from the leaves of *Psychotria viridis* Ruiz & Pav. and the scrapings of the stem of *Banisteriopsis caapi* (Spruce ex Griseb.) C.V.Morton, although over the years several modifications of this decoction have been developed. Currently, some natural or synthetic adulterants that can mimic the effects of the plants originally used are known. This decoction contains the hallucinogenic compound *N,N*-dimethyltryptamine (DMT) from *Psychotria viridis*, and β-carboline alkaloids such as harmine, tetrahydroharmine (THH) and harmaline, which come from *Banisteriopsis caapi*. DMT is the main psychoactive component of ayahuasca, since it acts as a serotonin receptor (5-HT1A/2A/2C) agonist and, when ingested alone, is metabolized by peripheral monoamine oxidase A (MAO-A), making it if harmless. However, together with the β-carboline alkaloids, it can reach the circulation and the central nervous system, by temporarily inhibiting MAO-A. Additionally, THH also inhibits serotonin reuptake enhancing the effects of DMT.

This synergy between compounds is known by the indigenous peoples for about 3000 years. Thus, originally, the tribes resorted to this beverage for therapeutic and religious purposes. It was also used by native healers to treat psychological disorders, stimulate visual creativity and creative thinking. More recently, non-indigenous religious entities in various countries, especially in Latin America, also used this beverage for their ceremonies, namely Barquinha, União do Vegetal and Santo Daime. These last two, currently spread to other continents. In recent decades, the popularity of ayahuasca has increased worldwide, being seen as a natural remedy used for millennia to cure various diseases. Thus, currently this beverage, despite continuing to be used in a traditional way, is also consumed recreationally all over the world, as well as used in modern medicine.

When consuming ayahuasca, vomiting, diarrhea and nausea often occur. In addition to these physical symptoms, changes in body temperature, pupil size and immunological, endocrine, and cardiovascular changes were observed. Psychological symptoms such as changes in perception (time, space, and the senses) and at the cognitive level have been also described. Additionally, connections with divine and mythical entities are also described, which is the aspect that motivates its consumption in a religious context. Despite all the effects described, over the years several beneficial properties have been pointed out to ayahuasca, even believing in its therapeutic potential in psychological illnesses, such as depression, anxiety, addiction, and psychological disorders. Additionally, beneficial properties in physiological treatments are also pointed out.

In recent decades, the consumption of psychoactive substances has been increasing, namely substances of natural origin, such as ayahuasca. This consumption is often incited by cultural motivations or by spiritual and religious beliefs, making it difficult to measure its worldwide use. According to the literature, the ayahuasca consumption of in controlled environments is not dangerous and is not associated with psychotic episodes. However, the expansion of its consumption has raised some concerns. Although the consumption of DMT source plants is not controlled, it has become a controlled substance in some countries, due to the increase in its demand. However, the legislation regulating psychoactive substances is very variable and may even be ambiguous.

Considering the potential of ayahuasca, the first objective of this thesis was to develop an analytical methodology for determine and quantify the main compounds present in ayahuasca decoctions, as well as to understand their behavior throughout the digestive process. The second objective was to carry out a phytochemical characterization of ayahuasca decoctions and to evaluate their potential antioxidant, anti-inflammatory, antimicrobial effects and their therapeutic properties in wound healing and cancer treatment.

Thus, initially decoctions, in sufficient quantity to carry out the entire study, of four individual plants commonly used in ayahuasca preparations (*P. viridis* (leaves), *Mimosa hostilis* Benth. (root), *B. caapi* (stem) and *Peganum harmala* L. (seeds)), a decoction of a commercial mixture and four decoctions of mixtures of two of the above plants (*P. viridis* and *B. caapi*, *P. viridis* and *Peganum harmala*, *Mimosa hostilis* and *B. caapi* and *M. hostilis* and *P. harmala*) were prepared. The samples were submitted to three microextraction procedures and later, an analytical method was developed and validated for the determination and quantification of the main compounds of these samples. The *Quick*, *Easy*, *Cheap*, *Effective*, *Rugged* and *Safe* (QuEChERS) technique proved to be the most promising and, therefore, was chosen for application in the samples. The analytical method showed to be linear between 0.16 and 10 μg/mL for β-carbolines and between 0.016 and 1 μg/mL for DMT, with determination coefficients (R^2) between 0.9968 and 0.9993. The limit of detection (LOD) and lower limit of quantification (LLOQ) were 0.16 μg/mL (0.016 μg/mL for DMT) and extraction efficiencies ranged from 60 to 88%.

The bioavailability and bioaccessibility of the same compounds were also evaluated *in vitro*. The bioaccessibility of the samples was assessed using an *in vitro* digestion process, and subsequently their bioavailability was evaluated using the colorectal adenocarcinoma cell line (Caco-2). After quantification, it was verified that the compounds (DMT, harmine, harmaline, harmol, harmalol and THH) were released from the matrix during the *in vitro* digestion process, becoming bioaccessible. Likewise, some

of these compounds, after being incubated with the cell monolayer, were absorbed, becoming bioavailable, without presenting a risk to its integrity.

The phytochemical profile of the samples was evaluated, and it was demonstrated that, in general, the samples have a high content of phenolic compounds and flavonoids. These results reflect the antioxidant and anti-inflammatory activities also determined. Additionally, the samples showed antimicrobial properties, with emphasis on the effect of *B. caapi* and *P. harmala* on the *A. baumannii* strain, which resulted in the inhibition of both biofilm formation and *quorum sensing*.

Ayahuasca samples also demonstrated their therapeutic potential, particularly in terms of wound healing. Using normal human dermal fibroblasts (NHDF), a scratch assay was performed, and it was found that only one sample showed cytotoxicity and the others promoted the migration of skin fibroblasts, without cutaneous absorption of DMT and β-carboline alkaloids. Likewise, the ayahuasca samples showed a great anticancer potential in Caco-2 cells, having been verified that after incubation, the samples significantly induced apoptosis while cell proliferation decreased significantly. There was also a significant reduction in oxidative stress with some samples, with a significant increase in the activity of antioxidant enzymes. Similarly, in gastric adenocarcinoma (AGS) cells, an increase in the induction of apoptosis and a reduction in oxidative stress were observed after incubation with the ayahuasca samples.

In conclusion, the main results of this thesis demonstrated that ayahuasca does indeed have a great therapeutic potential, with emphasis on its antimicrobial, antioxidant, anti-inflammatory, and healing actions, but above all its anticancer activity studied in two different cell lines. Also noteworthy was the development and validation of the analytical method where three miniaturized extraction techniques were compared and where the QuEChERS technique was applied to ayahuasca samples for the first time.

Keywords

Ayahuasca; DMT; β-carbolines; phytochemical characterization; analytical characterization; therapeutic properties

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List of Abbreviations

The following list includes all the abbreviations used in the eight publications that make up this doctoral thesis, corresponding to those used by the cited authors.

5-HT1A/2A/2C	Serotonin receptors
A. catechu	*Areca catechu* L.
A. nervosa	*Argyreia nervosa* (Burm.f.) Bojer
AAI	Antioxidant activity index
ABS	Absorbance
AGS	Gastric adenocarcinoma cell line
ATCC	American Type Culture Collection
ANOVA	Analysis of variance
B. caapi	*Banisteriopsis caapi*
BC	*Banisteriopsis caapi*
BHI-A	Brain Heart Infusion Agar
BHT	Butylated hydroxytoluene
BIAS	Mean relative error
BSA	Bovine serum albumin
C. edulis	*Catha edulis* (Vahl) Endl.
Caco-2	Colorectal adenocarcinoma cell line
cAMP	Cyclic adenosine monophosphate
CLSI	Clinical Laboratory and Standards Institute
CV	Coefficient of variation
D. stramonium	*Datura stramonium* L. test
D-SPE	Dispersive solid phase extraction
DAD	Diode array detector
DCFDA	2',7'-dichlorodihydrofluorescein diacetate
DHBA	3,4-dihydroxybenzylamine
DHE	Dihydroethidium
DLLME	Dispersive liquid-liquid microextraction
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DMT	*N,N*-dimethyltryptamine
DOE	Design of Experiments
DPPH	2,2-diphenyl-1-picrylhydrazyl
DTT	Dithiothreitol
EI	Electron impact
EMCDDA	European Monitoring Centre for Drug Addition
ESI	Electrospray ionization
FBS	Foetal bovine serum
FLD	Fluorescence detector
GABA	Gamma-aminobutyric acid
GAE	Gallic acid equivalents
GC	Gas chromatography
GC × GC	Two-dimensional gas chromatography
GC-MS/MS	Gas chromatography coupled to tandem mass spectrometry
GC/ToF-MS	Gas chromatography coupled to time-of-flight mass

	spectrometry
GPx	Glutathione Peroxidase
GSH	Glutathione
GSSG	Oxidized glutathione
GR	Glutathione reductase
HBSS	Hank's balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HESI	Heated electrospray
HPLC	High performance liquid chromatography
HPLC-DAD	High performance liquid chromatography coupled to diode array detector
HPLC-FLD	High-performance liquid chromatography coupled to fluorescence detection
HPLC-MS	High performance liquid chromatography coupled to mass spectrometry
HPLC-MS/MS	High performance liquid chromatography coupled to tandem mass spectrometry
HPLC-PDA	High performance liquid chromatography coupled to photodiode array detector
HPLC-UV	High performance liquid chromatography coupled to ultraviolet detector
HPTLC	High-performance thin-layer chromatography
HR	High resolution
IC_{50}	Half maximal inhibitory concentration
IS	Internal Standard
KOR	Kappa opioid receptors
L. williamsii	*Lophophora williamsii* (Lem. ex Salm-Dyck) J.M.Coult.
LB	Luria-Bertani
LC	Liquid chromatography
LC_{50}	Lethal Concentration 50
LC-MS/MS	Liquid chromatography coupled mass spectrometry in tandem
LC-Q/TOF-MS	Liquid chromatography quadrupole time-of-flight mass spectrometry
LDH	Lactate dehydrogenase
LLE	Liquid-liquid extraction
LLOQ	Lower limit of quantification
LOD	Limit of detection
LOQ	Limit of quantification
LSA	Lysergamide
LSD	Lysergic acid diethylamide
M. hostilis	*Mimosa hostilis*
M. officinarum	*Mandragora officinarum* Bertol.
M. speciosa	*Mitragyna speciosa* Korth.
MAO-A	Monoamine Oxidase A
MAO-B	Monoamine oxidase B
MEPS	Microextraction by packed sorbent
MH	*Mimosa hostilis*
MH+BC or	Mixture of *M. hostilis* and *B. caapi*

MHBC	
MH+PH or MHPH	Mixture of *M. hostilis* and *P. harmala*
MHB	Müeller–Hinton Broth
MIC	Minimum inhibitory concentration
MS	Mass spectrometry
MS/MS	Tandem Mass spectrometry
MSPD	Matrix solid phase dispersion
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
ND	Not detected
NF-kB	Nuclear factor-kB
NMDA	*N*-methyl-*D*-aspartatetype
NPD	Nitrogen–phosphorus detector
NPS	New psychoactive substances
NSCLC	Non-small cell lung cancer cells
OD	Optical density
P. harmala	*Peganum harmala*
P. methysticum	*Piper methysticum* G.Forst.
P. viridis	*Psychotria viridis*
PAMPA	Parallel artificial membrane permeability assay
PBS	Phosphate buffer solution
PBST	Phosphate buffer saline with 0.1% tween®
PH	*Peganum harmala*
pNA	p-nitroaniline
PP	Protein precipitation
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
PSA	Primary Secondary Amine
PV	*Psychotria viridis*
PV+PH or PVPH	Mixture of *P. viridis* and *P. harmala*
PV+BC or PVBC	Mixture of *P. viridis* and *B. caapi*
QC	Quality control
QE	Quercetin equivalents
QuEChERS	Quick, Easy, Cheap, Effective, Rugged and Safe
R^2	Coefficients of determination
RE	Relative error
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RSM	Response surface methodology
S. divinorum	*Salvia divinorum* Epling & Játiva
SD	Standard deviation
SEM	Scanning electron microscopy
SIGMAR1	Non-opioid intracellular receptor sigma 1
SH-SY5Y	Neuroblastoma cell line
SOD	Superoxide Dismutase
SPE	Solid-phase extraction
SPME	Microextraction in solid phase
T. iboga	*Tabernanthe iboga* Baill.

TEER	Transepithelial electrical resistance
ToF or Q/ToF	Time of flight
THH	Tetrahydroharmine
UDV	União do Vegetal
UHPLC	Ultrahigh-performance liquid chromatography
UPLC-ESI-TOF	Ultra-performance liquid chromatography coupled to electrospray ionization and time of flight detector
UHPLC-MS/MS	Ultra-high-performance liquid chromatography coupled to tandem mass spectrometry
UHPLC-Q/TOF-MS	Ultrahigh performance liquid chromatography-quadrupole time-of-flight mass spectrometry
UPLC-UV-MS	Ultra-performance liquid chromatography coupled to mass spectrometry and ultraviolet detector
UNODC	United Nations Office on Drug and Crime
UV-VIS	Ultraviolet-visible detector

Thesis overview

This doctoral thesis is divided into five chapters. The first chapter is an introductory chapter where general information about ayahuasca and the problem of the emergence of new natural psychoactive substances (NPS) is provided. Two review articles are included in this chapter. The first is a review of drugs of abuse of natural origin, where the main concerns about NPS are addressed, namely the rapid appearance and the lack of legislation, but also the toxicological aspects, therapeutic potential and the analytical methods developed for the quantification of its main compounds (Paper I). The second is a systematic review where the therapeutic properties, associated with ayahuasca and its consumption are addressed and discussed (Paper II).

The second chapter presents the overall objectives of this doctoral thesis.

The third chapter focuses on the research carried out throughout this project and includes six published studies. The first one (Paper III) describes the development, validation, and optimization of an analytical method, where initially three miniaturized extraction techniques are compared, finally choosing QuEChERS as the most promising technique for the study. The second published study (Paper IV) consists of the evaluation of the bioaccessibility and bioavailability of the main constituents of ayahuasca. For this, an *in vitro* simulated digestion process and a cell model that simulates the intestinal wall using the Caco-2 line were used. The third published study (Paper V) reports the evaluation of the phytochemical profile as well as the antioxidant, anti-inflammatory and antimicrobial properties of ayahuasca decoctions. The fourth published study (Paper VI) describes the *in vitro* evaluation of the healing potential of ayahuasca samples in NHDF cells. Finally, the fifth published study (Paper VII) describes the potential anticancer properties of ayahuasca samples in Caco-2 cells and the sixth published study (Paper VIII) consists of a brief description of the anticancer potential in AGS cells.

The studies (paper I to VIII) are presented as they appear in the respective publications, including the references.

The fourth chapter consists of a discussion and final reflection on the eight published articles. Finally, the fifth chapter presents the conclusions about the entire study carried out in this doctoral thesis.

Chapter 1

General Introduction

This chapter was published in:

Joana Gonçalves, Ângelo Luís, Eugenia Gallardo and Ana Paula Duarte. "*Psychoactive Substances of Natural Origin: Toxicological Aspects, Therapeutic Properties and Analysis in Biological Samples*" Molecules 2021, 26, 1397

Joana Gonçalves, Ângelo Luís, Eugenia Gallardo and Ana Paula Duarte. "*A Systematic Review on the Therapeutic Effects of Ayahuasca*" Plants 2023, 12, 2873

1.1. Paper I- Psychoactive Substances of Natural Origin: Toxicological Aspects, Therapeutic Properties and Analysis in Biological Samples

Abstract

The consumption of new psychoactive substances (NPSs) has been increasing, and this problem affects several countries worldwide. There is a class of NPSs of natural origin, consisting of plants and fungi, which have a wide range of alkaloids, responsible for causing relaxing, stimulating or hallucinogenic effects. The consumption of some of these substances is prompted by religious beliefs and cultural reasons, making the legislation very variable or even ambiguous. However, the abusive consumption of these substances can present an enormous risk to the health of the individuals, since their metabolism and effects are not yet fully known. Additionally, NPSs are widely spread over the internet, and their appearance is very fast, which requires the development of sophisticated analytical methodologies, capable of detecting these compounds. Thus, the objective of this work is to review the toxicological aspects, traditional use/therapeutic potential and the analytical methods developed in biological matrices in twelve plant species (*Areca catechu* L., *Argyreia nervosa* (Burm.f.) Bojer, ayahuasca, *Catha edulis* (Vahl) Endl., *Datura stramonium* L. test, *Lophophora williamsii* (Lem. ex Salm-Dyck) J.M.Coult., *Mandragora officinarum* Bertol., *Mitragyna speciosa* Korth., *Piper methysticum* G.Forst., *Psilocybe*, *Salvia divinorum* Epling & Játiva and *Tabernanthe iboga* Baill.).

Keywords: NPS of natural origin; psychoactive effects; toxicological aspects; traditional uses; analytical methodologies

1.1.1. Introduction

The use of drugs of abuse is a concern that has been increasing over the years. About 96 million individuals have already used drugs of abuse in the European Union, cannabis being the most used (27.4%), followed by cocaine (5.4%) and then ecstasy (4.1%) and amphetamines (3.7%) [1]. In recent years, a trend of new psychoactive substance (NPS) consumption has been reported. The European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) defines those compounds as “a new narcotic or psychotropic drug, in pure form or in preparation, that is not controlled by the United Nations Drug Conventions, but which may pose a public health threat comparable to that posed by substances listed in these conventions” [2]. NPSs have spread around the world, mainly because they are marketed on the internet in dark web forums, with different names, namely “bath salts”, “legal highs”, or “research chemicals” [3]. These substances are generally consumed because they are able to mimic the effects of more conventional drugs of abuse and because they are not detected in common screening methods [4–6]. However, the various risks

associated with the consumption of NPSs are described in the literature, as well as the resulting health problems [7–9].

Despite the term "new" referring to a recent appearance, the truth is that some of these compounds have existed for decades, but they only became available on the market more recently, and consequently, their commercialization is not yet regulated [3]. The constant appearance of these drugs (about 50 new per year) is a concern in terms of controlling their marketing [1]. Additionally, considering the health hazard presented by NPSs, the United Nations Office on Drugs and Crime (UNODC) and the EMCDDA have implemented early warning systems in order to detect these compounds [10,11].

NPSs may have a synthetic or natural origin, the most recognized synthetic NPS being synthetic cannabinoids, cathinones and opioids, piperazines, phenethylamines, designer benzodiazepines, indoalkylamines and arylcyclohexylamines [2–4]. NPSs of natural origin consist mainly of alkaloids naturally present in plants that, when consumed, allow the user to experience new sensations and different "mental states" [3,12]. These plants come mainly from South America and Asia but also from Africa and Russia [3] and, depending on their constituents, can trigger relaxing and/or sedative effects, such as *Areca catechu* and *Mitragyna speciosa*, hallucinogenic effects, as the constituents of ayahuasca, or stimulating effects, such as *Catha edulis* [3,13]. The consumption of preparations containing alkaloids of natural origin is often prompted by religious beliefs or cultural reasons, making it difficult to estimate the worldwide consumption of these substances [3]. For these reasons, the legislation that regulates these substances is quite variable and may even be ambiguous [2].

The metabolism of these substances is not fully studied, and therefore the resulting metabolites and their potential concentrations are not known [2,3]. Another gap that needs further study is the acute toxicity of many of these substances, which are also not completely known [2]. In fact, the symptoms described during intoxication with an NPS are confused with symptoms of consumption of other substances, namely medicines [2]. Thus, developing analytical methodologies is greatly important for the detection and quantification of potentially dangerous compounds present in these natural products. However, most developed analytical methods have focused on the detection of alkaloids naturally present in plant materials [14].

In this review, we sought to address the toxicological aspects of several psychoactive substances present in different plants, as well as some therapeutic properties/traditional uses. In addition, the analytical methods developed in biological samples aimed at the detection of psychoactive substances from the same plants were also discussed.

1.1.2. *Areca catechu* (Betel Quid)

Currently, *A. catechu* (Figure I.1.1A) is distributed in Africa, Europe and America, in spite of its main origin being Asia (Sri Lanka and Malaysia) [2]. The areca nut is the fruit produced by this plant, having been consumed for centuries as a traditional remedy or in rituals [15]. This fruit is normally chewed and can be consumed together with other substances in the form of a "betel quid" [15,16]. Areca nut is the fourth drug with the highest consumption rate worldwide, possibly

due to its stimulating, relaxing or aphrodisiac effects [15,16]. Arecoline (Figure I.1.1B) is the main psychoactive compound present in the fruit of *A. catechu* [2]. This compound is an alkaloid that works as a competitive inhibitor of gamma-aminobutyric acid (GABA) and as a non-selective nicotinic and muscarinic agonist [3,17,18]. Once in the body, arecoline quickly crosses the blood-brain barrier, exerting effects on the parasympathetic nervous system [3]. However, this fruit is addictive and can cause several adverse effects, namely on the digestive system and abstinence syndrome (insomnia, mood swings, irritability and anxiety) [3,15]. Other effects, such as severe extrapyramidal syndrome, asthma and myocardial infarction, have also been associated with the consumption of this fruit [19,20]. However, the use of this fruit for medicinal purposes has also been described, since antiquity, by Hindu and Buddhist peoples [21]. The consumption of areca nut has been associated with general properties such as satisfaction, well-being, psychostimulating effects, stress reduction, gum strengthening and breath sweetening [21]. Additionally, this fruit is used in the treatment of malaria, fever, hernia, hypertension, urinary stones and in the manufacture of formulations for the treatment of digestive diseases, diarrhea and indigestion [21]. Studies have also indicated that the consumption of areca nut is associated with antimicrobial [22,23], cardiovascular [23,24] and digestive effects [21,23–25]. In addition to the *A. catechu* fruit, roots and leaves were also traditionally used in medicine [21].

Figure I.1.1. *A. catechu* **(A)** and the main compound arecoline **(B).**

A. catechu and its fruit are not controlled substances, and therefore there is no legislation for their consumption in the United States of America and in the European Union [3]. Thus, several analytical methods, which allow the measurement of these substances, have been developed (Table I.1.1). Conventional samples, such as blood, continue to be used for the detection of the most diverse compounds, including arecoline [26]. Wu *et al.* [26] developed an analytical method where they proceeded to quantify arecoline in an LC-MS/MS equipment, using 1 mL of blood, obtaining a limit of detection (LOD) of 0.02 ng/mL and a limit of quantification (LOQ) of 0.5 ng/mL. Urine is

another biological matrix that has been used for the measurement of arecoline [27]. Pichini *et al.* [27] developed an analytical method in HPLC-MS equipment, where they used 1 mL of urine, having managed to quantify arecoline. In the same study, it was also possible to quantify the same compound in samples of meconium (1 g) and cord serum (1 mL) [27]. However, other alternative samples, such as teeth [28], saliva [29,30] and breast milk [31] have been also used in the development of new analytical methods for detecting arecoline. Pellegrini *et al.* [31] developed a method for the determination of arecoline in LC-MS/MS, using 1 mL of breast milk. This method had a LOD of 16 ng/mg and LOQ of 50 ng/mg [31].

Table I.1.1. Analytical methods for the determination of the main components of *Areca catechu.*

Compounds	Sample (amount)	Sample preparation	Analytical technique	Limits of detection	Limits of quantitation	Recovery (%)	Reference
Arecoline	Teeth (50 mg)	Pulverization and sonication (methanol)	LC-MS/MS (ESI); LC-HR-ToF-MS (ESI)	-	-	-	[28]
Arecoline	Saliva (950 mL)	LLE (ethylacetate)	HPLC-UV-VIS	-	-	-	[29]
Arecoline	Hair (50 mg)	Pulverization; alkaline digestion (NaOH 12 M) and LLE (cloroform/isopropanol (95:5, v/v)	LC-MS (ESI)	0.09 μg/g	0.3 μg/g	81.2 ± 2.6	[32]
Arecoline	Meconium (1000 mg), urine (1 mL) and cord serum (1 mL)	LLE (chloroform/isopropanol (95:5, v/v))	LC-MS (ESI)	0.0004-0.001 μg/g	0.001-0.005 μg/g	86.5-90.7	[27]
Arecoline	Breast milk (1 mL)	LLE (chloroform/isopropanol (95:5, v/v))	LC-MS/MS (ESI)	16 μg/L	50 μg/L	76.8 - 84.7	[31]
Arecoline, arecaidine and *N*-methylnipecotic acid	Saliva (0.05 mL)	PP (acetonitrile)	LC-MS/MS (ESI)	0.156 μg/L	1.25 μg/L	72.5 - 100.1	[30]

Caption: ESI (electrospray ionization); HPLC (high-performance liquid chromatography); HR (high resolution); LC (liquid chromatography); LLE (liquid-liquid extraction); MS (mass spectrometry); MS/MS (tandem mass spectrometry); PP (protein precipitation); ToF (time of flight); UV-VIS (ultraviolet-visible detector).

1.1.3. *Argyreia nervosa* (Adhoguda)

A. nervosa (Figure I.1.2A) is originally from India, but it is widely distributed in Europe, Africa and subtropical America [33]. This plant, also known as Adhoguda, Vidhara, Elephant Creeper, *Rivea corymbosa*, Hawaiian Baby Woodrose or Morning Glory or *Ipomoea violacea*, possesses psychoactive alkaloids in its seeds [3]. Isoergine (Figure I.1.2B) and lysergamide (LSA) (Figure I.1.2C) are the compounds responsible for the hallucinogenic properties of this plant, being able to induce effects similar to lysergic acid diethylamide (LSD), albeit with a lower intensity [34]. *A. nervosa* seeds have a total of ergoline alkaloids between 0.5% and 0.9%, of which 0.19% correspond to isoergine and 0.14% correspond to LSA [35]. The LSA exerts its effects by binding to dopamine D2 receptors and consequent inhibition of adenylate cyclase and reduction in the

production of cyclic adenosine monophosphate (cAMP) [36]. The consumption of this plant for medicinal purposes has also been described, namely as a diuretic and aphrodisiac [13]. Analgesic, anti-inflammatory, immunomodulatory, hepatoprotective and hypoglycemic properties have also been described [13,37]. *A. nervosa* roots are also used in the treatment of diseases of the central nervous system, rheumatism, gonorrhea and chronic ulcer. On the other hand, antimicrobial activity has been associated with the plant leaf [13,37]. In addition to the two alkaloids already mentioned, others have been also isolated from plants, namely erginine, ergometrine, lysergol, peniclavine, chanoclavin I, chanoclavin II, ergometrinine, elimoclavin and egine, but their effects are not yet known [38].

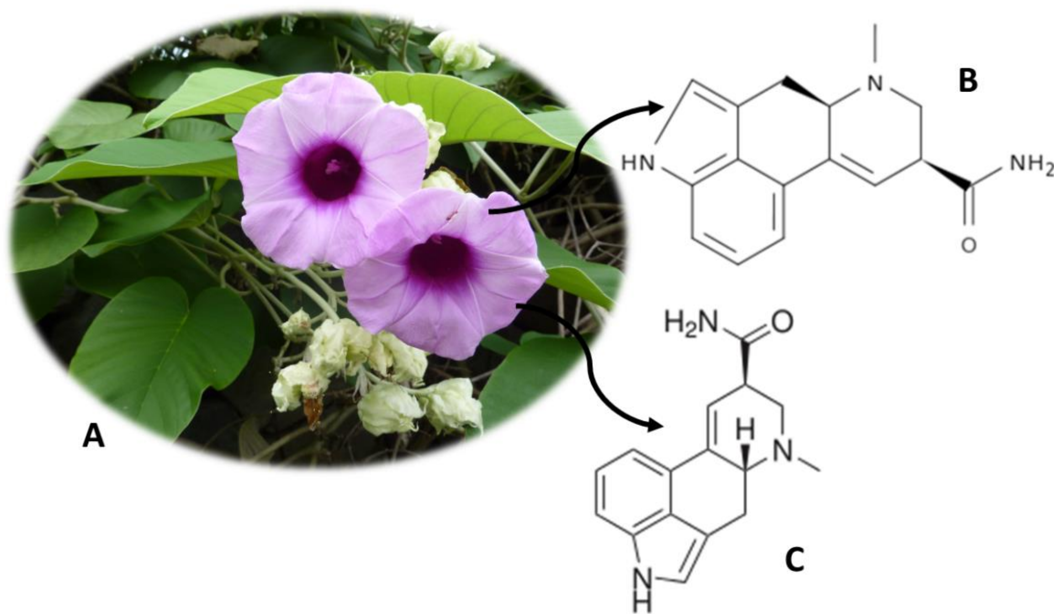


Figure I.1.2. *Argyreia nervosa* **(A)** and the main compounds isoergine **(B)** and lysergamide (LSA) **(C)**.

LSA is a controlled substance in some European countries, namely in the United Kingdom and Italy. It is also controlled in the United States of America, but the plant and its seeds are freely sold [3]. There are currently analytical methodologies developed that allow quantifying the LSA. Paulke *et al.* [39] developed an analytical method, with 1 mL of serum and urine, to detect and quantify LSA. The analytes were extracted using the SPE (solid-phase extraction) technique and quantified on HPLC-FLD equipment [39]. The method had detection and quantification limits of 0.05–0.15 ng/mL and 0.17 ng/mL, respectively, and recoveries between 69.4% and 78.8% [39].

1.1.4. Ayahuasca (“Hoasca”)

Ayahuasca is a word of Quechua origin, composed of two terms: "aya" and "waska", which mean "spirit" and "vine", respectively [40]. On its whole, the word ayahuasca means "rope of the soul", and it is also known as caapi, daime, hoasca, yagé and natema [3,40]. This term refers to a psychoactive drink, traditional in South America (Figure I.1.3A). More recently, it has been

imported into some countries in Europe and Asia [3,41,42]. ayahuasca consists of a brown, thick and oily liquid, the result of a decoction of shavings from the stem of *Banisteriopsis caapi* (*B. caapi*) and leaves of *Psychotria viridis* (*P. viridis*) (Figure I.1.3B) [41,42]. Additionally, other species of natural origin, which replace those already mentioned, can be used in the preparation of ayahuasca, namely *Brugmansia suaveolens*, *Psychotria carthagenensis*, *Nicotiana tabacum*, *Tabernaemontana* spp., *Brunfelsia* spp., *Datura suaveolens*, *Iochroma. fuchsioides*, *Malouetia tamarquina*, *Juanulloa* spp., *Peganum harmala*, among other products with hallucinogenic compounds [43].

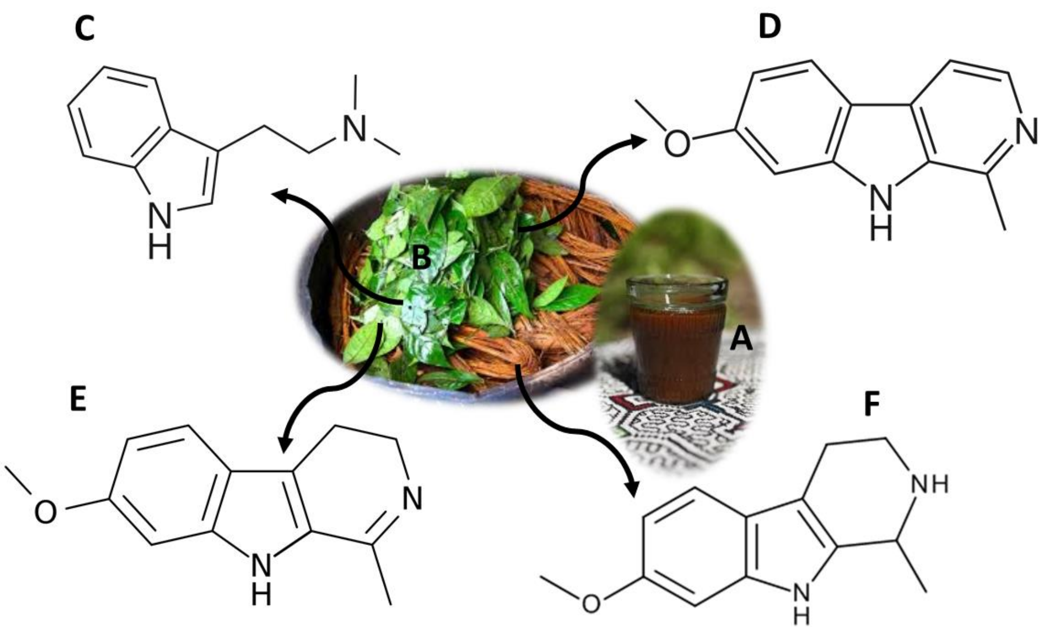


Figure I.1.3. Ayahuasca decoction **(A)**; *Banisteriopsis caapi* stem shavings and *Psychotria viridis* leaves used in the preparation of the ayahuasca beverage **(B)**; main compounds present in ayahuasca: DMT **(C)**, harmine **(D)**, harmaline **(E)** and THH **(F)**.

The effects of this psychoactive mixture are due to the synergy potential of *N,N*-dimethyltryptamine (DMT) (Figure I.1.3C), a hallucinogenic compound from *P. viridis*, and of the harmine (Figure I.1.3D), harmaline (Figure I.1.3E) and tetrahydroharmine (THH) (Figure I.1.3F), which are β-carbolinic alkaloids present in *B. caapi* [44,45]. DMT is a tryptamine that acts as an agonist for serotonin receptors (5-HT1A/2A/2C) [3]. When this compound is ingested alone, it undergoes metabolism by peripheral monoamine oxidase A (MAO-A), being inactive [46]. However, when DMT is ingested together with β-carbolinic alkaloids, it is able to penetrate the central nervous system, since it temporarily inhibits MAO-A [44,46–48]. In addition, THH also inhibits serotonin reuptake by increasing the effects of DMT [49]. Users describe visual hallucinations, with effects on temperature, pupil size and changes in the endocrine, cardiovascular and immune systems [3,50]. Side effects such as mydriasis, vomiting, hypertension, tachycardia, agitation, paranoia, anxiety and depression have also been described [3,50,51]. However, there are several studies that report therapeutic properties. Recently their proprieties were reported as antimicrobial and antioxidant agents [52], as well as their effect over dopaminergic neuron cells

[53]. Studies have shown that a single dose of ayahuasca leads to a rapid reduction in depressive symptoms, and this reduction is maintained for three weeks [54,55]. Other studies show that the consumption of this decoction results in a significant reduction in anxiety and panic [54,56]. The reduction of drug and alcohol abuse [57–59], attention problems [60] and decreased physical pain, fatigue, insomnia, irritability and obsession [61] have also been described.

Table I.1.2. Analytical methods for the determination of the main components of ayahuasca.

Compounds	Sample (amount)	Sample preparation	Analytical technique	Limits of detection	Limits of quantitation	Recovery (%)	Reference
5-OH-DMT, DMK, harmol, harmalol, NMT, 5-MeO-DMT, 2-MTHBC, THH, DMT-NO, harmine and harmaline	Urine (0.1 mL)	Dilution (90% water–0.1% formic acid:10% acetonitrile–0.1% formic acid) and enzyme hydrolysis (glucuronidase–sulfatase–acetate buffer)	LC-MS/MS (ESI)	0.04 – 0.57 µg/L	5 µg/L	-	[65]
DMT, harmine, harmaline, THH, harmol and harmalol	Plasma (1 mL)	LLE (n-pentane) and SPE (C_{18})	GC–NPD and HPLC–FLD	-	0.3-1.6 µg/L	74 - 87	[63]
DMT, harmine, harmaline, THH, harmol, harmalol, 5-OH-DMT, THH-OH, DMK, NMT, 5-MeODMT, 2-MTHBC, DMT-NO	Blood (0.2 mL)	PP (96-well plates), Dilution (formic acid (0.1% in water); formic acid (0.1% in acetonitrile))	LC–MS/MS (HESI)	0.09 – 0.45 µg/L	1.0 µg/L	60.28- 76.31	[62]
DMT, harmine, harmaline, THH	Plasma (1 mL)	SPE (C_{18})	LC–MS/MS (ESI)	0.1 µg/L	0.2 - 0.4 µg/L	88.4- 107.7	[64]
DMT	Hair (25 mg)	Hydrolysis (M3 reagent)	UHPLC - MS/MS (ESI)	0.01 - 0.02 µg/g	0.03 - 0.05 µg/g	79.6 - 97.4	[67]
DMT and DMT-NO	Urine (0.1 mL)	Dilution (97:3 water with 0.1% formic acid:acetonitrile with 0.1% formic acid)	LC-MS/MS (ESI)	-	5.0 µg/L	-	[66]

Caption: ESI (electrospray ionization); FLD (fluorescence detector); GC (gas chromatography); HESI (heated electrospray); HPLC (high-performance liquid chromatography); LC (liquid chromatography); LLE (liquid-liquid extraction); MS/MS (tandem mass spectrometry); NPD (nitrogen–phosphorus detector); PP (protein precipitation); SPE (solid-phase extraction); UHPLC (ultrahigh-performance liquid chromatography).

Ayahuasca has been used in religious rituals in the Amazon for centuries, and more recently by religious entities such as União do Vegetal (UDV) and Santo Daime [3,40]. DMT-containing substances are controlled in the United States of America and in some European countries [3]. However, the consumption of *P. viridis* and *B. caapi* is not controlled, and the use of ayahuasca for religious purposes is legal in the United States of America and Brazil [3]. There are currently several analytical methodologies that allow the detection and quantification of the compounds from ayahuasca and its metabolites (Table I.1.2). The samples of choice for the quantification of these compounds are the so-called conventional samples, namely blood [62], plasma [63,64] and urine [65,66]. Yritia *et al.* [63] and Oliveira *et al.* [64] developed analytical methods for the detection of DMT and β-carbolines, using 1 mL of plasma. Both methods used SPE [63,64] as a sample pre-treatment technique, and in the first study, a liquid-liquid extraction (LLE) was also performed [63]. Both studies showed good limits of detection and quantification, as well as good recoveries [63,64]. More recently, Pichini *et al.* [67] carried out a study, where they quantified DMT, using only 25 mg of hair. The hair sample was initially hydrolyzed with an M3 reagent, and HPLC-MS-MS equipment was used to quantify the analyte [67]. The LOD varied between 0.01 ng/mg and 0.02 ng/mg and the LOQ between 0.03 ng/mg and 0.05 ng/mg, with recoveries between 76.6% and 97.4% [67].

1.1.5. *Catha edulis* (Khat)

C. edulis (Figure I.1.4A) comes from some West African countries, as well as from Yemen, Ethiopia and the Arabian Peninsula [2]. This plant is often used as a drug of abuse since it allows to mimic the effects of synthetic cathinones but with a lower risk of intoxication, with no record of deaths associated with its consumption [2]. *C. edulis* is also called khat, qat and kafta, among others, and it is usually consumed in smoked form or by chewing fresh leaves [3]. The psychoactive components present in the leaves of this plant are S-(-)-cathinone (Figure I.1.4B), cathine ([S,S-(+)-norpseudoephedrine]) (Figure I.1.4C) and phenylpropanolamine (Figure I.1.4D). S-(-)-cathinone is photosensitive, and therefore it degrades easily with sun exposure, being the major compound in fresh khat leaves, but it is not found in older leaves [68]. After sun exposure, S-(-)-cathinone degrades into chatine and (-)-norephedrine, these being the compounds present mostly in the older leaves [68]. When consuming khat, the active compounds degrade not only into chatine and (-)-norephedrine but also into [R,S-(-)-norephedrine] and [R,R-(-)-norpseudoephedrine], compounds structurally similar to amphetamine [69]. Consumers of this plant describe effects such as hyperthermia, euphoria, increased breathing and sensory stimulation, excitation and anorexia. However, adverse effects such as violent behavior, schizophrenia, paranoia and psychosis, increased blood pressure, insomnia, tachycardia, irritability, migraine and sexual dysfunction have also been described [3].

The consumption and trade of khat leaves are not regulated by any international system, but the consumption and trade of cathinone and cathine are prohibited worldwide [3]. In some countries, khat is considered a controlled substance, namely Ireland, France, Germany, Denmark

and the United States of America. In the Netherlands, its trade is not prohibited, but is restricted and, in Canada, the possession of khat is allowed, but its import and trade are also illegal [3]. On the other hand, countries like Yemen, Somalia and Ethiopia allow the consumption of khat, since it is a cultural habit [3].

Table I.1.3. Analytical methods for the determination of the main components of *Catha edulis*.

Compounds	Sample (amount)	Sample preparation	Analytical technique	Limits of detection	Limits of quantitation	Recovery (%)	Reference
Cathinone, cathine, and phenylpropanolamine	Urine (0.2 mL)	SPE (C_8)	GC-MS (EI)	< 10µg/L	-	73 - 82	[72]
d-cathine (d-norpseudoehedrine), ephedrine, methcathinone, 1-(4-methoxyphenyl)-propan-2-amine, mephedrone, methedrone, 2,5-dimethoxy-4-methylamphetamine, 4-bromo-2,5-dimethoxyamphetamine, 2,5-dimethoxyphenethylamine, 4-bromo-2,5-dimethoxyphenethylamine, 4-iodo-2,5-dimethoxyphenethylamine, 2-[2,5-dimethoxy-4-(ethylthio)phenyl]ethanamine, 2,5-dimethoxy-4-isopropylthiophenethylamine and 2-[2,5-dimethoxy-4-(propylthio)phenyl]ethanamine	vitreous humor (0.1 mL), pericardial fluid (0.25 mL) and whole blood (0.25 mL)	SPE (Oasis® MCX)	GC-MS (EI)	5 µg/L	5 µg/L	100	[74]
Cathinone, methcathinone, ethcathinone, amfepramone, mephedrone, flephedrone, methedrone, methylone, butylone, cathine, norephedrine, ephedrine, pseudoephedrine, methylephedrine and methylpseudoephedrine	Blood (0.3 mL)	PP (methanol) and Ultrafiltration	LC-MS/MS (ESI)	0.5 - 3 µg/L	-	87 - 106	[70]
Cathinone, flephedrone, buphedrone, 4-MTA, α-PVP, methylone, 2C-P, ethylone, pentylone, MDPV and bromo-dragonFLY	whole blood (0.25 mL)	SPE (Oasis® MCX)	GC-MS	40 – 5 µg/L	40 – 5 µg/L	70.3 - 116.6	[75]
Cathine, cathinone, methcathinone and ephedrine	Oral fluid (0.5 mL)	LLE (ethyl acetate)	GC - MS (EI)	10.0 µg/L	20.0 µg/L	-	[73]

Caption: EI (electron impact); ESI (electrospray ionization); GC (gas chromatography); LC (liquid chromatography); LLE (liquid-liquid extraction); MS (mass spectrometry); MS/MS (tandem mass spectrometry); PP (protein precipitation); SPE (solid-phase extraction).

There are currently analytical methodologies developed that allow quantifying the compounds present in khat (Table I.1.3). Sørensen [70] developed an analytical method to quantify 15 compounds, using LC-MS/MS equipment with only 300 µL of blood. The sample was treated with methanol to precipitate proteins, and then it was filtered [70]. The analytical method showed

recoveries between 87% and 106% and a LOD between 0.5 ng/mL and 3 ng/mL [70]. In addition, samples such as plasma [71], urine [72] and oral fluid [73] were used to quantify these compounds. Mohamed *et al.* [73] used 500 µL of oral fluid to quantify cathine, methcathinone, cathinone and ephedrine. The samples were submitted to an LLE (ethyl acetate) extraction technique and subsequently analyzed by GC-MS. The analytical method showed a LOQ of 20 ng/mL and a LOD of 10 ng/mL [73].

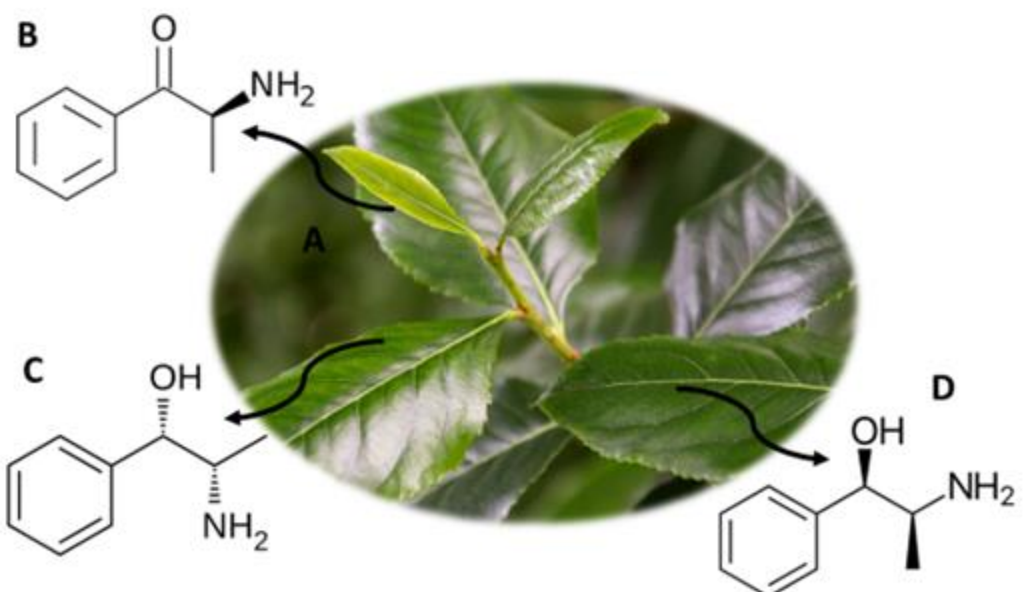


Figure I.1.4. *C. edulis* **(A)** and the main compounds S-(-)-cathinone **(B)**, cathine ([S,S-(+)-norpseudoephedrine]) **(C)** and phenylpropanolamine **(D)**.

1.1.6. *Datura stramonium* (Jimson Weed)

D. stramonium (Figure I.1.5A), originally from the United States of America, consists of a seasonal herb that grows naturally [2]. This species, also known as Jimson Weed, was traditionally used by the Pueblo Indians, due to its analgesic properties [76]. Moreover, in Western medicine, Jimson Weed was used to treat asthma [76]. This plant is usually consumed by eating its seeds or flowers intact, or in the form of an infusion of leaves or crushed seeds [77,78]. Dried leaves, flowers and seeds are also consumed in a smoked form, and there is also Asthmador™ powder available for consumption in smoked form or by inhalation [78,79]. The consumption of *D. stramonium* also causes hallucinogenic effects, which are due to the presence of the alkaloids scopolamine (Figure I.1.5B) and atropine (Figure I.1.5C) distributed throughout the plant [2]. These compounds are tertiary amines and therefore cross the blood-brain barrier rapidly [79]. Scopolamine acts at the level of the central nervous system, exerting antimuscarinic effects [80]. The effects of Jimson Weed consumption include tachypnea, delirium, psychomotor agitation, dilation of the pupils, blurred vision or photophobia [78,79,81]. Other effects, such as peripheral vasodilation, decreased thermoregulation, vomiting, constipation and difficulties in urinating, have been also described

[76,82]. At higher doses, respiratory depression and even cardiac arrest, seizures or hypoventilation may occur [79]. Some analytical methods have been developed to quantify the alkaloids scopolamine and atropine (Table I.1.4). These are discussed below.

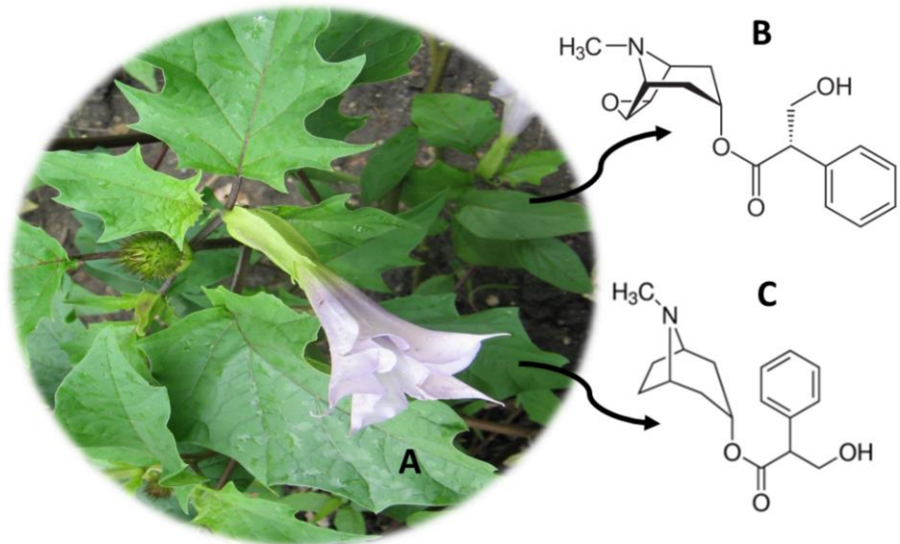


Figure I.1.5. Datura stramonium (A) and the main compounds scopolamine (B) and atropine (C).

1.1.7. *Mandragora officinarum* (Mandrake)

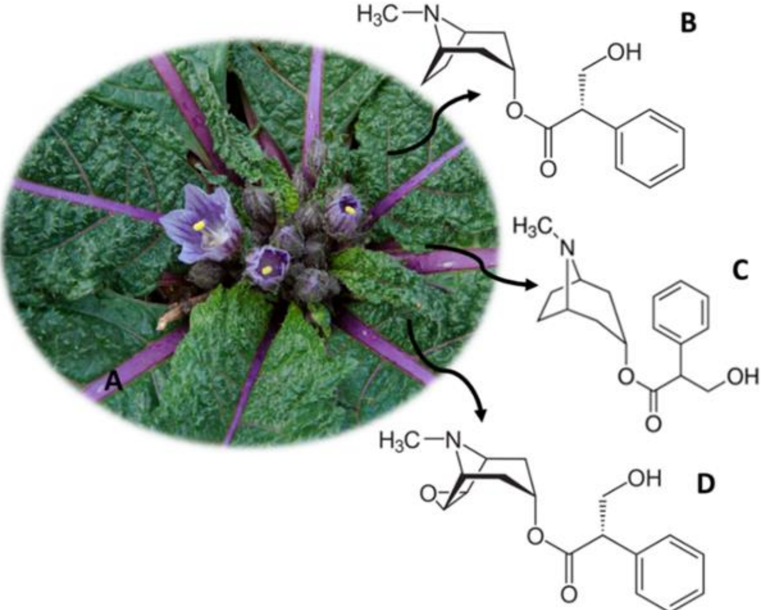


Figure I.1.6. *M. officinarum* **(A)** and the main compounds hyoscyamine **(B)**, atropine **(C)** and scopolamine **(D)**.

M. officinarum (Figure I.1.6A) is widely distributed worldwide, namely in Europe, North Africa, the Middle East and the Himalayas, however originating from the eastern Mediterranean [2]. This plant, also known as mandrake, has in the constitution of its seeds, roots, leaves and fruits,

hyoscyamine (Figure I.1.6B), atropine (Figure I.1.6C) and scopolamine (Figure I.1.6D), responsible for its healing, hallucinogenic and poisonous properties [83,84]. Since ancient times, mandrake was used as a surgical anesthetic in Rome and Greece [85]. It is also believed that this plant has aphrodisiac properties and its fruit increases fertility [85].

The consumption of mandrake can compromise the autonomic nervous system, resulting in an anticholinergic action and, consequently, reducing neuronal activity mediated by acetylcholine [86,87]. Thus, effects such as dry mouth, urinary retention, increased heart rate, mydriasis and decreased secretions are described [3,86,87]. In more extreme cases, its consumption can induce coma or even cause death [3]. Thus, the use of this plant is controlled both in the United States of America and in Europe, with the imposed measures being very restrictive [3].

Table I.1.4. Analytical methods for the determination of the main components of *Datura stramonium* and *Mandragora officinarum*.

Compounds	Sample (amount)	Sample preparation	Analytical technique	Limits of detection	Limits of quantitation	Recovery (%)	Reference
Hyoscyamine and scopolamine	Serum (0.5 mL) and Urine (0.5 mL)	SPE (Extrelut1)	GC-MS (EI)	5.0 µg/L	-	>80	[90]
Atropine, DMT, ephedrine, harmaline, harmine, ibogaine, LSA, psilocin, scopolamine and yohimbine	Urine (0.05 mL)	Dilution (distilled water)	LC–MS/MS (ESI)	2.0 – 10.0 µg/L	-	-	[91]
α-lobeline, α-solanine, aconitine, ajmaline, atropine, brucine, cephalomannine, colchicine, convallatoxin, cymarine, cytisine, digitoxin, digoxin, emetine, gelsemine, ibogaine, jervine, kavain, lanatoside C, lupanine, mitragynine, neriifolin, oleandrin, ouabain, paclitaxel, physostigmine, pilocarpine, podophyllotoxin, proscillaridin A, reserpine, retrorsine, ricinine, scopolamine, senecionine, sparteine, strophanthidin, strychnine, veratridine and yohimbine	Blood (1 mL)	SPE (HLB Oasis ®)	UHPLC-MS/MS (ESI)	0.1 - 1.6 µg/L	10 µg/L	33 - 106	[89]

Caption: EI (electron impact); ESI (electrospray ionization); GC (gas chromatography); LC (liquid chromatography); MS (mass spectrometry); MS/MS (tandem mass spectrometry); SPE (solid-phase extraction); UHPLC (ultrahigh-performance liquid chromatography).

There are currently analytical methodologies developed with different biological samples, which allow the quantification of atropine and scopolamine (Table I.1.4). Pietsch *et al.* [88] developed an analytical method with 1 mL of serum and urine to detect and quantify 13

compounds, namely scopolamine and atropine. The analytes were extracted using the SPE technique and quantified using HPLC-PDA and HPLC-UV equipment [88]. The method presented quantification limits of 0.3–94 ng/mL and recoveries between 23.7% and 86.9% [88]. In addition, Carlier *et al.* [89] quantified atropine and scopolamine, among other compounds, in a single analytical method. The SPE technique was used as a pre-treatment of the blood sample (1 mL), having subsequently been quantified in UHPLC-MS/MS equipment [89]. The method had a LOQ of 10 ng/mL and detection limits between 0.1 and 1.6 ng/mL [89].

1.1.8. *Lophophora williamsii* (Peyote)

L. williamsii (Figure I.1.7A), also known as Peyote, is a cactus from northern Mexico and the United States of America [92,93]. This plant was traditionally eaten in religious rituals, by indigenous peoples in the countries already mentioned [50]. Normally, the flesh of the fresh cactus is ingested, and it can also be dried and subsequently ingested or used to make teas [92,94]. *L. williamsii* contains a compound called mescaline [2-(3,4,5-trimethylphenyl) ethanamine] (Figure I.1.7B), a member of the phenylalkylamine class, responsible for the hallucinogenic properties of the plant [79,95]. This compound is also found for sale in the form of powder, which can be inflated or ingested orally [79]. Once consumed, mescaline accesses the central nervous system, acting at the level of serotonergic receptors 5-HT2 as an agonist of subtypes 5-HT2a, 5-HT2b and 5-HT2c [95].

The effects when consuming this plant include paranoia, compulsion, paresthesia, changes in color perception, headaches, mydriasis, spasms and psychomotor agitation [93,96,97]. Other effects at the cardiovascular, gastrointestinal and renal levels have been also described, namely hypertension and tachycardia, vomiting and decreased filtration rate at the glomerular level [93,98,99]. However, beneficial effects have been also described. One study demonstrated that *L. williamsii* extracts were effective in treating rheumatism, wounds, burns and snakebites [100]. Another study showed that this plant has antimicrobial properties against Staphylococcus aureus [100] Additionally, *L. williamsii* is used by some tribes to treat fever, labor pain, toothache, diabetes, blindness, breast pain and skin diseases [100].

Currently, substances containing mescaline are included in Annex I of the 1967 United Nations Convention on Drugs [100]. Given the effects of this plant, it is crucial to develop new analytical methodologies to detect mescaline and its metabolites in biological samples. Until now, methodologies have been developed using chromatography, namely in alternative samples such as hair [67]. Pichini *et al.* [67] developed an analytical method, using UHPLC-MS/MS equipment, to quantify several naturally occurring hallucinogens, including mescaline. For this purpose, 25 mg of hair was hydrolyzed with an M3 reagent. The method presented LOD values between 0.01 ng/mg and 0.02 ng/mg, LOQ between 0.03 ng/mg and 0.05 ng/mg and recoveries between 79.6% and 97.4% [67]. Another study by Beyer *et al.* [71] also allowed to quantify mescaline, using LC-MS/MS equipment with 1 mL of plasma, pre-treated with the SPE technique.

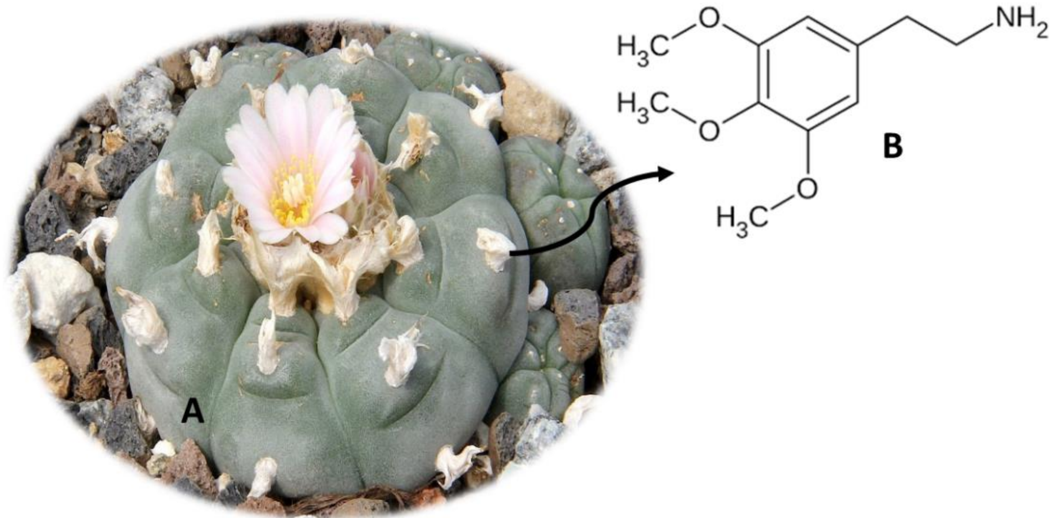


Figure I.1.7. *Lophophora williamsii* **(A)** and the main compound mescaline **(B)**.

1.1.9. *Mitragyna speciosa* (Kratom)

M. speciosa (Figure I.1.8A), also known as Kratom, appeared on the Asian continent, namely in countries like Biak, Malaysia and Thailand [2]. Currently, this plant is distributed in several regions of the world [3,12]. *M. speciosa* has been used for several years by rural workers and peasants in Asian people for reducing fatigue and increasing productivity at work, coughing, pain, fever, diarrhea, hypertension and diabetes. More recently, it began to be consumed in a recreational context in Europe and the United States of America [101–109]. Kratom leaves have been also used as a substitute for opium, as well as in morphine withdrawal treatments [12,50]. The preferred mode of consumption is chewing fresh leaves, but dried leaves can also be eaten or smoked [3,50]. Other forms of consumption of this plant include the preparation of teas and pastes by boiling the leaves for a long period [16]. Currently, there is greater ease in the consumption of this plant, since capsules, powders and drinks are available that can be easily purchased [3,110].

Kratom has psychoactive properties, which are due to the presence of about 40 alkaloids in the plant [3,50]. These compounds correspond to about 0.5% -1.5% of the compounds and their concentrations vary with the harvesting season, age and geographic lo-cation [3,111]. The most abundant psychoactive compound is mitragynine (Figure I.1.8B), corresponding to a total of 66.2% of the alkaloids content. However, the abundance of this compound in Malaysian plants was only 10% [12]. Other alkaloids with pharmacological activity were also detected, such as 7-hydroxmitraginine (Figure I.1.8C) and corinantheidine [110–114]. In addition, other alkaloids have been discovered that may contribute to pharmacological effects, namely corinantheidine, specioginine and paynantheine [3]. The alkaloids present in *M. speciosa* show high lipophilicity, crossing the blood-brain barrier, and a high affinity for opioid receptors [3]. Thus, 7-hydroxmitraginin binds to the supraspinous k-opioid and μ-opioid receptors, exerting their effects [3]. In addition to these, mitragynine binds to δ-opioid receptors, thereby exercising analgesic

effects [3,50]. Mitragynine is able to block Ca^{2+} channels, affecting the release of neurotransmitters [115,116]. Thus, antidepressant, antioxidant and anti-inflammatory properties have been associated with kratom consumption [117,118]. The use of this substance for substitution treatment in chronic opioid users has also been reported [3,50].

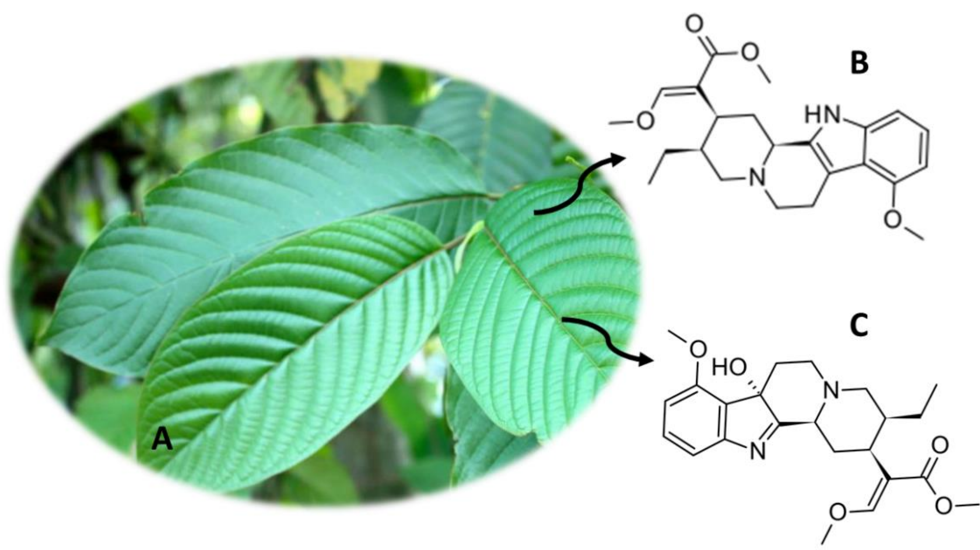


Figure I.1.8. *M. speciosa* **(A)** and the main compounds mitragynine **(B)** and 7-hydroxymitragynine **(C)**.

Table I.1.5. Analytical methods for the determination of the main components of *Mitragyna speciosa*.

Compounds	Sample (amount)	Sample preparation	Analytical technique	Limits of detection	Limits of quantitation	Recovery (%)	Reference
Mitragynine, 7-hydroxymitragynine, speciociliatine, speciogynine, and paynantheine	Urine (1 mL)	SPE (PolyChrom ClinII 3 cm^3 (35 mg))	LC-Q/TOF-MS	0.25-1 µg/L	0.5-1 µg/L	-	[122]
Mitragynine, 7-hydroxy-mitragynine, 5-desmethyl-mitragynine, 17-desmethyldihydromitragynine and mitraphylline	Urine (0.2 mL)	Hydrolysis (β-Glucuronidase) and LLE (methyl tert-butyl ether)	LC-MS/MS (ESI)	-	1 µg/L	-	[123]
Mitragynine, 16-carboxy mitragynine, and 9-*O*-demethyl mitragynine	Urine (1 mL)	Hydrolysis (β-glucuronidase/ arylsulfatase) and SPE (Bond Elut Certify (200 mg, 3 mL) and Abs Elut-Nexus SPE (60 mg, 3 mL))	LC-MS/MS (ESI)	-	1-50 µg/L	-	[121]
Mitragynine and 7-hydroxymitragynine	Urine (1 mL)	Dilution (water with 0.1% formic acid)	LC-MS/MS (ESI)	0.012- 0.069 µg/L	0.0356 – 0.215 µg/L	-	[124]

Caption: ESI (electrospray ionization); LC (liquid chromatography); LLE (liquid-liquid extraction); MS (mass spectrometry); MS/MS (tandem mass spectrometry); SPE (solid-phase extraction); Q/ToF (time of flight).

Adverse effects when consuming this plant include withdrawal and neonatal withdrawal syndrome, seizures, weight loss, dehydration, fatigue, insomnia, constipation and hyperpigmentation [50,103,105,119,120]. However, *M. speciosa* is not on the United Nations Drug Convention Schedule [3]. These compounds are controlled in the United States of America, New Zealand, Australia, Myanmar, Thailand, Malaysia and in some Euro-pean countries [3].

Thus, it is crucial to develop analytical methods to detect and quantify the compounds present in *M. speciosa* (Table I.1.5). Carlier *et al.* [89] developed an analytical method where they used 1 mL of blood, to detect mitragynine (among other compounds), using an UHPLC-MS/MS equipment. Lee *et al.* [121] developed an analytical method in LC-MS/MS, where they used SPE and enzymatic hydrolysis as a method of pre-treatment of the urine sample, to quantify 16-carboxy mitragynine, 9-*O*-demethyl mitragynine and mitragynine. More recently, Basiliere *et al.* [122] developed an analytical method using LC-Q/TOF-MS equipment for the quantification of mitragynine, 7-hydroxymitragynine, among other compounds. 1 mL of urine, pre-treated with SPE, was used, obtaining an LOD of 0.25-1 ng/mL and a LOQ of 0.5-1 ng/mL [122].

1.1.10. *Piper methysticum* (Kava)

Some parts of *P. methtysticum* (Figure I.1.9A) (roots and stems) are used in the manufacture of Kava, a psychotropic drink from the Pacific region [2]. Kava was consumed due to its therapeutic properties, namely in reducing fatigue and anxiety, relieving pain or inducing sleep [125]. Other treatments such as restlessness and anxiety were also associated with the consumption of Kava [3]. However, the use of this substance is associated with hepatotoxicity [125]. The effects of Kava are due to kavalactones, namely kavain (Figure I.1.9B), yangonin (Figure I.1.9C), desmethoxy-iangonin, 7,8-dihydrokavain, methysticin and 7,8- dihydromethysticin, to the derivatives of cinnamic acid, flavanones and chalcones [2]. These compounds act at the level of the central nervous system, inhibiting monoamine oxidase B, recapturing of noradrenaline and dopamine and interacting with γ-amino butyric acid [126].

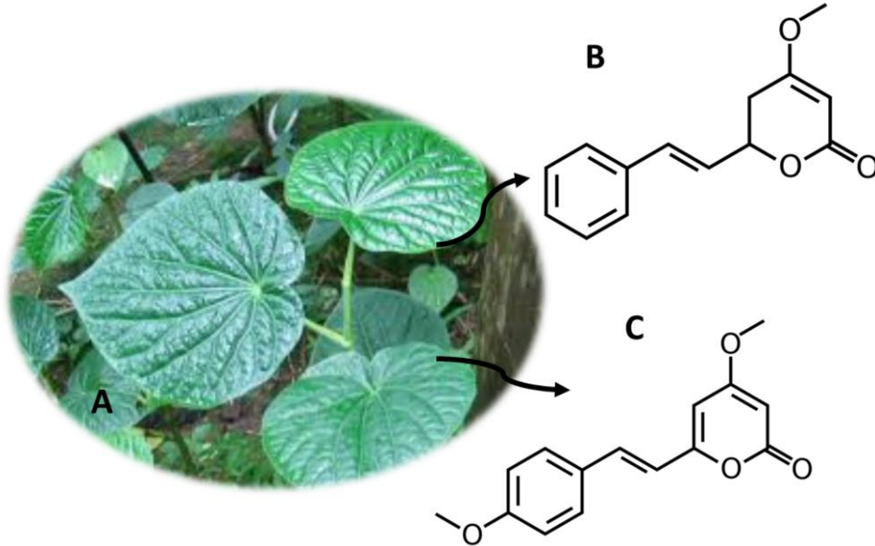


Figure I.1.9. *P. methysticum* **(A)** and the main compounds kavain **(B)** and yangonin **(C)**.

The sale of *P. methysticum* is controlled in Holland, Switzerland and France, and its sale and import are prohibited in the United Kingdom. In Poland, sales for human consumption are also prohibited. However, in most countries, this substance remains legal [3]. Thus, the development of analytical methods for the detection of these compounds is becoming increasingly important (Table I.1.6). Villain *et al.* [127] developed a method for the determination of kavain in GC-MS/MS, using between 29 and 50 mg of hair. The method had a LOD of 30 ng/g and a LOQ of 100 ng/g [127]. Another more recent study, carried out by Tarbah *et al.* [128], allowed to quantify 10 compounds, using between 21 and 253 mg of hair. The sample was initially decontaminated, then digested, using three different types of equipment for quantification: HPLC-DAD, LC-MS/MS and GC/TOF-MS [128].

Table I.1.6. Analytical methods for the determination of the main components of *Piper methysticum*.

Compounds	Sample (amount)	Sample preparation	Analytical technique	Limits of detection	Limits of quantitation	Recovery (%)	Reference
Kavain, dihydrokavain, methysticin, dihydromethysticin and desmethoxyyangonin	Urine (0.1 mL) and Plasma (0.1 mL)	PP (Methanol), LLE (ethyl acetate) and SPE (SOLA HRP cartridge)	UHPLC-MS/MS (HESI)	0.015 – 0.137 µg/L	0.0457 – 0.4165 µg/L	-	[129]
Kavain	Hair (29-50 mg)	Decontamination (methylene chloride) and digestion (methanol)	GC-MS/MS (EI)	0.030 µg/g	0.1 µg/g	-	[127]
Kavain, *p*-hydroxykavain, *p*-hydroxy-5,6-dehydrokavain and *p*-hydroxy-7,8-dihydrokavain	Blood (1 mL), Urine (1 mL) and Serum (1 mL)	LLE (dichlormethane: diethylether (7:3, v/v))	HPLC-DAD and LC-MS	1 µg/L	5 µg/L	91 - 97	[130]
Kavain, 7,8-dihydrokavain, yangonin, 5,6-dehydrokavain, 12-hydroxy-5,6-dehydrokavain, methysticin, 7,8-dihydromethysticin, 11-hydroxy-5,6-dehydrokavain, 12-hydroxykavain and 12-hydroxy-7,8-dihydrokavain	Hair (21 – 253 mg)	Decontamination (HPLC water, acetone and petroleum benzene) and digestion (methanol)	HPLC-DAD, LC-MS/MS (ESI) e GC/TOF-MS	-	-	-	[128]

Caption: DAD (diode array detector); EI (electron impact); ESI (electrospray ionization); GC (gas chromatography); HESI (heated electrospray); HPLC (high performance liquid chromatography); LC (liquid chromatography); LLE (liquid-liquid extraction); MS (mass spectrometry); MS/MS (tandem mass spectrometry); PP (protein precipitation); SPE (solid-phase extraction); ToF (time of flight); UHPLC (ultrahigh-performance liquid chromatography).

1.1.11. *Psilocybe* Genus (Magic Mushrooms)

Psilocybe (Figure I.1.10A) is a genus of hallucinogenic fungi, commonly known as magic mushrooms [131]. These species originate from certain regions of South America, but it is also possible to find them in Western Europe and in the United States of America [79,92]. The magic mushrooms were initially used in religious rituals, by the Aztec people in Mexico, persisting until today [50,79]. The active compounds present in these fungi are psilocybin (Figure I.1.10B) and psilocin (Figure I.1.10C), which consist of a substituted indolealkylamine [3,79]. There are about 190 species of mushrooms of the genus *Psilocybe*, which contain these two compounds responsible for the psychoactive effects of these fungi [131]. Mushrooms can be eaten after drying and making tea, but the most common route of consumption is by eating whole mushroom capsules [92]. After being consumed, psilocybin is converted into psilocin, and it acts as an agonist for the serotonergic receptors 5-HT1a and 5-HT2a, exerting its psychoactive effects [79]. In addition, these compounds can also increase the release of glutamate, which activates receptors such as N-methyl-d-aspartic acid receptors and α-amino-3hydroxy-5-methyl-4-isoxazolepropionic acid receptors [50,132]. The effects caused when consuming magic mushrooms can also be partially and indirectly mediated by dopamine [133].

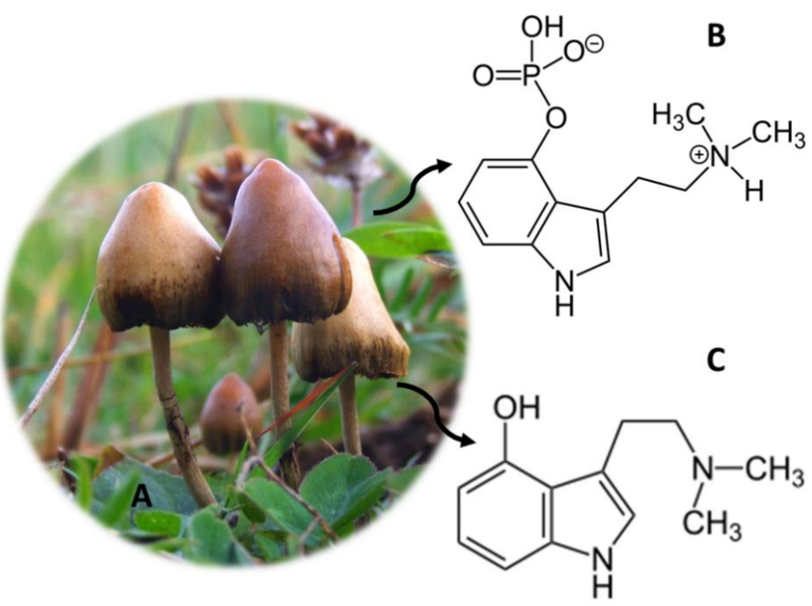


Figure I.1.10. *Psilocybe* mushrooms **(A)** and the main compounds psilocybin **(B)** and psilocin **(C)**.

The effects of *Psilocybe* consumption include changes in perception similar to drugs such as LSD, namely changes in visual and auditory perception [3]. Mystical experiences, tachycardia, headache, sweating, mydriasis, chills, nausea and increased body temperature are also associated with the consumption of magic mushrooms [134,135]. Other effects reported when consuming these substances are paranoia, dizziness and imbalance and abdominal pain, [79,135]. Moreover, it has been reported that psilocybin can be used to treat anxiety and resistant depression [136].

Psilocybe mushrooms are illegal all over the world [137]. However, in some countries the law is not consensual, namely in the Netherlands where the mushroom is illegal, but the Sclerotia truffle (philosopher's stone) is not [3]. Given the worldwide consumption of this substance, the development of new analytical methods that allow the determination of these compounds is crucial (Table I.1.7). Several samples were used to quantify the active compounds of *Psilocybe*, namely urine [91,138], serum [139] and hair [67]. Kamata *et al.* [139] developed a method for the quantification of psilocin glucuronide, where they used only 100 μL of serum. The sample was subjected to an enzymatic hydrolysis and deproteinization process, after which it was injected into LC-MS and LC-MS/MS equipment. The method showed 0.5 ng/mL LOD. The same authors had previously developed an analytical method for the quantification of psilocin glucuronide and psilocin, where they used the same volume of urine, the same equipment and the same LOD was obtained [138].

Table I.1.7. Analytical methods for the determination of the main components of the *Psilocybe* genus.

Compounds	Sample (amount)	Sample preparation	Analytical technique	Limits of detection	Limits of quantitation	Recovery (%)	Reference
Psilocin glucuronide and psilocin	Urine (0.1mL)	Enzymatic hydrolyses (β-glucuronidase), alkaline hydrolyses (potassium hydroxide) acid hydrolysis (concentrated hydrochloric acid) and deproteinization (methanol)	LC-MS (ESI) and LC-MS/MS (ESI)	0.5 μg/L	-	-	[138]
Psilocin glucuronide	Serum (0.1mL)	Enzymatic hydrolysis (β-glucuronidase) and deproteinization (methanol)	LC-MS (ESI) and LC-MS/MS (ESI)	0.5 μg/L	-	-	[139]
Mescaline, DMT, psilocin, psilocybin, salvinorin A	Hair (25 mg)	Hydrolysis (M3 reagent)	UHPLC-MS/MS (ESI)	0.01-0.02 μg/g	0.03-0.05 μg/g	79.6 - 97.4	[67]

Caption: ESI (electrospray ionization); LC (liquid chromatography); MS (mass spectrometry); MS/MS (tandem mass spectrometry); UHPLC (ultrahigh-performance liquid chromatography).

1.1.12. *Salvia divinorum* ("Hierba de Maria")

S. divinorum (Figure I.1.11A) originates from Oaxaca, a region in the northeast of the Sierra Mazateca, Mexico [140]. This psychoactive plant, also known as hierba de Maria, hojas de la Pastora, ska Maria, ska Pastora and magic mint, has been used for centuries by indigenous people because they believe it is the reincarnation of the Virgin Mary [12,141]. *S. divinorum* is consumed by chewing fresh or dried leaves. Dried leaves can be also smoked, and fresh leaves can be used to make tea [3]. The main psychoactive constituent of this plant is salvinorin A (Figure I.1.11B), but other compounds were also detected, such as salvinorins B (Figure I.1.11C), C, D, E and F, but these

do not have pharmacological activity [3,12]. Salvinorin A acts as a selective agonist for Kappa opioid receptors (KOR), thereby exerting its potent hallucinogenic effects [12,142]. A dose of between 200 μg and 500 μg is capable of inducing deep hallucinations with extraordinary illusions and a feeling of physical and mental displacement [143,144]. However, some studies conducted with *S. divinorum* and its bioactive compound salvinorin A have shown that it has some effects with therapeutic potential, such as drug addiction, pain treatment, neurological, gastrointestinal diseases and anti-inflammatory agent [145–156].

Despite its high potency, this substance is not included in any of the United Nations Drug Conventions' Schedules [3]. However, in Denmark, Latvia, Belgium, Lithuania, Sweden, Romania, Japan and Australia, these compounds are controlled. *S. divinorum* is considered an illegal drug in the United States of America, and its sale in Canada is also prohibited [3]. Other countries such as Germany, Poland, Croatia and Spain regulate its manufacture, and in Norway, Estonia and Finland this plant is legislated by the legislation of medicines [3]. Together with the consumption of *C. edulis* and *M. speciosa*, the consumption of *S. divinorum* is controlled by the United Nations Office on Drugs and Crime (UNODC) [3].

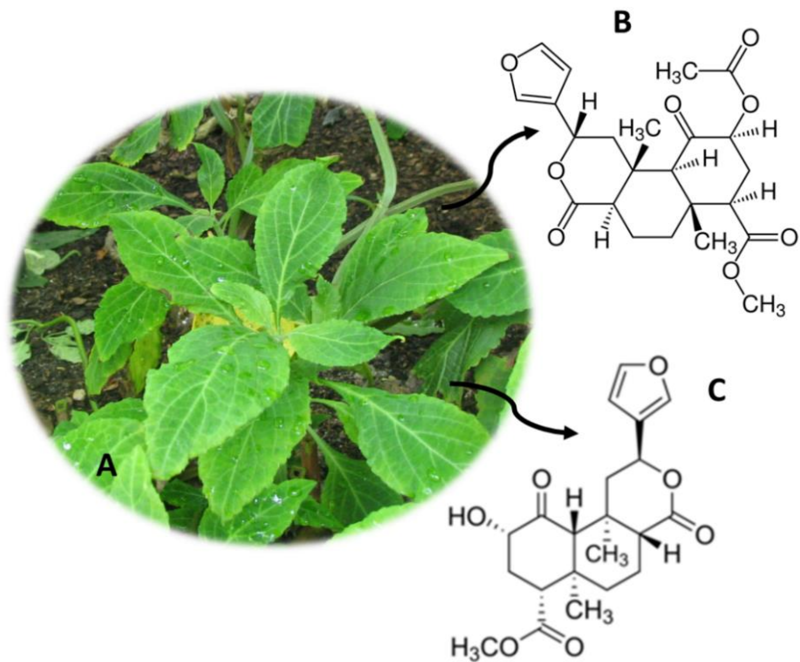


Figure I.1.11. *S. divinorum* **(A)** and the main compounds salvinorin A **(B)** and salvinorin B **(C)**.

The consumption of this substance has expanded worldwide and, therefore, several analytical methodologies for the detection of salvinorin A have emerged (Table I.1.8). Thus, several biological samples have been used for the development of these analytical methodologies, namely, urine [157–160], plasma [159,161], saliva [161], sweat [161], pericardial fluid [161], vitreous humor [161], blood [161] and hair [67]. Margalho *et al.* [161] quantified salvinorin A in pericardial fluid, vitreous humor, blood and plasma in the same analytical method. The quantity of the samples was reduced (100 μL-250 μL), being treated using the SPE technique [161]. Finally, the compounds were quantified using GC-MS equipment, and the method proved to be sensitive and selective,

presenting LOD and LOQ of 5 ng/mg [161]. Moreno *et al.* [160] quantified salvinorin A in urine samples (200 µL) using MEPS as the sample pre-treatment technique. The samples were analyzed by GC-MS/MS equipment, with good recoveries (71%–80%) and good LOD and LOQ (5 ng/mL and 20 ng/mL, respectively) [160].

Table I.1.8. Analytical methods for the determination of the main components of *Salvia divinorum*.

Compounds	Sample (amount)	Sample preparation	Analytical technique	Limits of detection	Limits of quantitation	Recovery (%)	Reference
Salvinorin A	Urine (20 mL)	LLE (chloroform) and SPME (85 µm polyacrylate fiber)	GC × GC – ToF-MS	4 - 200 µg/L	-	-	[157]
Salvinorin A	Urine (1 mL)	SPE (Waters Oasis ®HLB)	LC-MS (ESI)	5 µg/L	2.5 µg/L	-	[158]
Salvinorin A	Plasma (1 mL), Urine (1 mL), Saliva (1 mL) and Sweat (1 patch cut into little pieces)	LLE (chloroform/ isopropanol (9:1, v/v))	GC-MS (EI)	3 – 5 µg/L	10 – 15 µg/L	77,1 - 92,7	[159]
Salvinorin A	Pericardial fluid (0.25 mL), Vitreous humor (0.1 mL), Blood (0.25 mL) and Plasma (0.25 mL)	SPE (Waters Oasis®HLB)	GC-MS (EI)	5.0 µg/L	5.0 µg/L	-	[161]
Salvinorin A	Urine (0.2 mL)	MEPS (C_{18})	GC-MS/MS (EI)	5.0 µg/L	20 µg/L	71 - 80	[160]

Caption: EI (electron impact); ESI (electrospray ionization); GC (gas chromatography); GC × GC (two-dimensional gas chromatography); LC (liquid chromatography); LLE (liquid-liquid extraction); MEPS (microextraction by packed sorbent); MS (mass spectrometry); MS/MS (tandem mass spectrometry); SPE (solid-phase extraction); ToF (time of flight)

1.1.13. *Tabernanthe iboga* (Iboga)

T. iboga (FigureI.1.12A) is a shrub from Central and West Africa [94,162]. This plant has been consumed for centuries, in religious rituals of initiation into adulthood (Bwiti religion), in countries located in Central Africa and in the Congo basin [94,163]. The root barks of *T. iboga* contain psychoactive alkaloids, the majority of which are called ibogaine (Figure I.1.12B) [162]. This compound, which consists of a monoterpene-indole alkaloid, is consumed orally in the form of hydrochloride, extracts of alkaloids or by consumption of the dry root bark [162,164,165]. When consuming, users experience stimulating and aphrodisiac properties, trance, energization and increased alertness [94,163]. The consumption of this substance also causes hallucinations that, in contrast to common hallucinogens, are more intense and realistic when experienced with closed eyes [162]. Despite the structure of ibogaine is similar to other hallucinogens, this compound has a different mode of action [162]. So far, its mechanism of action is not fully known, but it is known that it is able to act as an agonist of σ2 receptors and an antagonist of nicotinic α3β4 acetylcholine receptors and as an antagonist at *N*-methyl-*D*-aspartatetype (NMDA) glutamate receptors [164,165].

Throughout history, the extract of *T. iboga* has been used for other purposes, namely for fatigue and depression [166]. Currently, ibogaine is used in opioid detoxification [94,162]. Thus, this compound is legal in most countries, however, in Switzerland, Belgium, Australia, Sweden, France, Denmark and the United States of America, it is illegal [162]. Currently, there are several methodologies for quantifying T. iboga compounds, namely using biological samples such as plasma [167], urine [88,91], blood [89] and serum [88]. Pietsch *et al.* [88] developed an analytical method where they determined, among other compounds, ibogaine. For this, they used HPLC-PDA and HPLC-UV equipment, using 1 mL of serum and 1 mL of urine, which were pre-treated with SPE [88]. Furthermore, Björnstad *et al.* [91] developed an analytical method where they quantified ibogaine and other compounds. This analytical method had a LOD between 2 ng/mL and 10 ng/mL and presented a very easy sample preparation technique [91]. Only 50 µL of urine were used, which were diluted and injected directly into LC-MS/MS equipment [91].

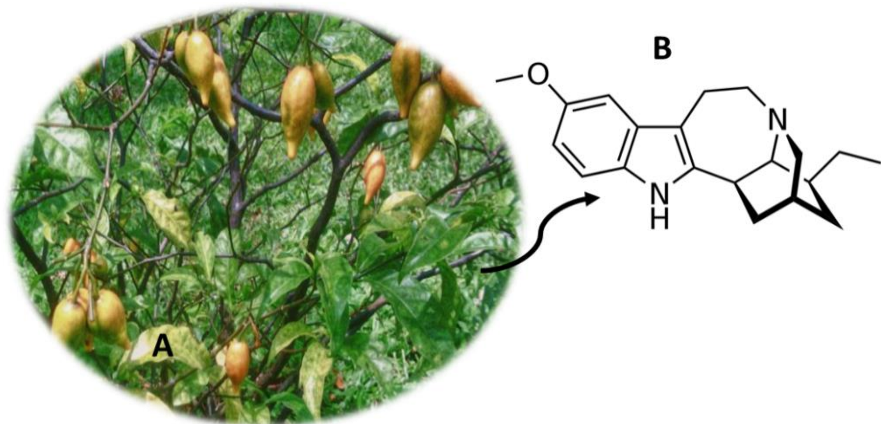


Figure I.1.12. *Tabernanthe iboga* **(A)** and the main compound ibogaine **(B)**.

1.1.14. Conclusions

Throughout this review, several plants/fungi that have psychoactive substances capable of inducing relaxing, stimulating or hallucinogenic effects were addressed. Toxicological aspects, some therapeutic properties and traditional uses were highlighted, as well as some of the analytical methods, developed in biological matrices, aimed at the detection of these substances.

Given the rapid emergence of these psychoactive substances in the abused drugs market, as well as the lack of legislation to control them, the development of new analytical methodologies is crucial. However, the lack of analytical standards to proceed with the development of chromatographic methods or the difficulty in finding plant species that allow scans of the compounds present in them constitute an enormous difficulty. Additionally, the fact that the compounds are usually present in very small amounts makes it even more difficult to develop and validate new methodologies, requiring the use of more sophisticated equipment, such as mass spectrometry detectors. Finally, the fact that the matrices of plant origin have several interferents

also constitutes a difficulty, since it is necessary to apply a pre-treatment step to the sample. This procedure makes the development of methods more expensive and requires the use of organic solvents. In the future, the use of miniaturized extraction techniques should be prioritized in order to achieve the development of more economical methods that aim to use lower volumes of organic solvents and, consequently, be more environment-friendly.

1.1.15 References

1. European Monitoring Centre for Drugs and Drug Addiction. EU Drug Markets Report 2019. **2019**, 1–260, doi:10.2810/766585.
2. Simão, A.Y.; Antunes, M.; Marques, H.; Rosado, T.; Soares, S.; Gonçalves, J.; Barroso, M.; Andraus, M.; Gallardo, E. Recent bionalytical methods for the determination of new psychoactive substances in biological specimens. *Bioanalysis* 2020, *12*, 1557–1595.
3. Lo Faro, A.F.; Di Trana, A.; La Maida, N.; Tagliabracci, A.; Giorgetti, R.; Busardò, F.P. Biomedical analysis of New Psychoactive Substances (NPS) of natural origin. *J. Pharm. Biomed. Anal.* 2020, *179*, 112945.
4. Guirguis, A. New psychoactive substances: a public health issue. *Int. J. Pharm. Pract.* 2017, *25*, 323–325.
5. Salomone, A.; Palamar, J.J.; Gerace, E.; Di Corcia, D.; Vincenti, M. Hair testing for drugs of abuse and new psychoactive substances in a high-risk population. *J. Anal. Toxicol.* 2017, *41*, 376–381.
6. Archer, J.R.H.; Mendes, F.; Hudson, S.; Layne, K.; Dargan, P.I.; Wood, D.M. Evaluation of long-term detection trends of new psychoactive substances in pooled urine from city street portable urinals (London, UK). *Br. J. Clin. Pharmacol.* 2020, *86*, 517–527.
7. Peacock, A.; Bruno, R.; Gisev, N.; Degenhardt, L.; Hall, W.; Sedefov, R.; White, J.; Thomas, K. V.; Farrell, M.; Griffiths, P. New psychoactive substances: challenges for drug surveillance, control, and public health responses. *Lancet* 2019, *394*, 1668–1684.
8. Meyer, M.R. New psychoactive substances: an overview on recent publications on their toxicodynamics and toxicokinetics. *Arch. Toxicol.* 2016, *90*, 2421–2444.
9. Evans-Brown, M.; Sedefov, R. New psychoactive substances: driving greater complexity into the drug problem. *Addiction* 2017, *112*, 36–38.
10. United Nations Office on Drugs and Crime. UNODC Early Warning Advisory (EWA) on New Psychoactive Substances (NPS) Available online: https://www.unodc.org/LSS/Home/NPS (accessed on Jan 28, 2021).
11. European Monitoring Centre for Drugs and Drug The EU Early Warning System on new psychoactive substances (NPS) Available online: https://www.emcdda.europa.eu/publications/topic-overviews/eu-early-warning-system_en (accessed on Jan 28, 2021).
12. Smith, J.P.; Sutcliffe, O.B.; Banks, C.E. An overview of recent developments in the analytical detection of new psychoactive substances (NPSs). *Analyst* **2015**, *140*, 4932–4948, doi:10.1039/c5an00797f.
13. Graziano, S.; Orsolini, L.; Rotolo, M.C.; Tittarelli, R.; Schifano, F.; Pichini, S. Herbal Highs: Review on Psychoactive Effects and Neuropharmacology. *Curr. Neuropharmacol.* 2016, *15*.
14. Souza, R.C.Z.; Zandonadi, F.S.; Freitas, D.P.; Tófoli, L.F.F.; Sussulini, A. Validation of an analytical method for the determination of the main ayahuasca active compounds and application to real ayahuasca samples from Brazil. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* 2019, *1124*, 197–203.
15. Peng, W.; Liu, Y.J.; Wu, N.; Sun, T.; He, X.Y.; Gao, Y.X.; Wu, C.J. *Areca catechu* L. (Arecaceae): A review of its traditional uses, botany, phytochemistry, pharmacology and toxicology. *J. Ethnopharmacol.* 2015, *164*, 340–356.
16. Gupta, P.C.; Warnakulasuriya, S. Global epidemiology of areca nut usage. *Addict. Biol.* 2002, *7*, 77–83.
17. Papke, R.L.; Horenstein, N.A.; Stokes, C. Nicotinic Activity of Arecoline, the Psychoactive Element of "Betel Nuts", Suggests a Basis for Habitual Use and Anti-Inflammatory Activity. *PLoS One* 2015, *10*, e0140907.
18. Coppola, M.; Mondola, R. Potential action of betel alkaloids on positive and negative symptoms of schizophrenia: A review. *Nord. J. Psychiatry* 2012, *66*, 73–78.
19. Taylor, R.F.H.; Al-Jarad, N.; John, L.M.E.; Barnes, N.C.; Conroy, D.M. Betel-nut chewing and asthma. *Lancet* 1992, *339*, 1134–1136.
20. Chen, Y.-C.; Lee, H.-C.; Lee, H.-H.; Su, H.-M.; Lin, T.-H.; Hsu, P.-C. Areca Nut Chewing Complicated with Non-Obstructive and Obstructive ST Elevation Myocardial Infarction 2017.
21. Salehi, B.; Konovalov, D.A.; Fru, P.; Kapewangolo, P.; Peron, G.; Ksenija, M.S.; Cardoso, S.M.; Pereira, O.R.; Nigam, M.; Nicola, S.; et al. Areca catechu—From farm to food and biomedical applications. *Phyther. Res.* 2020, *34*, 2140–2158.
22. Keshava Bhat, S.; Sarpangala, M.; Ashwin, D. Antilipidemic activity of arecanut, *Areca catechu* L.: A valuable herbal medicine. *Int. J. Herb. Med.* **2017**, *5*, 35–38.
23. Sreeramulu, D.; Reddy, C.V.K.; Chauhan, A.; Balakrishna, N.; Raghunath, M. Natural antioxidant activity of commonly consumed plant foods in India: Effect of domestic processing. *Oxid. Med. Cell. Longev.* **2013**, 1–12, doi:10.1155/2013/369479.
24. Wu, M.; Fang, M.; Hu, Y.; Wang, X. Four types of traditional Chinese medicine inducing epileptic seizures. *Seizure* **2012**, *21*, 311–315, doi:10.1016/j.seizure.2012.02.010.
25. Hamsar, M.N.; Ismail, S.; Mordi, M.N.; Ramanathan, S.; Mansor, S.M. Antioxidant activity and the effect of different parts of areca catechu extracts on Glutathione-S-Transferase activity in vitro. *Free Radicals Antioxidants*

2011, *1*, 28–33, doi:10.5530/ax.2011.1.6.
26. Wu, I.C.; Chen, P.H.; Wang, C.J.; Wu, D.C.; Tsai, S.M.; Chao, M.R.; Chen, B.H.; Lee, H.H.; Lee, C.H.; Ko, Y.C. Quantification of blood betel quid alkaloids and urinary 8-hydroxydeoxyguanosine in humans and their association with betel chewing habits. *J. Anal. Toxicol.* **2010**, *34*, 325–331, doi:10.1093/jat/34.6.325.
27. Pichini, S.; Pellegrini, M.; Pacifici, R.; Marcheil, E.; Murillo, J.; Puig, C.; Vall, O.; García-Algar, O. Quantification of arecoline (Areca nut alkaloid) in neonatal biological matrices by high-performance liquid chromatography/electrospray quadrupole mass spectrometry. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 1958–1964, doi:10.1002/rcm.1140.
28. Krais, S.; Klima, M.; Huppertz, L.M.; Auwärter, V.; Altenburger, M.J.; Neukamm, M.A. Betel Nut Chewing in Iron Age Vietnam? Detection of *Areca catechu* Alkaloids in Dental Enamel. *J. Psychoactive Drugs* **2017**, *49*, 11–17, doi:10.1080/02791072.2016.1264647.
29. Franke, A.A.; Li, X.; Custer, L.J.; Lai, J.F. Chemical Markers for Short- and Long-Term Areca Nut Exposure. *Subst. Use Misuse* **2020**, *55*, 1395–1402, doi:10.1080/10826084.2019.1630442.
30. Lee, H.H.; Chen, L.Y.; Wang, H.L.; Chen, B.H. Quantification of salivary arecoline, arecaidine and N-methylnipecotic acid levels in volunteers by liquid chromatography-tandem mass spectrometry. *J. Anal. Toxicol.* **2015**, *39*, 714–719, doi:10.1093/jat/bkv077.
31. Pellegrini, M.; Marchei, E.; Rossi, S.; Vagnarelli, F.; Durgbanshi, A.; García-Algar, Ó.; Vall, O.; Pichini, S. Liquid chromatography/electrospray ionization tandem mass spectrometry assay for determination of nicotine and metabolites, caffeine and arecoline in breast milk. *Rapid Commun. Mass Spectrom.* **2007**, *21*, 2693–2703, doi:10.1002/rcm.3137.
32. Marchei, E.; Durgbanshi, A.; Rossi, S.; Garcia-Algar, Ó.; Zuccaro, P.; Pichini, S. Determination of arecoline (areca nut alkaloid) and nicotine in hair by high-performance liquid chromatography/electrospray quadrupole mass spectrometry. *Rapid Commun. Mass Spectrom.* **2005**, *19*, 3416–3418, doi:10.1002/rcm.2183.
33. Austin, D.; Huáman, Z. A synopsis of Ipomoea (Convolvulaceae) in the Americas - Austin - 1996 - TAXON - Wiley Online Library. *Wiley Online Libr.* 1996.
34. Paulke, A.; Kremer, C.; Wunder, C.; Wurglics, M.; Schubert-Zsilavecz, M.; Toennes, S.W. Studies on the alkaloid composition of the Hawaiian Baby Woodrose Argyreia nervosa, a common legal high. *Forensic Sci. Int.* **2015**, *249*, 281–293, doi:10.1016/j.forsciint.2015.02.011.
35. Chao, J. -M; Der Marderosian, A.H. Ergoline alkaloidal constituents of hawaiian baby wood rose, *argyreia nervosa* (Burm. f.) bojer. *J. Pharm. Sci.* **1973**, *62*, 588–591, doi:10.1002/jps.2600620409.
36. Larson, B.T.; Harmon, D.L.; Piper, E.L.; Griffis, L.M.; Bush, L.P. Alkaloid binding and activation of D2 dopamine receptors in cell culture. *J. Anim. Sci.* **1999**, *77*, 942, doi:10.2527/1999.774942x.
37. Modi, A.J.; Khadabadi, S.S.; Deokate, U.A.; Farooqui, I.A.; Deore, S.L.; Gangwani, M.R. *Argyreia speciosa* Linn. f. : Phytochemistry, pharmacognosy and pharmacological studies. *J. Pharmacogn. Phyther.* **2010**, *2*, 34–42.
38. Weber, J.M.; Ma, T.S. Microchemical investigations of medicinal plants. XIV - Identification of the alkaloids in the leaves of Ipomoea violacea using preparative thin layer chromatography and solid probe mass spectrometry. *Mikrochim. Acta* **1976**, *65*, 227–242, doi:10.1007/BF01217830.
39. Paulke, A.; Kremer, C.; Wunder, C.; Toennes, S.W. Analysis of lysergic acid amide in human serum and urine after ingestion of *Argyreia nervosa* seeds. In Proceedings of the Analytical and Bioanalytical Chemistry; Springer, 2012; Vol. 404, pp. 531–538.
40. Simão; Gonçalves; Duarte; Barroso; Cristóvão; Gallardo Toxicological Aspects and Determination of the Main Components of Ayahuasca: A Critical Review. *Medicines* **2019**, *6*, 106, doi:10.3390/medicines6040106.
41. Andrade, T.S.; de Oliveira, R.; da Silva, M.L.; Von Zuben, M.V.; Grisolia, C.K.; Domingues, I.; Caldas, E.D.; Pic-Taylor, A. Exposure to ayahuasca induces developmental and behavioral alterations on early life stages of zebrafish. *Chem. Biol. Interact.* **2018**, *293*, 133–140, doi:10.1016/j.cbi.2018.08.001.
42. Gable, R.S. Risk assessment of ritual use of oral dimethyltryptamine (DMT) and harmala alkaloids. *Addiction* **2007**, *102*, 24–34, doi:10.1111/j.1360-0443.2006.01652.x.
43. Ramachandran, P.; Zhang, N.; McLaughlin, W.B.; Luo, Y.; Handy, S.; Duke, J.A.; Vasquez, R.; Ottesen, A. Sequencing the vine of the soul: Full chloroplast genome sequence of Banisteriopsis caapi. *Genome Announc.* **2018**, *6*, doi:10.1128/genomeA.00203-18.
44. Dos Santos, R.G.; Balthazar, F.M.; Bouso, J.C.; Hallak, J.E.C. The current state of research on ayahuasca: A systematic review of human studies assessing psychiatric symptoms, neuropsychological functioning, and neuroimaging. *J. Psychopharmacol.* **2016**, *30*, 1230–1247, doi:10.1177/0269881116652578.
45. dos Santos, R.G.; Osório, F.L.; Crippa, J.A.S.; Hallak, J.E.C. Antidepressive and anxiolytic effects of ayahuasca: A systematic literature review of animal and human studies. *Rev. Bras. Psiquiatr.* 2016, *38*, 65–72.
46. Riba, J.; Valle, M.; Urbano, G.; Yritia, M.; Morte, A.; Barbanoj, M.J. Human pharmacology of ayahuasca: Subjective and cardiovascular effects, monoamine metabolite excretion, and pharmacokinetics. *J. Pharmacol. Exp. Ther.* **2003**, *306*, 73–83, doi:10.1124/jpet.103.049882.
47. de Araujo, D.B.; Ribeiro, S.; Cecchi, G.A.; Carvalho, F.M.; Sanchez, T.A.; Pinto, J.P.; de Martinis, B.S.; Crippa, J.A.; Hallak, J.E.C.; Santos, A.C. Seeing with the eyes shut: Neural basis of enhanced imagery following ayahuasca ingestion. *Hum. Brain Mapp.* **2012**, *33*, 2550–2560, doi:10.1002/hbm.21381.
48. Riba, J.; Romero, S.; Grasa, E.; Mena, E.; Carrió, I.; Barbanoj, M.J. Increased frontal and paralimbic activation following ayahuasca, the pan-amazonian inebriant. *Psychopharmacology (Berl).* **2006**, *186*, 93–98, doi:10.1007/s00213-006-0358-7.
49. Morales-García, J.A.; De La Fuente Revenga, M.; Alonso-Gil, S.; Rodríguez-Franco, M.I.; Feilding, A.; Perez-Castillo, A.; Riba, J. The alkaloids of *Banisteriopsis caapi*, the plant source of the Amazonian hallucinogen Ayahuasca, stimulate adult neurogenesis *in vitro*. *Sci. Rep.* **2017**, *7*, 1–13, doi:10.1038/s41598-017-05407-9.

50. Hassan, Z.; Bosch, O.G.; Singh, D.; Narayanan, S.; Kasinather, B.V.; Seifritz, E.; Kornhuber, J.; Quednow, B.B.; Müller, C.P. Novel psychoactive substances-recent progress on neuropharmacological mechanisms of action for selected drugs. *Front. Psychiatry* 2017, *8*, 152.
51. Callaway, J.C.; Raymon, L.P.; Hearn, W.L.; McKenna, D.J.; Grob, C.S.; Brito, G.S.; Mash, D.C. Quantitation of *N,N*-dimethyltryptamine and harmala alkaloids in human plasma after oral dosing with ayahuasca. *J. Anal. Toxicol.* **1996**, *20*, 492–497, doi:10.1093/jat/20.6.492.
52. Gonçalves, J.; Luís, Â.; Gradillas, A.; García, A.; Restolho, J.; Fernández, N.; Domingues, F.; Gallardo, E.; Duarte, A.P. Ayahuasca Beverages: Phytochemical Analysis and Biological Properties. *Antibiotics* **2020**, *9*, 731, doi:10.3390/antibiotics9110731.
53. Simão, A.Y.; Gonçalves, J.; Gradillas, A.; García, A.; Restolho, J.; Fernández, N.; Rodilla, J.M.; Barroso, M.; Duarte, A.P.; Cristóvão, A.C.; et al. Evaluation of the Cytotoxicity of Ayahuasca Beverages. *Molecules* **2020**, *25*, 5594, doi:10.3390/molecules25235594.
54. de Osório, F.L.; Sanches, R.F.; Macedo, L.R.; dos Santos, R.G.; Maia-De-Oliveira, J.P.; Wichert-Ana, L.; de Araujo, D.B.; Riba, J.; Crippa, J.A.; Hallak, J.E. Antidepressant effects of a single dose of ayahuasca in patients with recurrent depression: A preliminary report. *Rev. Bras. Psiquiatr.* **2015**, *37*, 13–20, doi:10.1590/1516-4446-2014-1496.
55. Sanches, R.F.; De Lima Osório, F.; Santos, R.G.D.; Macedo, L.R.H.; Maia-De-Oliveira, J.P.; Wichert-Ana, L.; De Araujo, D.B.; Riba, J.; Crippa, J.A.S.; Hallak, J.E.C. Antidepressant effects of a single dose of ayahuasca in patients with recurrent depression a SPECT study. *J. Clin. Psychopharmacol.* **2016**, *36*, 77–81, doi:10.1097/JCP.0000000000000436.
56. Santos, R.G.; Landeira-Fernandez, J.; Strassman, R.J.; Motta, V.; Cruz, A.P.M. Effects of ayahuasca on psychometric measures of anxiety, panic-like and hopelessness in Santo Daime members. *J. Ethnopharmacol.* **2007**, *112*, 507–513, doi:10.1016/j.jep.2007.04.012.
57. Grob, C.S.; McKenna, D.J.; Callaway, J.C.; Brito, G.S.; Neves, E.S.; Oberlaender, G.; Saide, O.L.; Labigalini, E.; Tacla, C.; Miranda, C.T.; et al. Human psychopharmacology of hoasca, a plant hallucinogen used in ritual context in Brazil. *J. Nerv. Ment. Dis.* **1996**, *184*, 86–94, doi:10.1097/00005053-199602000-00004.
58. Thomas, G.; Lucas, P.; Capler, N.R.; Tupper, K.W.; Martin, G. Ayahuasca-assisted therapy for addiction: Results from a preliminary observational study in Canada. *Curr. Drug Abuse Rev.* **2013**, *6*, 30–42, doi:10.2174/15733998113099990003.
59. Oliveira-Lima, A.J.; Santos, R.; Hollais, A.W.; Gerardi-Junior, C.A.; Baldaia, M.A.; Wuo-Silva, R.; Yokoyama, T.S.; Costa, J.L.; Malpezzi-Marinho, E.L.A.; Ribeiro-Barbosa, P.C.; et al. Effects of ayahuasca on the development of ethanol-induced behavioral sensitization and on a post-sensitization treatment in mice. *Physiol. Behav.* **2015**, *142*, 28–36, doi:10.1016/j.physbeh.2015.01.032.
60. Da Silveira, D.X.; Grob, C.S.; de Rios, M.D.; Lopez, E.; Alonso, L.K.; Tacla, C.; Doering-Silveira, E. Ayahuasca in adolescence: A preliminary psychiatric assessment. *J. Psychoactive Drugs* **2005**, *37*, 129–133, doi:10.1080/02791072.2005.10399792.
61. Barbosa, P.C.R.; Cazorla, I.M.; Giglio, J.S.; Strassman, R.S. A six-month prospective evaluation of personality traits, psychiatric symptoms and quality of life in Ayahuasca-Naïve Subjects. *J. Psychoactive Drugs* **2009**, *41*, 205–212, doi:10.1080/02791072.2009.10400530.
62. McIlhenny, E.H.; Riba, J.; Barbanoj, M.J.; Strassman, R.; Barker, S.A. Methodology for determining major constituents of ayahuasca and their metabolites in blood. *Biomed. Chromatogr.* **2012**, *26*, 301–313, doi:10.1002/bmc.1657.
63. Yritia, M.; Riba, J.; Ortuño, J.; Ramirez, A.; Castillo, A.; Alfaro, Y.; de la Torre, R.; Barbanoj, M.J. Determination of *N, N*-dimethyltryptamine and β-carboline alkaloids in human plasma following oral administration of Ayahuasca. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2002**, *779*, 271–281, doi:10.1016/S1570-0232(02)00397-5.
64. Oliveira, C.D.R.; Okai, G.G.; Da Costa, J.L.; De Almeida, R.M.; Oliveira-Silva, D.; Yonamine, M. Determination of dimethyltryptamine and β-carbolines (ayahuasca alkaloids) in plasma samples by LC-MS/MS. *Bioanalysis* **2012**, *4*, 1731–1738, doi:10.4155/bio.12.124.
65. Mcilhenny, E.H.; Riba, J.; Barbanoj, M.J.; Strassman, R.; Barker, S.A. Methodology for and the determination of the major constituents and metabolites of the amazonian botanical medicine ayahuasca in human urine. *Biomed. Chromatogr.* **2011**, *25*, 970–984, doi:10.1002/bmc.1551.
66. Riba, J.; Mcilhenny, E.H.; Bouso, J.C.; Barker, S.A. Metabolism and urinary disposition of *N,N*-dimethyltryptamine after oral and smoked administration: A comparative study. *Drug Test. Anal.* **2015**, *7*, 401–406, doi:10.1002/dta.1685.
67. Pichini, S.; Marchei, E.; García-Algar, O.; Gomez, A.; Di Giovannandrea, R.; Pacifici, R. Ultra-high-pressure liquid chromatography tandem mass spectrometry determination of hallucinogenic drugs in hair of psychedelic plants and mushrooms consumers. *J. Pharm. Biomed. Anal.* **2014**, *100*, 284–289, doi:10.1016/j.jpba.2014.08.006.
68. Feyissa, A.M.; Kelly, J.P. A review of the neuropharmacological properties of khat. *Prog. Neuro-Psychopharmacology Biol. Psychiatry* 2008, *32*, 1147–1166.
69. Brenneisen, R.; Geisshüsler, S.; Schorno, X. Metabolism of cathinone to (−)-norephedrine and (−)-norpseudoephedrine. *J. Pharm. Pharmacol.* **1986**, *38*, 298–300, doi:10.1111/j.2042-7158.1986.tb04571.x.
70. Sørensen, L.K. Determination of cathinones and related ephedrines in forensic whole-blood samples by liquid-chromatography-electrospray tandem mass spectrometry. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2011**, *879*, 727–736, doi:10.1016/j.jchromb.2011.02.010.
71. Beyer, J.; Peters, F.T.; Kraemer, T.; Maurer, H.H. Detection and validated quantification of nine herbal phenalkylamines and methcathinone in human blood plasma by LC-MS/MS with electrospray ionization. *J. Mass Spectrom.* **2007**, *42*, 150–160, doi:10.1002/jms.1132.

72. Toennes, S.W.; Kauert, G.F. Excretion and Detection of Cathinone, Cathine, and Phenylpropanolamine in Urine after Kath Chewing. *Clin. Chem.* **2002**, *48*, 1715–1719, doi:10.1093/clinchem/48.10.1715.
73. Mohamed, K.M.; Al-Hazmi, A.H.; Alasiri, A.M.; Ali, M.E.S. A GC-MS Method for Detection and Quantification of Cathine, Cathinone, Methcathinone and Ephedrine in Oral Fluid. *J. Chromatogr. Sci.* **2016**, *54*, 1271–1276, doi:10.1093/chromsci/bmw082.
74. Margalho, C.; Castanheira, A.; Real, F.C.; Gallardo, E.; López-Rivadulla, M. Determination of "new psychoactive substances" in postmortem matrices using microwave derivatization and gas chromatography-mass spectrometry. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2016**, *1020*, 14–23, doi:10.1016/j.jchromb.2016.03.001.
75. Margalho,C.; Almeida, P.; Rosado, T.; Corte-Real, F.; Gallardo, E. Determination of New Psychoactive Substances in Whole Blood Using Microwave Fast Derivatization and Gas Chromatography/Mass Spectrometry. *J. Anal. Toxicol.* **2020**, *44*, 92–102, doi:10.1093/jat/bkz053.
76. Mahler, D.A. Anticholinergic poisoning from Jimson weed. *J. Am. Coll. Emerg. Physicians* 1976, *5*, 440–442.
77. Oerther, S.; Behrman, A.D.; Ketcham, S. Herbal hallucinations: Common abuse situations seen in the emergency department. *J. Emerg. Nurs.* **2010**, *36*, 594–596, doi:10.1016/j.jen.2010.07.018.
78. Forrester, M.B. Jimsonweed (*Datura stramonium*) exposures in Texas, 1998-2004. *J. Toxicol. Environ. Heal. - Part A Curr. Issues* **2006**, *69*, 1757–1762, doi:10.1080/15287390600631284.
79. Sutter, M.E.; Chenoweth, J.; Albertson, T.E. Alternative drugs of abuse. *Clin. Rev. Allergy Immunol.* 2014, *46*, 3–18.
80. Krenzelok, E.P. Aspects of datura poisoning and treatment. *Clin. Toxicol.* 2010, *48*, 104–110.
81. Gowdy, J.M. Stramonium intoxication: review of symptomatology in 212 cases. *JAMA J. Am. Med. Assoc.* **1972**, *221*, 585–587, doi:10.1001/jama.221.6.585.
82. Levy, R. Jimson seed poisoning - A new hallucinogen on the horizon. *J. Am. Coll. Emerg. Physicians* **1977**, *6*, 58–61, doi:10.1016/s0361-1124(77)80035-x.
83. Hanuš, L.O.; Řezanka, T.; Spížek, J.; Dembitsky, V.M. Substances isolated from Mandragora species. *Phytochemistry* 2005, *66*, 2408–2417.
84. Riley, S.C.E.; Blackman, G. Between prohibitions: Patterns and meanings of magic mushroom use in the UK. *Subst. Use Misuse* **2008**, *43*, 55–71, doi:10.1080/10826080701772363.
85. Suleiman, R.K.; Zarga, M.A.; Sabri, S.S. New withanolides from *mandragora officinarum*: First report of withanolides from the Genus Mandragora. *Fitoterapia* **2010**, *81*, 864–868, doi:10.1016/j.fitote.2010.05.013.
86. Frasca, T. Mandrake Toxicity. *Arch. Intern. Med.* **1997**, *157*, 2007, doi:10.1001/archinte.1997.00440380117012.
87. Vidal, F.; Templado, C.; Navarro, J.; Marina, S.; Egozcue, J. Meiotic and synaptonemal complex studies in a 14/21 translocation carrier. *Int. J. Androl.* **1982**, *5*, 21–26, doi:10.1111/j.1365-2605.1982.tb00229.x.
88. Pietsch, J.; Günther, J.; Henle, T.; Dreßler, J. Simultaneous determination of thirteen plant alkaloids in a human specimen by SPE and HPLC. *J. Sep. Sci.* **2008**, *31*, 2410–2416, doi:10.1002/jssc.200800128.
89. Carlier, J.; Guitton, J.; Romeuf, L.; Bévalot, F.; Boyer, B.; Fanton, L.; Gaillard, Y. Screening approach by ultra-high performance liquid chromatography-tandem mass spectrometry for the blood quantification of thirty-four toxic principles of plant origin. Application to forensic toxicology. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2015**, *975*, 65–76, doi:10.1016/j.jchromb.2014.10.028.
90. Namera, A.; Yashiki, M.; Hirose, Y.; Yamaji, S.; Tani, T.; Kojima, T. Quantitative analysis of tropane alkaloids in biological materials by gas chromatography-mass spectrometry. *Forensic Sci. Int.* **2002**, *130*, 34–43, doi:10.1016/S0379-0738(02)00302-X.
91. Björnstad, K.; Beck, O.; Helander, A. A multi-component LC-MS/MS method for detection of ten plant-derived psychoactive substances in urine. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2009**, *877*, 1162–1168, doi:10.1016/j.jchromb.2009.03.004.
92. Halpern, J.H.; Sewell, R.A. Hallucinogenic botanicals of America: A growing need for focused drug education and research. In Proceedings of the Life Sciences; Life Sci, 2005; Vol. 78, pp. 519–526.
93. Carstairs, S.D.; Cantrell, F.L. Peyote and mescaline exposures: A 12-year review of a statewide poison center database. *Clin. Toxicol.* **2010**, *48*, 350–353, doi:10.3109/15563650903586745.
94. Forsyth, B.; Machado, L.; Jowett, T.; Jakobi, H.; Garbe, K.; Winter, H.; Glue, P. Effects of low dose ibogaine on subjective mood state and psychological performance. *J. Ethnopharmacol.* **2016**, *189*, 10–13, doi:10.1016/j.jep.2016.05.022.
95. Halberstadt, A.L.; Geyer, M.A. Multiple receptors contribute to the behavioral effects of indoleamine hallucinogens. *Neuropharmacology* 2011, *61*, 364–381.
96. Hollister, L.E.; Hartman, A.M. Mescaline, lysergic acid diethylamide and psilocybin: Comparison of clinical syndromes, effects on color perception and biochemical measures. *Compr. Psychiatry* **1962**, *3*, 235–241, doi:10.1016/S0010-440X(62)80024-8.
97. Johnson, M.W.; Andrew Sewell, R.; Griffiths, R.R. Psilocybin dose-dependently causes delayed, transient headaches in healthy volunteers. *Drug Alcohol Depend.* **2012**, *123*, 132–140, doi:10.1016/j.drugalcdep.2011.10.029.
98. Hermle, L.; Fünfgeld, M.; Oepen, G.; Botsch, H.; Borchardt, D.; Gouzoulis, E.; Fehrenbach, R.A.; Spitzer, M. Mescaline-induced psychopathological, neuropsychological, and neurometabolic effects in normal subjects: Experimental psychosis as a tool for psychiatric research. *Biol. Psychiatry* **1992**, *32*, 976–991, doi:10.1016/0006-3223(92)90059-9.
99. Hollister, L.E.; Sjoberg, B.M. Clinical syndromes and biochemical alterations following mescaline, lysergic acid diethylamide, psilocybin and a combination of the three psychotomimetic drugs. *Compr. Psychiatry* **1964**, *5*, 170–178, doi:10.1016/S0010-440X(64)80030-4.
100. Dinis-Oliveira, R.J.; Pereira, C.L.; da Silva, D.D. Pharmacokinetic and Pharmacodynamic Aspects of Peyote and

Mescaline: Clinical and Forensic Repercussions. *Curr. Mol. Pharmacol.* **2018**, *12*, 184–194, doi:10.2174/1874467211666181010154139.
101. Singh, D.; Müller, C.P.; Vicknasingam, B.K.; Mansor, S.M. Social Functioning of Kratom *(Mitragyna speciosa)* Users in Malaysia. *J. Psychoactive Drugs* **2015**, *47*, 125–131, doi:10.1080/02791072.2015.1012610.
102. Singh, D.; Narayanan, S.; Vicknasingam, B. Traditional and non-traditional uses of Mitragynine (Kratom): A survey of the literature. *Brain Res. Bull.* 2016, *126*, 41–46.
103. Brown, P.N.; Lund, J.A.; Murch, S.J. A botanical, phytochemical and ethnomedicinal review of the genus Mitragyna korth: Implications for products sold as kratom. *J. Ethnopharmacol.* 2017, *202*, 302–325.
104. Vicknasingam, B.; Narayanan, S.; Beng, G.T.; Mansor, S.M. The informal use of ketum (*Mitragyna speciosa)* for opioid withdrawal in the northern states of peninsular Malaysia and implications for drug substitution therapy. *Int. J. Drug Policy* **2010**, *21*, 283–288, doi:10.1016/j.drugpo.2009.12.003.
105. Boyer, E.W.; Babu, K.M.; Adkins, J.E.; McCurdy, C.R.; Halpern, J.H. Self-treatment of opioid withdrawal using kratom (*Mitragynia speciosa* korth). *Addiction* **2008**, *103*, 1048–1050, doi:10.1111/j.1360-0443.2008.02209.x.
106. Cinosi, E.; Martinotti, G.; Simonato, P.; Singh, D.; Demetrovics, Z.; Roman-Urrestarazu, A.; Bersani, F.S.; Vicknasingam, B.; Piazzon, G.; Li, J.H.; et al. Following "the Roots" of Kratom (Mitragyna speciosa): The Evolution of an Enhancer from a Traditional Use to Increase Work and Productivity in Southeast Asia to a Recreational Psychoactive Drug in Western Countries. *Biomed Res. Int.* **2015**, *2015*, doi:10.1155/2015/968786.
107. Warner, M.L.; Kaufman, N.C.; Grundmann, O. The pharmacology and toxicology of kratom: from traditional herb to drug of abuse. *Int. J. Legal Med.* 2016, *130*, 127–138.
108. Suhaimi, F.W.; Yusoff, N.H.M.; Hassan, R.; Mansor, S.M.; Navaratnam, V.; Müller, C.P.; Hassan, Z. Neurobiology of Kratom and its main alkaloid mitragynine. *Brain Res. Bull.* 2016, *126*, 29–40.
109. Ahmad, K.; Aziz, Z. Mitragyna speciosa use in the northern states of Malaysia: A cross-sectional study. *J. Ethnopharmacol.* **2012**, *141*, 446–450, doi:10.1016/j.jep.2012.03.009.
110. Meireles, V.; Rosado, T.; Barroso, M.; Soares, S.; Gonçalves, J.; Luís, Â.; Caramelo, D.; Simão, A.; Fernández, N.; Duarte, A.; et al. *Mitragyna speciosa*: Clinical, Toxicological Aspects and Analysis in Biological and Non-Biological Samples. *Medicines* **2019**, *6*, 35, doi:10.3390/medicines6010035.
111. Shellard, E.J.; Phillipson, J.D.; Gupta, D. The Mitragyna species of Asia. XV. The alkaloids from the bark of *Mitragyna parvifolia* (Roxb.) Korth and a possible biogenetic route for the oxindole alkaloids. *Planta Med.* **1969**, *17*, 146–163, doi:10.1055/s-0028-1099840.
112. Ujvary, I. Kratom and Other Mitragynines: The Chemistry and Pharmacology of Opioids from a Non-Opium Source. *J. Addict. Res. Ther.* **2015**, *06*, 1–2, doi:10.4172/2155-6105.1000214.
113. Hassan, Z.; Muzaimi, M.; Navaratnam, V.; Yusoff, N.H.M.; Suhaimi, F.W.; Vadivelu, R.; Vicknasingam, B.K.; Amato, D.; von Hörsten, S.; Ismail, N.I.W.; et al. From Kratom to mitragynine and its derivatives: Physiological and behavioural effects related to use, abuse, and addiction. *Neurosci. Biobehav. Rev.* 2013, *37*, 138–151.
114. Yusoff, N.H.M.; Mansor, S.M.; Müller, C.P.; Hassan, Z. Opioid receptors mediate the acquisition, but not the expression of mitragynine-induced conditioned place preference in rats. *Behav. Brain Res.* **2017**, *332*, 1–6, doi:10.1016/j.bbr.2017.05.059.
115. Lu, J.; Wei, H.; Wu, J.; Jamil, M.F.A.; Tan, M.L.; Adenan, M.I.; Wong, P.; Shim, W. Evaluation of the Cardiotoxicity of Mitragynine and Its Analogues Using Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes. *PLoS One* **2014**, *9*, e115648, doi:10.1371/journal.pone.0115648.
116. Matsumoto, K.; Yamamoto, L.T.; Watanabe, K.; Yano, S.; Shan, J.; Pang, P.K.T.; Ponglux, D.; Takayama, H.; Horie, S. Inhibitory effect of mitragynine, an analgesic alkaloid from Thai herbal medicine, on neurogenic contraction of the vas deferens. *Life Sci.* **2005**, *78*, 187–194, doi:10.1016/j.lfs.2005.04.042.
117. Utar, Z.; Majid, M.I.A.; Adenan, M.I.; Jamil, M.F.A.; Lan, T.M. Mitragynine inhibits the COX-2 mRNA expression and prostaglandin E 2 production induced by lipopolysaccharide in RAW264.7 macrophage cells. *J. Ethnopharmacol.* **2011**, *136*, 75–82, doi:10.1016/j.jep.2011.04.011.
118. Farah Idayu, N.; Taufik Hidayat, M.; Moklas, M.A.M.; Sharida, F.; Nurul Raudzah, A.R.; Shamima, A.R.; Apryani, E. Antidepressant-like effect of mitragynine isolated from *Mitragyna speciosa* Korth in mice model of depression. *Phytomedicine* **2011**, *18*, 402–407, doi:10.1016/j.phymed.2010.08.011.
119. Nelsen, J.L.; Lapoint, J.; Hodgman, M.J.; Aldous, K.M. Seizure and Coma Following Kratom (*Mitragynina speciosa* Korth) Exposure. *J. Med. Toxicol.* **2010**, *6*, 424–426, doi:10.1007/s13181-010-0079-5.
120. Davidson, L.; Rawat, M.; Stojanovski, S.; Chandrasekharan, P. Natural drugs, not so natural effects: Neonatal abstinence syndrome secondary to "kratom." *J. Neonatal. Perinatal. Med.* **2019**, *12*, 109–112, doi:10.3233/NPM-1863.
121. Lee, M.J.; Ramanathan, S.; Mansor, S.M.; Yeong, K.Y.; Tan, S.C. Method validation in quantitative analysis of phase I and phase II metabolites of mitragynine in human urine using liquid chromatography-tandem mass spectrometry. *Anal. Biochem.* **2018**, *543*, 146–161, doi:10.1016/j.ab.2017.12.021.
122. Basiliere, S.; Bryand, K.; Kerrigan, S. Identification of five Mitragyna alkaloids in urine using liquid chromatography-quadrupole/time of flight mass spectrometry. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2018**, *1080*, 11–19, doi:10.1016/j.jchromb.2018.02.010.
123. Le, D.; Goggin, M.M.; Janis, G.C. Analysis of Mitragynine and Metabolites in Human Urine for Detecting the Use of the Psychoactive Plant Kratom. *J. Anal. Toxicol.* **2012**, *36*, 616–625, doi:10.1093/jat/bks073.
124. Fu, H. A Mass Spectrometric Study of Kratom Compounds by Direct Infusion Electrospray Ionization Triple Quadrupole Mass Spectrometry. *Detection* **2016**, *04*, 66–72, doi:10.4236/detection.2016.43009.
125. Pantano, F.; Tittarelli, R.; Mannocchi, G.; Zaami, S.; Ricci, S.; Giorgetti, R.; Terranova, D.; Busardò, F.; Marinelli, E. Hepatotoxicity Induced by "the 3Ks": Kava, Kratom and Khat. *Int. J. Mol. Sci.* **2016**, *17*, 580, doi:10.3390/ijms17040580.

126. Teschke, R.; Qiu, S.X.; Lebot, V. Herbal hepatotoxicity by kava: Update on pipermethystine, flavokavain B, and mould hepatotoxins as primarily assumed culprits. *Dig. Liver Dis.* 2011, *43*, 676–681.
127. Villain, M.; Cirimele, V.; Tracqui, A.; Ricaut, F.X.; Ludes, B.; Kintz, P. Testing for kavain in human hair using gas chromatography-tandem mass spectrometry. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2003**, *798*, 351–354, doi:10.1016/j.jchromb.2003.09.055.
128. Tarbah, F.; Barguil, Y.; Müller, C.; Rickert, A.; Weinmann, W.; Nour, M.; Kintz, P.; Daldrup, T. Chromatographic hair analysis for natural kavalactones and their metabolites. A preliminary study. *Ann. Toxicol. Anal.* **2013**, *25*, 109–119, doi:10.1051/ata/2013044.
129. Wang, Y.; Eans, S.O.; Stacy, H.M.; Narayanapillai, S.C.; Sharma, A.; Fujioka, N.; Haddad, L.; McLaughlin, J.; Avery, B.A.; Xing, C. A stable isotope dilution tandem mass spectrometry method of major kavalactones and its applications. *PLoS One* **2018**, *13*, doi:10.1371/journal.pone.0197940.
130. Tarbah, F.; Mahler, H.; Kardel, B.; Weinmann, W.; Hafner, D.; Daldrup, T. Kinetics of kavain and its metabolites after oral application. In Proceedings of the Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences; Elsevier, 2003; Vol. 789, pp. 115–130.
131. Halpern, J.H. Hallucinogens and dissociative agents naturally growing in the United States. In Proceedings of the Pharmacology and Therapeutics; Pergamon, 2004; Vol. 102, pp. 131–138.
132. Vollenweider, F.X.; Kometer, M. The neurobiology of psychedelic drugs: Implications for the treatment of mood disorders. *Nat. Rev. Neurosci.* 2010, *11*, 642–651.
133. Harries, A.D.; Evans, V. Sequelae of a “magic mushroom banquet.” *Postgrad. Med. J.* **1981**, *57*, 571–572, doi:10.1136/pgmj.57.671.571.
134. Wittmann, M.; Carter, O.; Hasler, F.; Cahn, B.R.; Grimberg, U.; Spring, P.; Hell, D.; Flohr, H.; Vollenweider, F.X. Effects of psilocybin on time perception and temporal control of behaviour in humans. *J. Psychopharmacol.* **2007**, *21*, 50–64, doi:10.1177/0269881106065859.
135. Griffiths, R.R.; Johnson, M.W.; Richards, W.A.; Richards, B.D.; McCann, U.; Jesse, R. Psilocybin occasioned mystical-type experiences: Immediate and persisting dose-related effects. *Psychopharmacology (Berl).* **2011**, *218*, 649–665, doi:10.1007/s00213-011-2358-5.
136. Vargas, A.S.; Luís, Â.; Barroso, M.; Gallardo, E.; Pereira, L. Psilocybin as a New Approach to Treat Depression and Anxiety in the Context of Life-Threatening Diseases—A Systematic Review and Meta-Analysis of Clinical Trials. *Biomedicines* **2020**, *8*, 331, doi:10.3390/biomedicines8090331.
137. Pellegrini, M.; Rotolo, M.C.; Marchei, E.; Pacifici, R.; Saggio, F.; Pichini, S. Magic truffles or Philosopher’s stones: a legal way to sell psilocybin? *Drug Test. Anal.* **2013**, *5*, 182–185, doi:10.1002/dta.1400.
138. Kamata, T.; Nishikawa, M.; Katagi, M.; Tsuchihashi, H. Optimized glucuronide hydrolysis for the detection of psilocin in human urine samples. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2003**, *796*, 421–427, doi:10.1016/j.jchromb.2003.08.030.
139. Kamata, T.; Nishikawa, M.; Katagi, M.; Tsuchihashi, H. Direct detection of serum psilocin glucuronide by LC/MS and LC/MS/MS: Time-courses of total and free (unconjugated) psilocin concentrations in serum specimens of a “magic mushroom” user. *Forensic Toxicol.* **2006**, *24*, 36–40, doi:10.1007/s11419-006-0006-2.
140. Valdés, L.J.; Díaz, J.; Paul, A.G. Ethnopharmacology of ska María Pastora (*Salvia divinorum*, Epling AND Játiva-M.). *J. Ethnopharmacol.* **1983**, *7*, 287–312, doi:10.1016/0378-8741(83)90004-1.
141. Medana, C.; Massolino, C.; Pazzi, M.; Baiocchi, C. Determination of salvinorins and divinatorins in *Salvia divinorum* leaves by liquid chromatography/multistage mass spectrometry. *Rapid Commun. Mass Spectrom.* **2006**, *20*, 131–136, doi:10.1002/rcm.2288.
142. Roth, B.L.; Baner, K.; Westkaemper, R.; Siebert, D.; Rice, K.C.; Steinberg, S.A.; Ernsberger, P.; Rothman, R.B. Salvinorin A: A potent naturally occurring nonnitrogenous κ opioid selective agonist. *Proc. Natl. Acad. Sci. U. S. A.* **2002**, *99*, 11934–11939, doi:10.1073/pnas.182234399.
143. Paudel, M.K.; Shirota, O.; Sakamoto, S.; Morimoto, S.; Tanaka, H. An immunochromatographic assay for rapid etection of salvinorin A. *J. Immunoass. Immunochem.* **2017**, *38*, 438–448, doi:10.1080/15321819.2017.1324797.
144. Paudel, M.K.; Shirota, O.; Sasaki-Tabata, K.; Tanaka, H.; Sekita, S.; Morimoto, S. Development of an Enzyme Immunoassay Using a Monoclonal Antibody against the Psychoactive Diterpenoid Salvinorin A. *J. Nat. Prod.* **2013**, *76*, 1654–1660, doi:10.1021/np400358n.
145. Braida, D.; Limonta, V.; Capurro, V.; Fadda, P.; Rubino, T.; Mascia, P.; Zani, A.; Gori, E.; Fratta, W.; Parolaro, D.; et al. Involvement of κ-Opioid and Endocannabinoid System on Salvinorin A-Induced Reward. *Biol. Psychiatry* **2008**, *63*, 286–292, doi:10.1016/j.biopsych.2007.07.020.
146. Braida, D.; Capurro, V.; Zani, A.; Rubino, T.; Viganò, D.; Parolaro, D.; Sala, M. Potential anxiolytic- And antidepressant-like effects of salvinorin A, the main active ingredient of *Salvia divinorum*, in rodents. *Br. J. Pharmacol.* **2009**, *157*, 844–853, doi:10.1111/j.1476-5381.2009.00230.x.
147. Ebner, S.R.; Roitman, M.F.; Potter, D.N.; Rachlin, A.B.; Chartoff, E.H. Depressive-like effects of the kappa opioid receptor agonist salvinorin A are associated with decreased phasic dopamine release in the nucleus accumbens. *Psychopharmacology (Berl).* **2010**, *210*, 241–252, doi:10.1007/s00213-010-1836-5.
148. Fichna, J.; Dicay, M.; Lewellyn, K.; Janecka, A.; Zjawiony, J.K.; MacNaughton, W.K.; Storr, M.A. Salvinorin A has antiinflammatory and antinociceptive effects in experimental models of colitis in mice mediated by KOR and CB1 receptors. *Inflamm. Bowel Dis.* **2012**, *18*, 1137–1145, doi:10.1002/ibd.21873.
149. Hanes, K.R. Antidepressant effects of the herb *salvia divinorum*: A case report [12]. *J. Clin. Psychopharmacol.* **2001**, *21*, 634–635, doi:10.1097/00004714-200112000-00025.
150. Kivell, B.M.; Ewald, A.W.M.; Prisinzano, T.E. Salvinorin A analogs and other kappa-opioid receptor compounds as treatments for cocaine abuse. In *Advances in Pharmacology*; Academic Press Inc., 2014; Vol. 69, pp. 481–511.
151. McCurdy, C.R.; Sufka, K.J.; Smith, G.H.; Warnick, J.E.; Nieto, M.J. Antinociceptive profile of salvinorin A, a

structurally unique kappa opioid receptor agonist. *Pharmacol. Biochem. Behav.* **2006**, *83*, 109–113, doi:10.1016/j.pbb.2005.12.011.
152. Su, D.; Riley, J.; Armstead, W.M.; Liu, R. Salvinorin a pretreatment preserves cerebrovascular autoregulation after brain hypoxic/ischemic injury via extracellular signal-regulated kinase/mitogen-activated protein kinase in piglets. *Anesth. Analg.* **2012**, *114*, 200–204, doi:10.1213/ANE.0b013e31823a5d36.
153. Xin, J.; Zhang, Y.; He, Z.; Wang, Z. Highly selective non-opioid kappa opioid receptor (KOR) agonist salvinorin A protects against forebrain ischemia-induced brain injury in rats. *Brain Res.* **2016**, *1637*, 168–176, doi:10.1016/j.brainres.2016.02.024.
154. Zhang, Y.; Butelman, E.R.; Schlussman, S.D.; Ho, A.; Kreek, M.J. Effects of the plant-derived hallucinogen salvinorin A on basal dopamine levels in the caudate putamen and in a conditioned place aversion assay in mice: Agonist actions at kappa opioid receptors. *Psychopharmacology (Berl).* **2005**, *179*, 551–558, doi:10.1007/s00213-004-2087-0.
155. Cruz, A.; Domingos, S.; Gallardo, E.; Martinho, A. A unique natural selective kappa-opioid receptor agonist, salvinorin A, and its roles in human therapeutics. *Phytochemistry* **2017**, *137*, 9–14, doi:10.1016/j.phytochem.2017.02.001.
156. Silva, A.; Simão, A.; Gonçalves, J.; Caramelo, D.; Soares, S.; Luís, Â.; Rosado, T.; Barroso, M.; Duarte, A.P.; Gallardo, E. *Salvia Divinorum*: Beyond Its Therapeutic Uses. In *Salvia: Chemistry and Effects*; Villalobos, J.R.R., Ed.; Nova Publisher: New York, 2020; Vol. 1, pp. 175–211 ISBN 978-1-53617-819-7.
157. Barnes, B.B.; Snow, N.H. Analysis of Salvinorin A in plants, water, and urine using solid-phase microextraction-comprehensive two-dimensional gas chromatography-time of flight mass spectrometry. *J. Chromatogr. A* **2012**, *1226*, 110–115, doi:10.1016/j.chroma.2011.09.056.
158. McDonough, P.C.; Holler, J.M.; Vorce, S.P.; Bosy, T.Z.; Magluilo, J.; Past, M.R. The Detection and Quantitative Analysis of the Psychoactive Component of *Salvia divinorum*, Salvinorin A, in Human Biological Fluids Using Liquid Chromatography-Mass Spectrometry*. *J. Anal. Toxicol.* **2008**, *32*, 417–421, doi:10.1093/jat/32.6.417.
159. Pichini, S.; Abanades, S.; Farré, M.; Pellegrini, M.; Marchei, E.; Pacifici, R.; De La Torre, R.; Zuccaro, P. Quantification of the plant-derived hallucinogen Salvinorin A in conventional and non-conventional biological fluids by gas chromatography/mass spectrometry after *Salvia divinorum* smoking. *Rapid Commun. Mass Spectrom.* **2005**, *19*, 1649–1656, doi:10.1002/rcm.1970.
160. Moreno, I.; Da Fonseca, B.; Oppolzer, D.; Martinho, A.; Barroso, M.; Cruz, A.; Queiroz, J.A.; Gallardo, E. Analysis of Salvinorin A in urine using microextraction in packed syringe and GC-MS/MS. *Bioanalysis* **2013**, *5*, 661–668, doi:10.4155/bio.13.18.
161. Margalho, C.; Gallardo, E.; Castanheira, A.; Vieira, D.N.; López-Rivadulla, M.; Real, F.C. A validated procedure for detection and quantitation of salvinorin a in pericardial fluid, vitreous humor, whole blood and plasma using solid phase extraction and gas chromatography-mass spectrometry. *J. Chromatogr. A* **2013**, *1304*, 203–210, doi:10.1016/j.chroma.2013.07.031.
162. Alper, K.R.; Stajić, M.; Gill, J.R. Fatalities Temporally Associated with the Ingestion of Ibogaine. *J. Forensic Sci.* **2012**, *57*, 398–412, doi:10.1111/j.1556-4029.2011.02008.x.
163. Paškulin, R.; Jamnik, P.; Danevčič, T.; Koželj, G.; Krašovec, R.; Krstić-Milošević, D.; Blagojević, D.; Štrukelj, B. Metabolic plasticity and the energy economizing effect of ibogaine, the principal alkaloid of *Tabernanthe iboga*. *J. Ethnopharmacol.* **2012**, *143*, 319–324, doi:10.1016/j.jep.2012.06.039.
164. Alper, K.R. Ibogaine: A review. *Alkaloids Chem. Biol.* 2001, *56*, 1–38.
165. Glick, S.D.; Maisonneuve, I.M.; Szumlinski, K.K. Mechanisms of action of ibogaine: Relevance to putative therapeutic effects and development of a safer iboga alkaloid congener. *Alkaloids Chem. Biol.* 2001, *56*, 39–53.
166. Goutarel, R.; Gollnhofer, O.; Sillans, R. Pharmacodynamics and therapeutic applications of iboga and ibogaine. *Psychedelic Monogr.* **1993**, *6*, 71–111.
167. Kontrimavičiut, V.; Larroque, M.; Briedis, V.; Margout, D.; Bressolle, F. Quantitation of ibogaine and 12-hydroxyibogamine in human plasma by liquid chromatography with fluorimetric detection. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2005**, *822*, 285–293, doi:10.1016/j.jchromb.2005.06.018.

1.2. Paper II- A Systematic Review on the Therapeutic Effects of Ayahuasca

Abstract

Traditional therapies, resorting to the use of plants, have acquired a great demand over the years, both for economic reasons and the preference for natural treatments. Some studies suggest that ayahuasca may have beneficial properties in treating some physical and psychological imbalances. Thus, we carried out a systematic review of studies published up to December 2022, where these themes were addressed. The search was carried out in the PubMed database, and only studies written in English and published in peer-reviewed journals were included. Thus, 228 publications were identified, of which 66 were included in the present study. The reviewed studies suggest that ayahuasca may have beneficial effects on various physical and psychological conditions, namely in the treatment of depression, anxiety and various diseases of the neurobiological system, as well as anti-inflammatory and antimicrobial properties, demonstrating its therapeutic potential. The number of studies that address this issue has also been growing, demonstrating interest in the search for alternative treatments. However, to the best of our knowledge, this is the first systematic review where all the findings of therapeutic effects associated with the consumption of ayahuasca are reviewed.

Keywords: ayahuasca; therapeutic properties; natural treatments; systematic review

1.2.1. Introduction

Traditional therapies have been known since ancient civilizations [1]. This practice has been preserved over the centuries, with current knowledge derived from thousands of years of trial-and-error experiments by humans [2], which allowed for distinguishing and using the adequate species for the intended purposes [3], as well as isolating the first drugs used in modern medicine. The number of publications on the therapeutic potential of these plants or natural substances (e.g., psilocybin), including their use in patients nonresponsive to conventional approaches, is increasing every year.

Ayahuasca is an ancient beverage that has been used for centuries by indigenous peoples in the northeast of the Amazon [4–6]. Originally, the tribes resorted to this beverage for therapeutic purposes and divine rituals [7–10]. It was also used by native healers to cure psychological disorders and stimulate creative thinking and visual creativity [11]. More recently, non-indigenous religious entities from countries such as Brazil, Peru, Colombia and Ecuador also resorted to this decoction for their rituals, namely Barquinha, União do Vegetal (UDV) and Santo Daime [4,12]. These last two have now spread to the United States, Asia, Africa and some European countries [12]. In recent decades, the popularity of ayahuasca has increased outside the Amazon region. It is

often seen as a natural remedy that has been used for millennia to cure various ailments [13]. Thus, currently, this beverage, despite continuing to be used in a traditional way, is also consumed recreationally worldwide, as well as used in modern medicine [13].

The word ayahuasca comes from the language of the Andean region, from Quechua, and means "vine of the dead" or "vine of the soul" [11,12]. This word derives from the terms "aya", meaning "soul" or "dead spirit", and "waska" or "huasca" meaning "rope" or "vine" [6,7,12]. However, over time, other terms have also been used to refer to this drink, namely daime, hoasca, caapi, nate, natema and yajé, among others [5,6]. This term refers to a psychoactive beverage prepared from *Psychotria viridis* (*P. viridis*) leaves and *Banisteriopsis caapi* (*B. caapi*) stem scrapings, with an oily, thick appearance and a brownish color [14,15]. However, over the years, several variations in this decoction have been developed, and currently, several adulterants are known [7,9,10]. Presently, more than one hundred different plants used in the preparation of ayahuasca have been documented, namely *Nicotiana tabacum*, *Tabernaemontana* spp., *Datura suaveolens*, *Iochroma fuchsioides*, *Malouetia tamarquina*, *Brugmansia suaveolens*, *Psychotria carthagenensis*, *Brunfelsia* spp., *Juanulloa* spp. and *Peganum harmala*, among others [1,4]. Additionally, the use of synthetic analogs is also described, namely moclobemide, harmine freebase/HCl and tetrahydroharmine freebase/HCl [6,8,16,17].

This psychoactive decoction owes its effects to the presence of *N,N*-dimethyltryptamine (DMT), from *P. viridis*, and β-carbolines (harmaline, harmine and tetrahydroharmine (THH)), from *B. caapi* [1]. DMT is a tryptamine with an agonistic function at serotonergic receptors (5-HT1A/2A/2C), which, when ingested alone, is metabolized by peripheral monoamine oxidase A (MAO-A) being inactivated [5,18]. On the other hand, when this substance is ingested together with β-carboline alkaloids, MAO-A is temporarily inhibited, and DMT can access the bloodstream and central nervous system, exerting its psychoactive effects [1,18–20]. The effects of DMT are further enhanced by the ability of THH to inhibit serotonin reuptake [11].

Physically, ayahuasca consumption is commonly characterized by vomiting, nausea and diarrhea [21]. Other alterations in the endocrine, cardiovascular and immune systems, as well as pupil size and body temperature, have also been verified [5,22]. On the other hand, with regard to psychological effects, users describe changes in the perception of time and space, visual and auditory changes, and also alterations at the cognitive level [1]. Religious experiences, such as connections with mythical entities or Gods, are also frequently reported [23]. Despite this, there are several studies that describe the therapeutic properties associated with the consumption of ayahuasca. Several studies point to the benefits of consuming this decoction at a psychological level, namely in cases of anxiety, depression, psychological disorders or addiction [1,13,24,25]. Other benefits, such as healing, anti-inflammatory and antimicrobial properties, have been pointed out to ayahuasca extracts [1,7].

In the last decades, the consumption of ayahuasca has undergone a great expansion all over the world, raising a lot of curiosity and interest from a therapeutic point of view [7,11]. Given the current interest in therapies of natural origin and traditional medicine, it is increasingly important to know the bioactive effects of plant species, namely those that can be applied as therapies or

alternative treatments. Thus, in the present work, we systematically reviewed studies where bioactive properties associated with ayahuasca were investigated.

1.2.2. Results and Discussion

1.2.2.1. Study Selection

Figure I.2.1 illustrates the flow diagram with the different phases of the systematic review and respective screenings.

After performing the literature search, 228 separate references were obtained. Given the overlapping coverage between the different keywords used in the search, 68 of the publications found were duplicates. Thus, 160 publications were submitted to the first screening phase for abstract reading. After the first phase, 94 studies were excluded according to the exclusion criteria. The remaining 66 publications were submitted to the second screening phase, and after reading each publication in full, they were included in the present study.

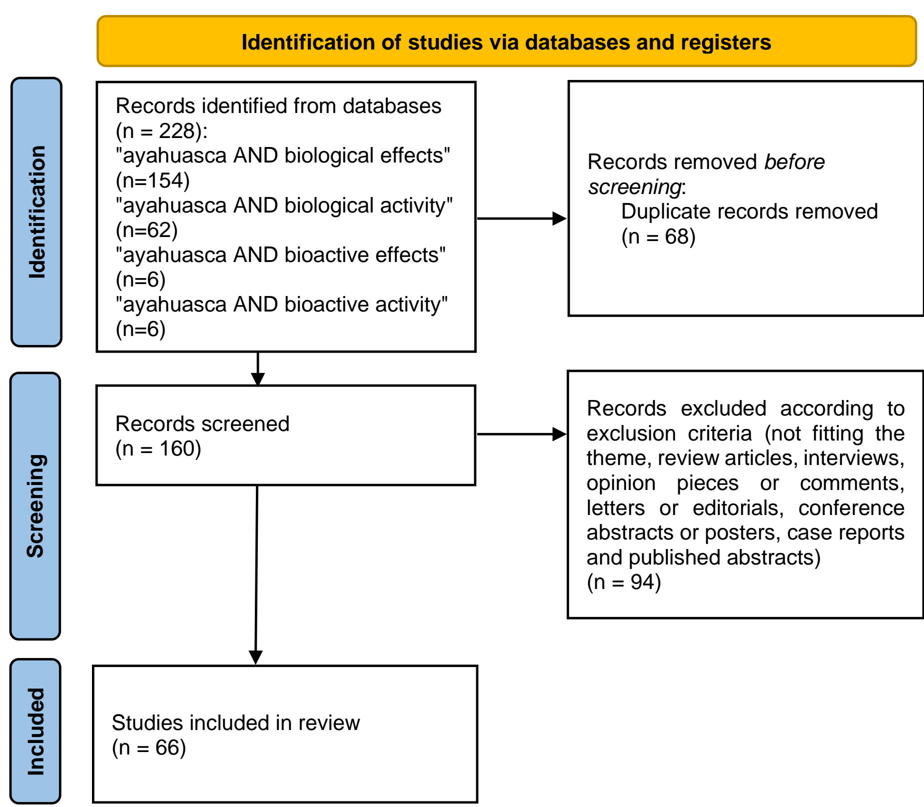


Figure I.2.1. Flow-diagram illustrating the different phases of the systematic review.

The studies were classified according to the properties under study. The chosen studies included 27 publications on psychological/psychiatric effects, 2 studies on antimicrobial properties, 3 publications on anti-inflammatory properties, 5 studies on toxicity and toxicological effects, 9

studies on different potential therapeutic properties, 13 publications that addressed effects at the physiological level and 7 about effects at the level of metabolism. The principal characteristics of the included studies in this systematic review are summarized in Supplementary Table I.2.S1.

1.2.2.2. Effects Associated with Mental Health and Psychological Well-Being

Psychedelic drugs have gained interest in recent years, particularly in the treatment of psychological disorders [26]. Thus, several studies have been developed in order to find possible ways to improve anxiety and depression symptoms. A study developed by Silva *et al.* [27] evaluated the anxiolytic and antidepressant potential of ayahuasca in an animal model of neuroinflammation. For this, 80 male rats (90 days old) were used, divided into control groups and those with neuroinflammation induced by the application of lipopolysaccharide. Anxiety behavioral parameters were assessed by open field tests, and depressive-like behaviors were assessed by forced swimming [27]. When analyzing the results, there was a reduction in anxiety and depression behaviors, concluding that ayahuasca has an anxiolytic and antidepressant potential in this animal model of neuroinflammation [27]. Also, Correa-Netto *et al.* [28] investigated the effects of ayahuasca consumption on anxiety and memory in mice. For this purpose, after the last intake of ayahuasca, the animals were subjected to the open field, elevated plus maze and Morris water maze [28]. The results demonstrated that ayahuasca consumption did not affect exploration with open arms in the elevated plus maze and locomotion in the open field [28]. However, there was an increase in risk assessment behavior in the group aged between 21 and 35 days [28]. With regard to the Morris water maze, no change in spatial memory acquisition was verified [28]. However, in animals aged between 35 and 63 days, there was a reduction in the time spent in the platform quadrant [28]. Thus, the results suggested that exposure to ayahuasca in mice in childhood promoted anxiety. On the other hand, in adolescence, it promoted memory impairment; however, after reaching adulthood, these changes were not verified [28]. Studies in humans have also been described, namely the study by Sanches *et al.* [29], whose objective was to evaluate the potential antidepressant of ayahuasca and investigate its effects on regional cerebral blood flow. This clinical trial was carried out in a psychiatric inpatient unit, and an oral dose of ayahuasca (2.2 mL/kg) was administered to 17 patients with depression [29]. In order to evaluate the evolution of the patients, blood perfusion was performed after eight hours of administration using single photon emission tomography. The Hamilton Rating Scale for Depression, the Brief Psychiatric Rating Scale, the Montgomery–Åsberg Depression, the Young Mania Rating Scale, and Clinician-Administrated Dissociative States Scale were performed during acute effects and 1, 7, 14, and 21 days after ingestion of ayahuasca [29]. The results obtained indicated that the consumption of ayahuasca was associated with a significant decrease in scores on scales that assess depression. Additionally, increased psychoactivity and in-creased blood perfusion in brain regions were associated with the regulation of emotions and mood, suggesting that ayahuasca may have antidepressant properties [29]. Also, Santos *et al.* [30] investigated the effects of ayahuasca on panic, anxiety and hopelessness in members of a religion that uses this substance (Santo Daime). Thus, a double-

blind, placebo-controlled study was carried out in which participants, who had already consumed this substance for at least ten years, were evaluated regarding their level of anxiety (state-anxiety and trait-anxiety), panic and hopelessness [30][30]. The participants were evaluated 1 h after consumption and the results showed that while they were under the acute effects of ayahuasca, there was no change in their state of anxiety [30][30]. However, states of panic and hopelessness decreased [30][30]. In another study developed by Mian *et al.* [31], the contribution of behavioral activation and mindfulness to the antidepressant effects of ayahuasca consumption was evaluated. To this end, 152 individuals were evaluated on changes in the above-mentioned parameters [31]. The analysis of results allowed us to verify that mindfulness was shown to have a strong association with the reduction in depression severity and behavioral activation a moderate association [31]. Changes in depressive symptoms were also seen on the subscales of the Five Facet Mindfulness Questionnaire and Experiences Questionnaire, and a significant improvement in Behavioral Activation for the Depression Scale-Short Form [31]. Ayahuasca consumption has been associated with curing and overcoming addictions, namely in a study carried out by Nolli *et al.* [32]. They used Wistar rats and evaluated whether the ingestion of ayahuasca would lead to a decrease in the ingestion of ethanol in the animals after exposure to it. Additionally, the work aimed to investigate the effects of ayahuasca on relevant neural activity in ethanol dependence [32]. Thus, the animals had access to ethanol for eight weeks, receiving ayahuasca at three different doses of ayahuasca (0.5×, 1× or 2× the dose taken during an ayahuasca ritual), naltrexone or water (control group). Another naive group only had access to water. The results revealed that the groups treated with naltrexone and ayahuasca did not lead to a decrease in ethanol intake [32]. With regard to neural activity, it was found that ethanol led to a significant decrease in cFos expression in the medial orbital cortex after treatment with naltrexone and 0.5× ayahuasca, with the latter reaching levels not significantly different from the naïve group [32]. On the other hand, ethanol led to increased cFos expression in the ventral orbital cortex in the 1× ayahuasca-treated group, in the lateral orbital cortex in the 2× ayahuasca-treated group, and in the nucleus accumbens in the naltrexone-treated group [32]. This increase was also observed in the region of the medial orbital cortex in the groups treated with naltrexone, ayahuasca 1×, ayahuasca 2× and in the control [32]. Also, Talin *et al.* [33] qualitatively analyzed the addiction recovery experience after ayahuasca rituals. The work was based on observation of participants in ayahuasca communities and subsequent interviews of participants with histories of substance misuse [33]. The results made it possible to verify that the effectiveness of ayahuasca in the treatment of addiction combines different dimensions (somatic, symbolic and collective), with the orientation given during the ritual being fundamental for success [33]. Thus, the authors concluded that the form and care employed play a key role in the success of addiction recovery. Additionally, inclusion in a community plays an important role in therapeutic potential [33]. Another investigation, carried out by Loizaga-Velder *et al.* [34], also evaluated the use of ayahuasca in the treatment of addictions. Thus, after interviewing 13 therapists who use ayahuasca for that purpose, 14 individuals who had undergone ayahuasca-assisted therapy for addictions and 2 specialist researchers were used. They found that this substance can be a tool in the treatment of substance dependence and the prevention of relapses [34]. However, the success of the treatment was conditioned by a series of variables [34]. Peláez [35] also evaluated the impact

of ayahuasca consumption on personality traits of former substance users. For this, a control group and a group treated with ayahuasca were used, and it was verified that the latter presented significantly greater results in the traits of impulsivity, compassion, attachment and spiritual acceptance [35]. The same was verified in the dimensions of self-transcendence and search for novelty [35]. As far as it is known, these results may be related to the self-reflective and transcendent ritual experiences of ayahuasca, which helps in the reconstruction of personal goals, social bonds and the general projection of life [35].

Grief also seems to be improved through the ayahuasca experience. González *et al.* [36] developed a study where they explored the effects of ayahuasca consumption on grief. To this end, they designed a study that measured the level of grief and experiential avoidance in 30 people who participated in peer support groups compared to 30 people who took ayahuasca [36]. The results indicate that ayahuasca consumers showed benefits in some psychological and interpersonal dimensions since the level of grief was lower on the Present Feelings Scale of the Revised Texas Grief Inventory [36]. This group also described, in an open-ended question, biographical memories, emotional release and experiences of contact with the deceased [36].

Some studies describe that other important parameters related to well-being can also be influenced by the consumption of ayahuasca, namely changes in personality, concentration and tolerability, among others. Uthaug *et al.* [37] carried out a study whose objective was to evaluate the subacute and long-term effects of ayahuasca on well-being and cognitive thinking style, as well as to evaluate its influence on the degree of ego dissolution. The 57 participants performed the ayahuasca ritual and were evaluated the day after it, after 4 weeks and after finishing the ritual [37]. This study led to the conclusion that ayahuasca leads to improvements in affection and thinking style since the results showed that convergent thinking improved after the ceremony [37]. Likewise, life satisfaction and mindfulness increased the day after the ceremony, which was not significant after 4 weeks [37]. Levels of depression and stress were significantly lower after the ceremony and for the next 4 weeks [37]. There was also a significant correlation between changes in affection, life satisfaction and mindfulness and the level of ego dissolution achieved during the ceremony [37]. However, the same authors developed another study, this time a placebo-controlled naturalist observational study, where the influence of ayahuasca and the setting on changes in mental health was evaluated. Thus, evaluations were carried out on 30 participants before and after retreat sessions with ayahuasca [38]. The results revealed that the beneficial effects on the mental health of ayahuasca consumers may be related to non-pharmacological factors (placebo response) but also to pharmacological factors related to the use of ayahuasca [38]. Otherwise, Soler *et al.* [39] carried out a study where they explored the psychological mechanisms underlying the benefits of ayahuasca consumption. Thus, 25 individuals were evaluated before and after 24 h of ingestion of ayahuasca, using the Five Faces Mindfulness Questionnaire and the Experiences Questionnaire [39]. The results showed that ayahuasca ingestion led to a reduction in internal reactivity and in the processing of experience judgments and a significant increase in the ability to decentralize. These results were compatible with extensive mindfulness practice and corroborated the therapeutic potential of ayahuasca in increasing mindfulness capabilities [39]. Also, Harris *et al.* [40] questioned 177 individuals after using ayahuasca and found that ayahuasca users ate healthier

diets, reduced alcohol intake, enjoyed improved mood and greater self-acceptance, and felt more loving and compassionate in their relationships. Seventy-four percent of respondents said they had a relationship guided and supported by the spirit of ayahuasca [40]. Barbosa *et al.* [41] psychologically evaluated 28 individuals one to four days before and one to two weeks after their first consumption of ayahuasca in the religious groups União do Vegetal and Santo Daime. In order to assess the state of mental health, a structured psychiatric scale was used to raise variables about attitudes toward the experience [41]. Subsequently, the second evaluation was carried out, where the state of mental health was evaluated according to the phenomenology of altered states of consciousness [41]. The results indicated positive expectations regarding the ritual, with tranquillity, visual phenomena, insights, numinosity and a distressing reaction being the most notable experiences after ayahuasca consumption [41]. There was a significant decrease in psychiatric symptoms in the Santo Daime group; however, in the experiences with both religious groups, changes in serenity, vivacity/joy and assertiveness were reported [41]. In order to assess tolerability, Riba *et al.* [42] carried out a single-blind crossover placebo-controlled clinical trial in which they evaluated the psychological effects and tolerability of ayahuasca. Thus, three increasing doses of ayahuasca were administered to six volunteers with previous experience in the use of ayahuasca [42]. The results revealed that the effects at the psychological level start after 30 to 60 min of consumption, reaching a peak between 60 and 120 min and ceasing after 240 min [42]. Ayahuasca was found to lead to significant dose-dependent increases in five of the six subscales used [42]. It was also possible to verify that at the cardiovascular level, ayahuasca was well tolerated, and systolic blood pressure increased [42]. During the study, one of the volunteers voluntarily withdrew from the study after experiencing anxiety and intense dysphoria with transient disorientation at the intermediate dose [42].

Mindfulness and emotional stability are of great importance for psychological wellbeing. Domínguez-Clavé *et al.* [43] developed an observational study with the aim of examining the effects of ayahuasca on capabilities related to mindfulness and emotional regulation. To this end, they had 45 volunteers who participated in an ayahuasca session who were evaluated (before and after 24 h of the session) regarding emotional dysregulation (Difficulties in the Emotional Regulation Scale) and mindfulness traits (Five Facet Mindfulness Questionnaire) [43]. The results showed that participants improved in emotional non-acceptance, emotional interference and lack of control, as well as in a state of consciousness and decentration. Significant improvements were also observed in emotional interference and lack of control but not in mindfulness abilities [43]. This study suggests that ayahuasca has therapeutic potential in regulating mindfulness and emotion regulation. Also, Franquesa *et al.* [44] explored the relationship between ayahuasca consumption and decentralization, values and the self by conducting a comparative study between individuals with and without ayahuasca experience. The results obtained indicate that individuals with ayahuasca experience scored less in fulfilment of life, living values, self in close relationships, self in social relationships and general self [44]. However, they obtained better results for positive self and decentralization [44].

Other studies indicate that creativity and creative thinking are also influenced by ayahuasca. Kuypers *et al.* [26] evaluated the effects of ayahuasca on creative thinking by

performing creativity tests before and during the effect of ayahuasca in 26 participants of spiritual workshops. Tests performed included the Image Concept Test, which assesses divergent and convergent thinking, and the pattern/Line Meaning Test, which assesses divergent thinking [26]. The results of the image concept test showed significant changes, inferring that ayahuasca consumption modified divergent (increased) and convergent thinking (decreased) [26]. This study led to the conclusion that ayahuasca ingestion enhances divergent creative thinking and increases psychological flexibility, which allows for facilitated psychotherapeutic interventions [26]. Also, Frecska *et al.* [45] studied the psychometric measures of creativity, after the disappearance of the acute effects, in ayahuasca ceremonies. Additionally, they investigated the appearance of entoptic phenomena during the expression of creativity [45]. Thus, forty participants in ayahuasca rituals were tested using Torrance Tests of Creative Thinking before and two days after the completion of two weeks of rituals [45]. The study had a control group composed of twenty-one individuals who performed the same tests [45]. When analyzing the results, it was verified that the ingestion of ayahuasca led to a significant increase in phosphenic and original responses; however, this increase already occurred in the baseline [45]. Thus, these results suggest that visual creativity and entoptic activity may increase after the acute effects of ayahuasca consumption [45].

Other studies also describe changes in spirituality and temporal reproduction. Weiss *et al.* [46] studied the association between the ceremonial use of ayahuasca and changes in personality traits. To this end, they resorted to the participation of individuals who attend spiritual and ayahuasca healing centers, evaluated in three moments: before, after and over three months after consuming ayahuasca [46]. The results of changes in personality and the moderation of these changes by covariates were evaluated by linear mixed models [46]. Thus, it was possible to verify that neuroticism was the major alteration; however, acute experiences and purgative experiences and moderation of personality change by baseline personality were also observed [46]. On the other hand, Campagnoli *et al.* [47] developed a double-blind study where they evaluated the effects of ayahuasca at two concentrations in a ritualistic context, using temporal reproduction tasks in participants with experience in ayahuasca consumption. For this, nine volunteers were asked to ingest ayahuasca in two different doses (low concentration or ritualistic concentration) and at two different moments in the ritual; they then performed the task of listening to 20 s of musical stimuli and played it immediately [47]. The results made it possible to verify that there is less temporal distortion in the participants who consumed ayahuasca [47]. Trichter *et al.* [48] further evaluated the influence of participation in an ayahuasca ceremony on spirituality and novice participants. Thus, participants in an ayahuasca ritual were compared with non-participants in rituals, using the Peak Experience Profile, the Spiritual Well-being Scale and the Mysticism Scale [48]. When analyzing the results, it was possible to verify that there were no significant increases in the Spiritual Well-being Scale and in the Mysticism Scale; however, it was verified that the increase in the score in the Peak Experience Profile was accompanied by higher scores in the Spiritual Well-being Scale and in the Mysticism Scale [48].

Studies to investigate the effects of ayahuasca in terms of psychological well-being are the most common, with very promising results, especially in the treatment of disorders such as depression and anxiety. However, positive results have been found in the various other purposes,

which influence well-being, reported above. The summary of effects associated with mental health and psychological well-being is described in Table I.2.1.

Table I.2.1. Summary of the results obtained for effects associated with mental health and psychological well-being.

Studied Sample	Main Results	Reference
Ayahuasca beverage	Reduction in anxiety and depression behaviors	[27]
	Increased anxiety (childhood), memory impairment (adolescence), unchecked changes (adult)	[28]
	Decreased depression, increased psychoactivity, increased blood perfusion in regions that regulate emotions and mood	[29]
	No change in anxiety, decrease in panic and hopelessness	[30]
	Mindfulness reduced the severity of depression and depressive symptoms	[31]
	No decrease in ethanol intake. Ethanol increased cFos expression after being treated with ayahuasca	[32]
	The effectiveness of ayahuasca in treating addiction depends on guidance during the ritual	[33]
	Potential substance addiction treatment and relapse prevention	[34]
	Improved impulsivity, compassion, attachment and spiritual acceptance, self-transcendence and novelty seeking.	[35]
	Benefits in some psychological and interpersonal dimensions, biographical memories, emotional release and contact experiences with the deceased	[36]
	Improvements in affect and thinking style, life satisfaction and mindfulness. Decreased depression and stress. Correlation between changes in affect, life satisfaction and mindfulness and the level of ego dissolution	[37]
	placebo-related beneficial mental health effects	[38]
	Decreased internal reactivity and judgmental processing of experiences. Increased decentralization capacity	[39]
	Increased healthier diets, reduced alcohol intake, improved mood, self-acceptance and relationships	[40]
	Improved tranquillity, visual phenomena, insights, numinosity. Decreased psychiatric symptoms. Increased serenity, vivacity/cheerfulness and assertiveness	[41]
	Improved psychological effects. Tolerability at the cardiovascular level.	[42]
	Improvement in emotional non-acceptance, emotional interference, lack of control, state of consciousness and decentralization. No change in mindfulness abilities.	[43]
	Decrease in the values of achievement in life, living valued, self in close relationships, self in social relationships and general self. Improved results for positive self and decentralization	[44]
	Enhancement of divergent creative thinking and increased psychological flexibility	[26]
	Increased phosphenic and original responses, visual creativity and entoptic activity	[45]
	Alteration of neuroticism and moderation of personality	[46]
	Decreased temporal distortion	[47]
	Increased Peak Experience Profile and Spiritual Well-being at Mysticism	[48]

1.2.2.3. Antimicrobial Properties

The resistance of microorganisms to conventionally used drugs is a major current concern [7]. Thus, the search for alternatives, namely of natural origin, has been awakening interest among the scientific community [7]. During the literature search, two studies were found describing the

antimicrobial properties of ayahuasca. In a study developed by Bussmann *et al.* [49], the minimum inhibitory concentration (MIC) of several plant extracts, namely *B. caapi*, and their antibacterial properties against Gram-positive and Gram-negative bacteria were evaluated. After determining the MIC values, it was verified that the ethanolic extracts of *B. caapi* showed interesting activity against *E. coli*. The same extracts were tested against S. aureus, obtaining a MIC value of 1 mg/mL [49]. In another study developed by Gonçalves *et al.* [7], the antimicrobial properties of nine plant extracts were evaluated against four Gram-positive bacterial strains (*S. aureus*, *B. cereus*, *L. monocytogenes* and *E. faecalis*) and four Gram-negative strains (*A. baumannii*, *P. aeruginosa*, *E. coli* and S. typhimurium). The results obtained during the disk diffusion test showed that six samples (*P. viridis*, *B. caapi*, *Peganum harmala* (*P. harmala*), *Mimosa hostilis* (*M. hostilis*) and a mixture of *M. hostilis* and *P. harmala* inhibited the bacterial growth in all strains [7]. The least susceptible strain was *E. faecalis*, with a range of inhibition diameters between 6.00 mm and 10.13 mm [7]. Regarding the resazurin microtitration method, it was verified that the samples that generally showed better MIC values were the samples of *B. caapi* and *P. harmala* [7]. With regard to anti-quorum sensing properties, only the *M. hostilis* sample did not inhibit violacein production and, consequently, the quorum sensing. Regarding the anti-biofilm activity, it was verified that in the presence of the samples of *B. caapi* and *P. harmala* the biofilm formation did not occur in the *A. baumanni* strain [7].

The results obtained in both studies (Table I.2.2) are indicative that ayahuasca has antimicrobial effects and may be used to combat pathogenic microorganisms responsible for various infections.

Table I.2.2. Summary of the results obtained for effects associated with antimicrobial properties.

Studied Sample	Main Results	Reference
141 plant species	Inhibition of growth of *E. coli* and *S. aureus*	[49]
***P. viridis*, *B. caapi*, *M. hostilis*, *P. harmala* and a commercial mixture of beverages**	Inhibition of bacterial growth in all strains. Anti-quorum sensing properties. Anti-biofilm activity in two samples on the same strain.	[7]

1.2.2.4. Anti-Inflammatory Properties

Inflammation is a protective reaction developed in response to harmful stimuli; however, in the long term, it can result in the development of chronic diseases [50]. Thus, the search for compounds that inhibit the development of the inflammatory response has been increasing. Gonçalves *et al.* [7] evaluated the anti-inflammatory activity by the protein denaturation inhibition method in nine samples. It was verified that the samples with a lower IC_{50} value presenting, therefore, a better anti-inflammatory activity were the extracts of *P. harmala*, *M. hostilis*, a mixture of *M. hostilis* and *P. harmala*, and a commercial mixture [7]. Also, Galvão-Coelho *et al.* [51] performed a double-blind, placebo-controlled clinical trial of ayahuasca in 45 healthy controls and 28 patients with treatment-resistant depression. During the study, it was evaluated whether ayahuasca consumption alters inflammation biomarkers, namely C-reactive protein and interleukin 6, and the correlation with serum levels of cortisol and brain-derived neurotrophic factor was

established [51]. For this purpose, blood samples were collected before and 48 h after ingestion of the substance under study in order to verify the concentration of inflammatory biomarkers. After analyzing the results, it was possible to verify that before treatment, the group of patients with depression had higher levels of C-reactive protein than the control group. A significant negative correlation was also observed between C-reactive protein and serum cortisol levels [51]. After treatment with ayahuasca, there was a significant reduction in C-reactive protein levels [51]. In another study carried out by Liu *et al.* [50], the anti-inflammatory effects of β-carboline alkaloids present in the plant P. harmala, commonly used in the preparation of ayahuasca, were investigated using a nuclear factor-kB (NF-kB) reporter assay [50]. The results indicate that harmol and harmine were able to inhibit NF-kB transactivity, with the latter inhibiting NF-kB transactivity induced by tumor necrosis factor and lipopolysaccharides and nuclear translocation in macrophage RAW264.7 cells mouse [50]. There was also a decrease in NF-kB mRNA and protein levels downstream of inflammatory cytokines. In the same study, a lipopolysaccharide-stimulated mouse model was also used, where harmine decreased the serum levels of tumor necrosis factor-α, interleukin-6, interleukin-1b and prevented lung inflammation [50]. Thus, these results are indicative that harmine can exert an anti-inflammatory effect by inhibiting the NF-kB signaling pathway [50]. Although, so far, few studies have evaluated the anti-inflammatory properties associated with ayahuasca, the results indicate that both the ayahuasca extracts [7,51] and some β-carbolines [50] present in it can decrease factors that contribute to inflammation. The summary of the anti-inflammatory properties is described in Table I.2.3.

Table I.2.3. Summary of the results obtained for effects associated with anti-inflammatory properties.

Studied Sample	Main Results	Reference
***P. viridis*, *B. caapi*, *M. hostilis*, *P. harmala* and a commercial mixture beverages**	Presentation of anti-inflammatory activity	[7]
Ayahuasca beverage	Negative correlation between C-reactive protein and serum cortisol levels. Decreased levels of C-reactive protein and correlation between greater reductions in C-reactive protein and less depressive symptoms	[51]
***P. harmala* beverage**	Harmine showed anti-inflammatory effects by inhibiting the NF-kB signaling pathway	[50]

1.2.2.5. Other Therapeutic Effects

The therapeutic potential of ayahuasca for various conditions and diseases has been investigated over the years [32]. Most studies that report the therapeutic potential of ayahuasca are related to the effects at the neurological level. Katchborian-Neto *et al.* [52] developed a study whose objective was to evaluate the potential of neuroprotection conferred by ayahuasca on the cell viability of SH-SY5Y neuroblastoma in an *in vitro* model of Parkinson's disease. Thus, initially, the cytotoxicity of the crude extracts of *B. caapi* and *P. viridis* and their hydroethanolic and alkaloid fractions was evaluated using an MTT assay (48 h and 72 h), and then the chemical composition of the samples was analyzed using ultra-performance liquid chromatography coupled to electrospray

ionization and time of flight detector (UPLC-ESI-TOF) [52]. The main alkaloids were quantified by UPLC-MS/MS [52]. After analyzing the results, it was possible to verify that the samples did not present cytotoxicity *in vitro*, and in three samples, the cell viability increased after 48 h [52]. It was also verified that the crude extracts and the alkaloid fractions presented a neuroprotective effect after 72 h of exposure [52]. On the other hand, the hydroalcoholic fractions showed the same neuroprotection results, but at both times tested (48 h and 72 h) [52]. The β-carbolines and monoterpene indole alkaloids were shown to be correlated with this property, while harmine, despite showing potent neuroprotective action in 72 h, did not present a correlation with the neuroprotection profile [52]. The authors concluded that the lowest doses stimulated cell proliferation and/or had the most effective neuroprotective profile [52]. Morales-Garcia *et al.* [53] proceeded to develop an *in vitro* and *in vivo* study, where they evaluated the potential neurogenic effect of DMT. The results demonstrated that DMT administration promotes newly generated neurons in the granular zone through activation of the adult neurogenic niche (the sub granular zone of the dentate gyrus of the hippocampus) [53]. Additionally, it was found that the mice used in the study, treated with DMT, performed better in memory tests than the animals in the control group [53]. Thus, the study concluded that treatment with DMT leads to the proliferation of neural stem cells and the migration of neuroblasts, which promotes the generation of new neurons in the hippocampus [53]. This may be indicative of increased adult neurogenesis and improved learning tasks and spatial memory [53]. Another study developed by Samoylenko *et al.* [54] evaluated the potential for preventing neurological disorders, such as Parkinson's disease, by evaluating the MAO inhibitory activities of *B. caapi* constituents. Thus, after fractionation and isolation of *B. caapi* constituents, their inhibitory activity on catechol-*O*-methyl transferase, MAO-A, MAO-B, butyrylcholinesterase and acetylcholinesterase was evaluated. Additionally, the cytotoxic and antioxidant activities of the extract and isolated compounds were also evaluated [54]. Results revealed that harmine and harmaline were able to strongly inhibit MAO-A and MAO-B *in vitro*, and (-)-epicatechin and (-)-procyanidin showed potent antioxidant and moderate MAO-B inhibitory activity [54]. The study made it possible to verify that the stem extract of *B. caapi* could help in the treatment of parkinsonism and other neurodegenerative disorders [54]. Also, in order to assess the influence of ayahuasca on Parkinson's disease, Schwarz *et al.* [55] investigated the activity of *B. caapi* extract, harmine and harmaline. Thus, the effects of plant extract and compounds on rat liver MAO-A and MAO-B activity were evaluated [55]. The results showed that harmaline achieves concentration-dependent inhibition of MAO-A but demonstrated little activity on MAO-B [55]. It was also verified that *B. caapi* extract, harmine and harmaline led to a significant increase in [3H] dopamine. The results demonstrated that the extract of *B. caapi* could be promising in the treatment of Parkinson's Disease since the discovery that harmine and harmaline stimulate the release of dopamine is a new discovery [55]. Bouso *et al.* [56] also investigated the neuropsychological performance in executive function and working memory after acute ingestion of ayahuasca. For this, 24 participants were evaluated in the execution of the Stroop, Sternberg and Tower of London tasks, in a usual environment, before and after ingestion of ayahuasca [56]. The results showed that there was an increase in errors in the performance of the Sternberg task; on the other hand, the reaction times in the performance of the Stroop task decreased [56]. No participant

showed alterations after ingestion of ayahuasca [56]. Regarding the results of the Tower of London, there was a significant increase for experienced users in resolution and execution times and in the number of movements performed [56]. The study concluded that acute ingestion of ayahuasca decreased stimulus–response interference and impaired working memory [56]. However, it was also concluded that greater previous exposure to ayahuasca resulted in a decrease in disability, so the continued use of this substance may be related to neuromodulatory or compensatory effects on executive function [56].

Other therapeutic effects, such as skin healing and overcoming eating disorders, among others, have also been reported. Gonçalves *et al.* [1] evaluated the healing potential of nine plant extracts used in the preparation of ayahuasca in the NHDF cell line. Thus, a wound-healing assay was carried out, and later a Parallel artificial membrane permeability assay, in order to understand whether the compounds were absorbed by the skin fibroblasts [1]. The results allowed for verification that all samples promoted the migration of skin fibroblasts, not being absorbed through the skin [1]. Thus, the results revealed that the ayahuasca samples presented a great healing potential [1]. On the other hand, Lafrance *et al.* [57] developed a study where they explored the therapeutic potential of ayahuasca in eating disorders. To this end, 16 individuals with eating disorders were interviewed about their experiences with ayahuasca consumption [57]. The results of the interviews demonstrated that after the beginning of the participation in ayahuasca ceremonies, there were reductions in thoughts and symptoms related to eating disorders [57]. Other improvements in terms of reductions in anxiety, depression, self-injury, suicide and problematic substance use have also been described [57]. Participants also described that the ceremonial context enhanced the results and reduced the risks and damage that may have occurred [57]. Also, Santos *et al.* [58] evaluated the sensitization and acute tolerance of ayahuasca in repeated doses through a double-blind, crossover and placebo-controlled clinical trial. Thus, participants received a lactose placebo 4 h later, one dose of ayahuasca (control) or two doses of ayahuasca 4h apart (treated) [58]. Subsequently, cardiovascular, autonomic, neurophysiological, cellular immunity and neuroendocrine measurements were performed. The results showed that, after the second dose of ayahuasca, there was a significant decrease in growth hormone and a reduction in heart rate and systolic blood pressure [58]. However, no differences were observed for autonomic, neurophysiological or immunological effects [58]. Thus, it was concluded that there was a significant tolerance to the secretion of growth hormone and less cardiovascular activation [58]. There was no sensitization or tolerance in the remaining variables [58]. Halpern *et al.* [59] also investigated the effects of ayahuasca on members of the Santo Daime Church in the United States of America. Thus, 32 individuals were examined regarding the extent of their participation in the Church, which health benefits or harms they attribute to ayahuasca, what they like least and most about it, drug use schedule, psychological factors, data on childhood conduct disorder, physical examination and demographic information [59]. The results made it possible to verify that church attendance occurs once a week and that individuals are healthy [59]. Additionally, 24 of the subjects were drug or alcohol-dependent, with 22 in remission whose motivation is church attendance [59]. Similarly, 19 participants described already having a psychiatric disorder, with 8 describing that onset of remission was achieved with church participation [59].

The use of ayahuasca for therapeutic purposes has been studied mainly in terms of neurological effects, with very interesting results obtained so far [52–56]. However, other interesting therapeutic applications have also been proven [57–59], and its potential for skin healing has recently been proven [1]. The summary of the therapeutic effects is described in Table I.2.4.

Table I.2.4. Summary of the results obtained for effects associated with other therapeutics effects.

Studied Sample	Main Results	Reference
***P. viridis*, *B. caapi* extracts and harmine and DMT**	Neuroprotective effect on crude extracts and hydroalcoholic fractions. Stimulation of cell proliferation and neuroprotection profile at lower doses	[52]
DMT	Increased proliferation of neural stem cells, migration of neuroblasts, promoting the generation of new neurons. Increased adult neurogenesis and improved learning tasks and spatial memory.	[53]
***B. caapi* extracts**	Therapeutic potential of *B. caapi* stem extract in the treatment of Parkinsonism and other neurodegenerative disorders	[54]
***B. caapi* extract, harmine and harmaline**	Therapeutic potential of *B. caapi* extract in Parkinson's disease	[55]
***P. viridis*, *B. caapi*, *M. hostilis*, *P. harmala* and a commercial mixture beverages**	Potential wound-healing effect	[1]
Ayahuasca beverage	Decreased working memory. Decreased disability by promoting neuromodulatory or compensatory effects on executive function	[56]
	Reductions in thoughts and symptoms related to eating disorders, anxiety, depression, self-harm, suicide and problematic substance use	[57]
	Significant decrease in growth hormone, heart rate and systolic blood pressure. No differences in autonomic, neurophysiological or immunological effects	[58]
	Remission of individuals dependent on substances or with a psychiatric disorder after participation in the church	[59]

1.2.2.6. Effects on Metabolism

Ayahuasca has been studied in terms of its metabolism and its potential influence on it. Mello *et al.* [60] evaluated liver biochemical parameters in order to verify whether ayahuasca consumption influences them. For this purpose, serum was collected from 22 volunteers who had been regular consumers of ayahuasca for at least one year [60]. The results revealed that there were no significant changes in lactate dehydrogenase, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, creatinine, urea, bilirubin and gamma glutamyl transferase, indicating that seemingly ayahuasca consumption does not affect liver function [60]. On the other hand, Madrid-Gambin *et al.* [61] developed a study whose objective was to evaluate the human metabolomic signature, its connection with the subjective effects and plasma concentrations of alkaloids after the consumption of ayahuasca. Thus, plasma samples were collected from 23 individuals before and after consumption of the substance [61]. Subsequently, a metabolomics analysis, an assessment of the subjective experience with Ayahuasca using the 5-Dimensional

Altered States of Consciousness Rating Scale, and an integrated network analysis to determine the alkaloids present in the plasma were performed [61]. After analyzing the results, it was possible to verify that the consumption of ayahuasca altered several large neutral amino acids, decreased 2-acyl-glycerol endocannabinoids and increased N-acyl-ethanolamine endocannabinoids [61]. There was deregulation in several pathways involved in neurotransmission (synthesis of serotonin and dopamine), and some endocannabinoids and hexosylceramides were directly associated with ayahuasca alkaloids [61]. It was also possible to verify that the majority of large neutral amino acids were inversely associated with the nine 5-Dimensional Altered States of Consciousness Rating Scale. Thus, the authors concluded that subjective effects may be associated with large neutral amino acids, helping to understand the metabolic fingerprint and mechanism of action associated with ayahuasca consumption [61]. On the other hand, alkaloid concentrations do not seem to be related to these subjective effects or metabolism [61]. Riba *et al.* [62] evaluated the brain bioavailability of ayahuasca, as well as the time course of its effects. For this purpose, topographic quantitative electroencephalography was used in 18 volunteers after ingesting doses of ayahuasca equivalent to 0.6 and 0.85 mg of DMT/kg of body weight [62]. It was possible to verify that there was a decrease in power in the frequency bands, mainly in the theta band [62]. It was further observed that total centroid activity increased [62]. These data support 5-HT2 and dopamine receptor agonism in the effects developed on the central nervous system by ayahuasca [62]. The same authors developed a double-blind, placebo-controlled clinical trial, where they studied the pharmacokinetic profile of ayahuasca, as well as its effects on the cardiovascular level and on the urinary excretion of monoamine metabolites [18]. Thus, 18 volunteers orally received a placebo or ayahuasca containing 0.6 and 0.85 mg of DMT/kg of body weight [18]. The results showed that the higher dose caused a significant increase in diastolic blood pressure, while heart rate and systolic blood pressure increased but not significantly [18]. An increase in the urinary excretion of normetanephrine was also seen, but the levels of deaminated monoamine metabolites did not decrease [18]. These results, associated with reduced plasma levels of harmine, suggest that this compound has a predominantly peripheral metabolism (gastrointestinal and hepatic) [18]. Pharmacological effects were also evaluated by Schenberg *et al.* [63], who developed a study that aimed to understand the pharmacological mechanisms of action. Additionally, the correlations at the neuronal level of the modified states of consciousness associated with the consumption of ayahuasca were also studied. Thus, the compounds of ayahuasca and its metabolites in the systemic circulation were quantified, as well as an electroencephalogram recording in 20 individuals with experience in the consumption of ayahuasca after its consumption [63]. It was found that initially, there was a reduction in power in the alpha band in the brain, followed by an increase in power in the slow and fast range [63]. The first reported effects were seen in the left parieto–occipital cortex; on the other hand, the slow increase in power was observed in the left fronto-temporal, right frontal and left centro–parieto–occipital cortices [63]. The rapid increase in power was seen in the left fronto-temporal, right frontal and right parieto–occipital cortices and the left centro–parieto–occipital cortices [63]. Associated with these effects are the levels of DMT, harmine, harmaline and tetrahydroharmine and some of their metabolites [63]. These results may help in the interpretation of the cognitive and emotional effects associated with ayahuasca consumption [63]. Also, Brierley

et al. [64] investigated the pharmacological mechanism, as well as the acute effects of harmine, on electrically evoked dopamine efflux parameters in the nucleus accumbens during cocaine consumption. Thus, fast cyclic voltammetry was applied to brain slices of Wistar rats in order to assess dopamine efflux in the core and shell accumbens [64]. The results indicated an increase in dopamine flux in the concha accumbens after harmine administration; however, no effect on the nucleus accumbens and reuptake in the other sub-regions [64]. The MAO inhibitor (moclobemide) had no effect on dopamine efflux, and the effect of harmine was attenuated by ketanserine (5-HT(2A/2C) antagonist) [64]. The results are indicative that harmine leads to an increase in dopamine efflux by a mechanism dependent on the presynaptic 5-HT(2A) receptor, not dependent on the activity of MAO inhibitors [64]. Thus, we may be looking at a therapy that could contribute to the therapeutic efficacy of ayahuasca for cocaine dependence [64]. Finally, Santos *et al.* [65] evaluated the impact of ayahuasca in terms of autonomic, neuroendocrine and immunomodulatory effects, through a double-blind randomized crossover clinical trial. The study included a placebo group, a positive control (20 mg D-amphetamine) and a group that ingested ayahuasca (1.0 mg DMT/kg body weight) [65]. Taking ayahuasca triggered subjective and neurophysiological effects absent in the positive control [65]. In both the positive control and ayahuasca groups, there was an increase in pupil diameter, cortisol levels and changes in lymphocyte subpopulations [65]. However, in the group that consumed ayahuasca, there was an increase in the levels of prolactin and natural killer cells; on the contrary, the amounts of CD4 and CD3 decreased [65]. The authors concluded that ayahuasca consumption stimulated neuroendocrine and immunomodulatory function and led to the appearance of sympathomimetic effects [65].

Table I.2.5. Summary of the results obtained for effects associated with effects on metabolism.

Studied Sample	Main Results	Reference
Ayahuasca beverage	No changes in liver function	[60]
	Changes in several large neutral amino acids, decrease in 2-acyl-glycerol endocannabinoids and increase in N-acyl-ethanolamine endocannabinoids. Dysregulation of various neurotransmission pathways. Subjective effects associated with large neutral amino acids. Alkaloid concentrations unrelated to subjective effects or metabolism	[61]
	Decreased power in frequency bands. Increased total centroid activity. Support of 5-HT2 and dopamine receptor agonism in ayahuasca-induced effects	[62]
	Increased diastolic blood pressure, heart rate and systolic blood pressure. Increased urinary excretion of normetanephrine without decrease in deaminated monoamine metabolite levels. Results indicative of a predominantly peripheral metabolism of harmine	[18]
	Decreased alpha-band potency in the brain and increased slow- and fast-range potency associated with levels of DMT, harmine, harmaline and tetrahydroharmine and some of their metabolites	[63]
	Stimulation of neuroendocrine and immunomodulatory function and led to the appearance of sympathomimetic effects	[65]
Harmine	Increased dopamine flux in the concha accumbens after harmine administration. harmine leads to an increase in dopamine efflux by a presynaptic 5-HT(2A) receptor-dependent mechanism. Possible therapy for cocaine addiction	[64]

Thus far, studies on the effects of ayahuasca on metabolism have made it possible to understand how it works in the body [18,61,63,64]. Some effects arising therefrom, namely at the autonomic, neuroendocrine and immunological level [65], and cardiovascular and excretory levels [18], have also been investigated, allowing us to understand the effects caused by the consumption of ayahuasca at this level. The summary of the effects on metabolism is described in Table I.2.5.

1.2.2.7. Physiological Effects

Over the years, the most diverse effects have been attributed to the consumption of ayahuasca, namely at the neurobiological level. Andrade *et al.* [14] investigated the effect of ayahuasca consumption on the neurobehavior and embryonic development of zebrafish. The animals were exposed for 96 h to amounts ranging between 0 and 1000 mg/L in order to assess toxicity, and the effects on locomotion activity were tracked through the ZebraBox video system after 120 and 144 h of exposure to amounts of 0 to 20 mg/L [14]. The results obtained were similar to those carried out in mammals, revealing that the LC50 of ayahuasca was 236.3 mg/L and that exposure caused significant damage, namely decreased locomotor activity, loss of balance, delay in hatching, accumulation of red blood cells and edema [14]. Also, Dakic *et al.* [66] investigated, *in vitro*, the effect of harmine in human neural progenitor cells derived from pluripotent stem cells. The results indicated that harmine strongly inhibited a regulator of cell proliferation and brain development, the dual-specific tyrosine phosphorylation-regulated kinase [66]. The effect of harmine analogs was further tested, and a tyrosine kinase inhibitor was found to induce the proliferation of human neural progenitor cells similarly to harmine [66]. These results may help explain how harmine induced cell proliferation *in vitro* [66]. On the other hand, Riba *et al.* [67] developed a study where they investigated the alterations in the spatial distribution of the brain electrical activity induced by ayahuasca. For this purpose, low-resolution electromagnetic tomography was performed on 18 volunteers after the administration of ayahuasca (0.85 mg of DMT/kg of body weight) or a placebo [67]. Subjective effects were measured using the Hallucinogen Rating Scale [67]. The results indicated significant differences in low-resolution electromagnetic tomography between the ayahuasca group and the placebo group [67]. Additionally, there were increases in all six Hallucinogen Rating Scales [67]. It was also possible to verify that there was a decrease in power density in the theta, alpha-2, delta and beta-1 frequency bands, the latter three being found predominantly in the temporo-parieto-occipital junction, and the first in the temporomedial and lateral cortex in the frontomedial regions [67]. The authors were able to conclude that the psychological effects caused by ayahuasca are due to the involvement of the unimodal and heteromodal association cortex and the limbic structures [67]. The same authors also investigated the effects of ayahuasca on regional cerebral blood flow [20]. To this end, they performed a double-blind, randomized clinical trial, where 15 volunteers received a dose of ayahuasca equivalent to 1.0 mg DMT/kg of body weight or placebo [20]. At 100–110 min after drug administration, regional cerebral blood flow was measured using single photon emission tomography [20]. The results showed that after ayahuasca consumption, there was an increase in blood perfusion in areas implicated in subjective feeling states, somatic awareness and emotional

arousal (right hemisphere of the anterior insula and anterior/frontomedial cingulate cortex) [20]. The same results were verified in the left amygdala/parahippocampal gyrus [20]. Thus, the results suggest that ayahuasca can interact with neural systems involved in interoception and emotional processing, playing a role in the serotonergic neurotransmission of these processes [20]. Finally, Viole *et al.* [68] evaluated the entropy of the brain's functional connectivity by means of functional magnetic resonance imaging. Thus, the aforementioned technique was applied to the brain of human beings at rest in a normal waking state or after an alteration of consciousness due to the consumption of ayahuasca [68]. An increase in the Shannon entropy of the distribution of degrees of the networks in the ayahuasca consumer group was verified. These results are in accordance with the entropic brain hypothesis, which was also intended to be evaluated in this study [68].

Other effects at the physiological level have also been reported by several studies. Alvarenga *et al.* [69] evaluated the sexual performance of male Wistar rats deprived of sleep after acute consumption of ayahuasca. For this, the animals were subjected to sleep deprivation for 96 h, after which they were administered ayahuasca at 250, 500 and 1000 µg/mL [69]. A control group, where a saline solution was administered instead of ayahuasca, was also used [69]. After measuring hormone concentrations and sexual behavior, ayahuasca was found to significantly decrease sexual performance, although the lower dose increased sexual performance in sleep-deprived rats [69]. On the other hand, the 500 µg/mL dose demonstrated a detrimental effect on sexual response compared to the control group [69]. Regarding the measurement of hormone concentrations, it was found that corticosterone remained unchanged, while an increase in testosterone occurred in rats deprived of sleep with saline solution [69]. The authors were able to conclude that ayahuasca ingestion, combined with sleep deprivation, suggested a decrease in sexual performance [69]. Another study developed by Barbanoj *et al.* [70] aimed to investigate the influence of daytime consumption of ayahuasca on sleep parameters. Thus, 22 volunteers participated in the study and were administered an ayahuasca dose of 1 mg of DMT/kg body weight (study group), 20 mg of D-amphetamine (positive control) or a placebo (control) [70]. Subsequently, spectral analysis of sleep, polysomnography and sleep quality were evaluated [70]. The results obtained in the polysomnography allowed for verification that ayahuasca inhibits rapid eye movement sleep, increasing the onset latency as the positive control [70]. It was also verified that ayahuasca did not induce deterioration in the quality of sleep, nor interruptions in the beginning or during sleep [70]. Regarding spectral analysis, it was found that ayahuasca led to an increase in potency in the high-frequency range and potency of slow wave sleep [70]. Thus, it was possible to conclude that ayahuasca can interfere with sleep [70]. Once again, Riba *et al.* [71] developed a double-blind, balanced crossover project in order to evaluate the acute effects of ayahuasca in the suppression of P50 auditory evoked potential and prepulse inhibition of startle, as well as its modulatory actions in sensory and sensorimotor gating measures. The study had 18 volunteers who were given a placebo or ayahuasca in amounts of 0.6 mg and 0.85 mg DMT/kg of body weight [71]. The results showed that ayahuasca consumption did not lead to significant changes in prepulse inhibition of startle, habituation rate or startle response; however, there was a significant (dose-dependent) decrease in P50 suppression [71]. Thus, the authors could conclude that there were no effects on sensorimotor

gating; however, the suppression of P50 indicated a decreasing effect of ayahuasca on sensory gating [71].

Table I.2.6. Summary of the results obtained for effects associated with physiological effects.

Studied Sample	Main Results	Reference
Harmine	Inhibition of the regulator of cell proliferation and brain development by harmine. Induction of proliferation of human neural progenitor cells by a harmine analog	[66]
Ayahuasca beverage	Decreased locomotor activity, loss of balance, delayed hatching, accumulation of red blood cells and edema	[14]
	Significant differences in low-resolution electromagnetic tomography between groups. Decreased power density in the theta, alpha-2, delta and beta-1 frequency bands. Possible involvement of the unimodal and heteromodal association cortex and limbic structures in the psychological effects caused by ayahuasca.	[67]
	Increased blood perfusion in areas implicated in subjective feeling states, somatic awareness, emotional arousal and left amygdala/parahippocampal gyrus. Possible association of ayahuasca with the neurotransmission of neural systems involved in interoception and emotional processing.	[20]
	Increase in the Shannon entropy of the degree distribution of networks	[68]
	Ayahuasca ingestion, combined with sleep deprivation, decreased sexual performance	[69]
	Inhibition of rapid eye movement sleep by increasing onset latency. No induction of deterioration in sleep quality nor interruption of sleep. Increased power in the high-frequency range and slow-wave sleep power	[70]
	No significant changes in prepulse inhibition of startle, habituation rate or startle response. Significant decrease in P50 suppression. No effects on sensorimotor gating, but decreasing effect on sensory gating	[71]
	Prevention of the development of behavioral sensitization caused by ethanol. Block in the expression of sensitization. Inhibition and reversal of behaviors associated with ethanol dependence.	[72]
	Stretching and flattening of vascular smooth muscle cells. Changes in the arrangement and distribution of collagen and elastic fibers	[73]
	Potential therapeutic effect of ayahuasca in the treatment of alcohol dependence	[74]
	Increased dominance time under standard binocular rivalry conditions such as binocular rivalry	[75]
	Increased percept length, and decreased rivalry switching rates	[76]

Oliveira-Lima *et al.* [72] investigated the effects of ayahuasca on hyperlocomotion and spontaneous locomotor activity, normally caused by ethanol consumption, as well as locomotor sensitization and counter sensitization in mice. After analyzing the results obtained, it was found that ayahuasca managed to prevent the development of behavioral sensitization caused by ethanol [72]. Also, in counter-sensitization, where, after sensitization, ayahuasca was administered for eight days, there was a block in the expression of sensitization [72]. Thus, ayahuasca has been shown to inhibit and reverse behaviors associated with ethanol dependence [72]. Another group investigated the acute and chronic effects of ayahuasca on the structural parameters of the aorta in rats [73]. The results were indicative that ayahuasca administration caused stretching and flattening of vascular smooth muscle cells [73]. Alterations in the arrangement and distribution of collagen and elastic fibers were also verified [73]. However, these results are still premature [73]. Serra *et al.* [74]

also carried out a study whose objective was to evaluate the effect of ayahuasca on ethanol self-administration as well as the role of 5-HT2A receptors in these effects. For this purpose, mice with previous access to ethanol were used, which were later treated with ayahuasca or with a 5-HT2A receptor antagonist [74]. It was found that after treatment with ayahuasca, the expression of ethanol self-administration decreased, as well as ethanol intake and preference [74]. Additionally, administration of the 5-HT2A receptor antagonist was found to block the effects of ayahuasca on ethanol consumption without significantly attenuating ethanol self-administration [74]. These results support the potential of ayahuasca as therapies for the treatment of alcohol dependence [74]. Finally, Frecska *et al.* [75] evaluated the effect of ayahuasca on dichotic stimulus alternation. Thus, 10 volunteers, participants in ayahuasca ceremonies, performed binocular rivalry tests before and after ingestion of ayahuasca [75]. It was found that the consumption of the substance under study led to an increase in the time of dominance both in standard conditions of binocular rivalry and with binocular rivalry, with periods of dominance being longer when drinking the beverage [75]. These results may be related to slow visual processing and the increase in dominance may be the result of hallucinogen induced alteration of gamma oscillations in the visual pathways [75]. Later, the same research group tested the alternation of perception in ayahuasca ceremonial participants, in order to examine whether alteration of interhemispheric function occurs under the influence of hallucinogens [76]. The results indicated an increase in the length of a percept, but a decrease in rivalry alternation rates [76]. Thus, the results are in agreement with previous studies that support the occurrence of interhemispheric fusion in altered states of consciousness [76].

The most studied physiological effects are, once again, those associated with neurobiology [14,20,66–68]. However, it is worth highlighting some interesting studies that looked into different but equally important parameters, such as the influence of ayahuasca on sleep parameters [70] or on sexual performance [69]. The summary of the physiological effects is described in Table I.2.6.

1.2.2.8. Toxicological Effects and Toxicity

The plants used in traditional medicine are taken as safe; however, some can be toxic, especially depending on doses, making it crucial to investigate their possible toxicity [77]. Kummrow *et al.* [77] developed a study where they evaluated the mutagenicity of two ayahuasca samples and two individual drinks from *P. viridis* and *B. caapi* plants, using the Salmonella/microsome assay with TA98 and TA100. Additionally, harmine and harmaline have also been tested [77]. The results obtained indicated that both ayahuasca drinks were mutagenic for TA98 and TA100. Also, harmaline and *B. caapi* drink showed mutagenicity for TA98. On the other hand, the *P. viridis* drink and harmine were not mutagenic [77]. In another study developed by Colaço *et al.* [78], the potentially toxic effects of ayahuasca were evaluated *in vivo*. For this purpose, rats were chronically exposed to the drink, and the levels of monoamines (serotonin, dopamine and norepinephrine), their metabolites and the brain-derived neurotrophic factor in the brain were evaluated [78]. Thus, female and male Wistar rats were used, divided into five groups: control group (water administration), fluoxetine group (administration of fluoxetine (selective serotonin reuptake inhibitor antidepressant)), Aya0.5 group (administration of ayahuasca 0.5 × the ritualistic

dose), Aya1 group (administration of ayahuasca 1 × the ritualistic dose) and Aya2 group (administration of ayahuasca 2 × the ritualistic dose). The results showed that ayahuasca was safe for the rats, with serotonin levels significantly increased in the Aya2 group and its 5-HIAA metabolite significantly decreased in the fluoxetine group [78]. Regarding dopamine and the HVA metabolite, no significant changes were observed between experimental groups; however, the DOPAC metabolite increased significantly in the Aya1 and Aya2 groups, the DOPAC/dopamine turnover was significantly higher in the Aya2 group, and the HVA/DOPAC ratio was significantly lower in the male groups Aya0.5, Aya1 and Aya2 [78]. Regarding norepinephrine, it was not detected, and its metabolite did not change significantly [78]. With regard to brain-derived neurotrophic factors in the hippocampus, there was a significant increase in the FLX and Aya2 female groups [78]. Also, Pic-Taylor *et al.* [79] carried out a study in female Wistar rats to assess the acute toxicity of ayahuasca, following the OECD Guide 423/2001 protocol. Thus, ayahuasca doses of 30 × and 50 × that of the dose taken during a religious ritual were administered by gavage, and the animals were observed for 14 days [79]. The behavior of the animals was evaluated by carrying out open field, elevated plus maze and forced swimming tests. After analyzing the results, it was possible to verify that the groups treated with ayahuasca significantly decreased locomotion in the open field test and elevated plus maze tests compared to the control [79]. Regarding the forced swimming test, the rats treated with ayahuasca swam more. Additionally, this group also showed greater neuronal activation in all brain areas involved in serotonergic neurotransmission [79]. No permanent damage has been detected, although there may be some brain damage [79]. In another study developed by Motta *et al.* [80], Wistar rats were also used in order to assess the development of maternal and fetal toxicity. The rats were divided into five groups: a control and four groups with four different doses of ayahuasca (1 ×, 2 ×, 4× and 8 × the typical dose taken by an adult during an ayahuasca ritual). After analyzing the results, it was possible to verify that the control group and the two groups with the lowest dose of ayahuasca survived; however, the groups where ayahuasca was administered 4 × and 8 × only survived 56% and 48%, respectively, presenting kidney damage [80]. Additionally, the group receiving the 8 × dose showed neuronal losses in the hippocampus and raphe nuclei, intrauterine growth retardation, induced embryonic deaths, and increased occurrence of fetal anomalies [80]. The group whose administered dose was 2 × showed a neuronal loss in the CA1 zone of the hippocampus [80]. At non-lethal doses, ayahuasca increased embryonic death and the incidence of fetal anomalies (soft tissue and skeleton) [80]. These results suggest that ayahuasca may have developmental toxicity and that its use by pregnant women may pose risks to the fetus [80]. Finally, Simão *et al.* [81] carried out a study with the objective of studying the cytotoxic effects of five preparations of ayahuasca infusions in dopaminergic immortalized cell lines. After evaluating the cytotoxicity, the results suggested that a concentration of 10 μM of harmine and THH, induce cytotoxicity in the N27 cell line [81]. The compound harmaline also induced cytotoxicity at a concentration of 80 μM, contrary to DMT, which did not showed cytotoxicity in the range of concentrations tested (0.0008 to 1 μM) [81]. Regarding the decoction extracts, it was found that *P. harmala* showed cytotoxicity at concentrations corresponding to 16 and 80 μM of harmaline, *B. caapi* showed cytotoxicity at concentrations corresponding to 10 μM of harmine and a commercial mixture showed cytotoxicity

at the corresponding concentrations of 10 μM THH [81]. The total protein was also quantified and the values agreed with those obtained in the cytotoxicity assays (when cell viability decreased, the same was verified in the total protein contents) [81]. Some of these studies indicated that ayahuasca consumption is safe [78], not leading to permanent damage [79]. However, alterations or lack of knowledge about the doses usually consumed could have serious consequences, namely at the level of dopaminergic neurons [81]. The results obtained in the study developed by Motta *et al.* [80], where several negative consequences associated with the consumption of ayahuasca during pregnancy were verified, since, although not advised, the decision to consume will be made by the woman herself. The summary of the toxicological effects and toxicity is described in Table I.2.7.

Table I.2.7. Summary of the results obtained for effects associated with toxicological effects and toxicity.

Studied Sample	Main Results	Reference
***P. viridis* and *B. caapi* beverages; harmine and harmaline**	Mutagenicity induced by ayahuasca beverages for TA98 TA100. Mutagenicity induced by harmaline and *B. caapi* drink for TA98. Drink of *P. viridis* and harmine without mutagenicity	[77]
Ayahuasca beverage	Increased serotonin (Aya2) levels and decreased 5-HIAA metabolite. No changes for dopamine and HVA metabolite, but increased DOPAC metabolite (Aya1 and Aya2), increased DOPAC/dopamine turnover (Aya2) and decreased HVA/DOPAC ratio in male groups (Aya0.5, Aya1 and Aya2). Increased brain-derived neurotrophic factor in female groups (FLX and Aya2).	[78]
	Decreased locomotion in the open field test elevated plus maze tests. Increased movement in the forced swimming test and increased neuronal activation in all brain areas involved in serotonergic neurotransmission. No permanent damage	[79]
	56% and 48% survival for 4× and 8× ayahuasca with kidney damage. Neuronal losses in the hippocampus and raphe nuclei, intrauterine growth retardation, induced embryonic deaths and increased occurrence of fetal anomalies	[80]
***P. viridis, B. caapi*, *M. hostilis*, *P. harmala* and a commercial mixture beverages**	Induction of cytotoxicity in the N27 cell line by harmine, harmaline and THH and by extracts of *P. harmala*, *B. caapi* and a commercial mixture. No cytotoxicity for DMT.	[81]

Given the presented studies, it was possible to verify that for some decades, interest in the therapeutic effects associated with ayahuasca has aroused much interest. It is clear that most studies focus on psychological/psychiatric disorders, and, in fact, many results point to the use of ayahuasca as a possible treatment, being depression and anxiety the most studied topics. However, several studies were carried out on ayahuasca rituals or those that resort to individuals who are regular consumers of ayahuasca and who have religious beliefs in this beverage. Thus, these results should be considered with caution, as they may sometimes present this bias. Despite this, many other studies are clinical trials controlled by a placebo and, therefore, present more reliable results. However, despite the positive results, the mechanism by which ayahuasca acts is still not completely understood, from a molecular point of view, nor which are its most promising components to be used as future anxiolytics or antidepressants. The same can be seen in the treatment of addictions using ayahuasca. It must be considered that ayahuasca, despite all the

beneficial effects associated, is a psychoactive substance, so we may be compensating for one addiction with another. The same can be considered for other reported therapeutic effects.

Contrary to what usually happens with other substances, most of the studies dealing with ayahuasca are clinical trials or used by volunteers. However, it would be important to understand and complete the findings of these studies with other primary studies, namely *in vitro* and *in vivo* assays. Although it is undeniable that, in fact, ayahuasca presents some benefit, it would be important to study and understand what makes this possible.

1.2.3. Materials and Methods

1.2.3.1. Data Acquisition

We intend to identify the works published until December 2022, when the biological or bioactive properties/effects associated with ayahuasca were evaluated.

1.2.3.2. Search Strategy

Electronic searches were performed in PubMed (up to December 2022). The following keywords were used: "ayahuasca AND biological effects"; "ayahuasca AND biological activity"; "ayahuasca AND bioactive effects" and "ayahuasca AND bioactive activity".

1.2.3.3. Eligibility Criteria

For the purposes of this review, *in vitro*, *in vivo*, clinical trials or studies that used ayahuasca extracts to study biological and bioactive properties with therapeutic purposes were included. Studies that used the ayahuasca beverage in its original form, or prepared with other analogues, were considered. Additionally, studies that used plant extracts used in the preparation of ayahuasca (original or altered) were also included. Exclusion criteria consisted of not fitting the theme, review articles, interviews, opinion pieces or comments, letters or editorials, conference abstracts or posters, case reports and published abstracts. Only articles written in English were included.

1.2.3.4. Study Selection and Data Extraction

Following the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) recommendations [82–84], two authors independently screened all titles and abstracts based on the defined inclusion criteria. Subsequently, the full text of each potentially eligible article was obtained and screened to support its inclusion in this systematic review. Any disagreement about study eligibility was solved through discussion between authors.

According to the PRISMA methodology [82–84], two authors independently reviewed and extracted the data using a prespecified protocol. In cases of discordance, a third author was consulted to analyze discrepancies in data extraction.

1.2.4. Conclusions

Treatments based on products of natural origin have gained prominence over the years. Despite the wide variety of drugs currently available, some trigger serious adverse effects, which can limit the daily lives of users. Additionally, despite global development, there are still many people in need and without access to basic health care, finding in plant species the only available cure.

In general, the available studies indicate that the therapeutic use of ayahuasca can be effective and bring benefits in some conditions, the most evident in terms of psychological disorders. In fact, studies that evaluate the therapeutic effects of ayahuasca in terms of emotional well-being and mental health are the most common and with the most evident results. However, the treatment of substance imbalances and addictions has also had some focus with regard to ayahuasca consumption. The interest and demand for favorable results associated with the consumption of ayahuasca has also been growing, as evidenced by the increase in publications addressing the topic. Other approaches, particularly at the level of molecular biology and microbiology, have also shown that ayahuasca may have promising effects in combating pathologies associated with microorganisms and in the treatment of dermal lesions or neurodegenerative diseases. It is notorious that most of the available studies were performed on volunteers in rituals or clinical trials, which is surprising when compared with the small number of existing *in vivo* and *in vitro* reports. However, it should also be noted that many of the existing articles are observational studies, and many were carried out on volunteers who participate in religious rituals. Thus, we have to consider that some of the results may present a bias justified by the spiritual and religious beliefs of the volunteers, constituting an important limitation of the results presented. Additionally, more studies at the molecular level are needed to understand the possible therapeutic effects of this substance better.

Supplementary Material

Table I.S1. Main characteristics of the included studies in this systematic review.

Reference	Authors	Year	Type of study	Study object (sample size (if applicable))	Studied compound/extract	Methodology	Evaluated parameters
1	Gonçalves *et al.*	2022	*In vitro*	NHDF cells	*P. viridis*, *B. caapi*, *M. hostilis*, *P. harmala* and a commercial mixture beverages	MTT assay; Wound-Healing assay; Parallel Artificial Membrane Permeability assay	Wound-Healing Potential
7	Gonçalves *et al.*	2020	*In vitro*	*Staphylococcus aureus*, *Bacillus* cereus, *Listeria monocytogenes*,	*P. viridis*, *B. caapi*, *M. hostilis*, *P. harmala* and a commercial mixture	Folin–Ciocalteu colorimetric method; aluminum chloride colorimetric method; DPPH Scavenging Assay; -Carotene	Antioxidant, Anti-Inflammatory and Antimicrobial activity

				Enterococcus faecalis, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Salmonella* Typhimurium	beverages	Bleaching Test; protein denaturation inhibition assay; Disc Diffusion Assay; Resazurin Microtiter Method; Anti-Quorum Sensing Properties: Solid Diffusion Assay; Anti-Biofilm Activity evaluation by scanning electron microscopy	
14	Andrade *et al.*	2018	*In vivo*	Zebrafish (not specified)	Ayahuasca beverage	Fish embryo toxicity test; behavioral assessment	Embryo development effects and neurobehavior
18	Riba *et al.*	2003	Clinical trial	Human volunteers (18)	Ayahuasca beverage	Visual analog scales; Hallucinogen Rating Scale; Addiction Research Center Inventory; Cardiovascular Measures; Pharmacokinetic Analysis	Subjective and cardiovascular effects and alkaloid pharmacokinetics
20	Riba *et al.*	2006	Clinical trial	Human volunteers (15)	Ayahuasca beverage	Hallucinogen Rating Scale; Addiction Research Center Inventory; single photon emission tomography imaging	Regional cerebral blood Flow effects
26	Kuypers *et al.*	2016	Ayahuasca ritual	Human volunteers (26)	Ayahuasca beverage	Pattern/line meanings test; picture concept test; visual analog scales	Creative thinking effects
27	Silva *et al.*	2022	*In vivo*	Rats (80)	Ayahuasca beverage	Open field test; forced swimming test	Behavioral response in neuroinflammation
28	Correa-Netto *et al.*	2017	*In vivo*	Mice (8-12/group)	Ayahuasca beverage	Behavioral tests; Open field test; elevated plus maze tasks; Morris water maze	Memory and anxiety effects
29	Sanches *et al.*	2016	Clinical trial	Human volunteers (17)	Ayahuasca beverage	Hamilton Rating Scale for Depression, the Montgomery-Åsberg Depression Rating Scale; Brief Psychiatric Rating Scale; Young Mania Rating Scale; Clinician Administered Dissociative States Scale	Anti-depressive potentials and its effects on regional cerebral blood flow
30	Santos *et al.*	2007	Observational study	Human volunteers (9)	Ayahuasca beverage	Standard questionnaires to evaluate state-anxiety, trait-anxiety, panic-like and hopelessness	Psychometric measures of anxiety, panic-like and hopelessness effects
31	Mian *et al.*	2019	Observational study	Human volunteers (152)	Ayahuasca beverage	Behavioral Activation for Depression Scale-Short Form; Five Facet Mindfulness Questionnaire; Experiences Questionnaire; Center for Epidemiological Sciences Depression Short Form	Contribution of mindfulness and behavioral activation in anti-depressant effects
32	Nolli *et al.*	2020	*In vivo*	Wistar rats (64)	Ayahuasca beverage	cFos immunohistochemistry	Treatment to decrease ethanol intake; neural activity effects
33	Talin *et al.*	2017	Observational study	Human volunteers (not specified)	Ayahuasca beverage	Long-term fieldwork and participant observation in ayahuasca communities	Interactive ritual contexts support on the healing effort
34	Loizaga-Velder *et al.*	2014	Observational study	Human volunteers (not specified)	Ayahuasca beverage	Review of therapeutic projects; interviews with therapists who apply ayahuasca in the treatment of addictions; interviews with expert researchers on the topic; interviews with individuals who had undergone ayahuasca-assisted therapy for addiction in diverse treatment settings	Substance dependence treatment

35	Peláez *et al.*	2020	Observational study	Human volunteers (14)	Ayahuasca beverage	Scale Temperament and Character Inventory–Revisited; Cloninger's model	Personality traits
36	González *et al.*	2019	Observational study	Human volunteers (60)	Ayahuasca beverage	General Characteristics Bereavement Questionnaire; Texas Revised Inventory of Grief; Acceptance and Action Questionnaire; Ethical Considerations	Grief therapy
37	Uthaug *et al.*	2018	Ayahuasca ritual	Human volunteers (57)	Ayahuasca beverage	Picture concept task; Depression, Anxiety, and Stress Scale-21; Satisfaction with Life Scale; Five Facets Mindfulness Questionnaire; Ego Dissolution Inventory	Well-being and cognitive thinking style effects; Depend on the degree of ego dissolution effects
38	Uthaug *et al.*	2021	Clinical trial	Human volunteers (30)	Ayahuasca beverage	Multifaceted empathy test; Ego Dissolution Inventory; the 5-Dimensional Altered States of Consciousness Rating Scale; Depression, Anxiety, and Stress Scale 21; Brief Symptom Inventory 18; Five Facets Mindfulness Questionnaire	Mental health changes
39	Soler *et al.*	2016	Observational study	Human volunteers (25)	Ayahuasca beverage	Five Facets Mindfulness Questionnaire; Experiences Questionnaire	Psychological mechanisms underlying the beneficial effects
40	Harris *et al.*	2012	Observational study	Human volunteers (177)	Ayahuasca beverage	Qualitative questionnaire	Effects on joy in life, relationship to the sacred and toxic feelings
41	Barbosa *et al.*	2005	Observational study	Human volunteers (28)	Ayahuasca beverage	Clinical Interview Schedule-Revised Edition; Sociodemographic profile; Inventory of intrinsic religious beliefs profiles; Inventory of expectancies/motivations; Phenomenological mapping of the altered states of Consciousness; Behavioral changes inventory	Psychological aspects in mental health
42	Riba *et al.*	2001	Clinical trial	Human volunteers (6)	Ayahuasca beverage	Visual analogue scales; Spanish adaptations of the Hallucinogen Rating Scale; Addiction Research Center Inventory; Tolerability measures	Psychological effects and tolerability
43	Domínguez-Clavé *et al.*	2019	Observational study	Human volunteers (45)	Ayahuasca beverage	Difficulties in Emotion Regulation Scale; mindfulness traits (Five Facet Mindfulness Questionnaire–Short Form; Experiences Questionnaire	Emotion regulation and mindfulness-related abilities effects
44	Franquesa *et al.*	2018	Observational study	Human volunteers (122)	Ayahuasca beverage	Brief Symptom Inventory 18 scale; Psychoticism scale of the Symptoms Assessment-45; Experiences Questionnaire; Engaged Living Scale; Experiencing of Self Scale	Link between Decentering, Values and Self
45	Frecska *et al.*	2012	Ayahuasca ritual	Human volunteers (61)	Ayahuasca beverage	Torrance Tests of Creative Thinking	Creativity and expression of creativity
46	Weiss *et al.*	2021	Ayahuasca ritual	Human volunteers (256)	Ayahuasca beverage	Changes in personality traits by the Five-Factor model; demographic characteristics, baseline personality, and acute post-ayahuasca experiences	Personality changes
47	Campagnoli *et al.*	2020	Ayahuasca ritual	Human volunteers (9)	Ayahuasca beverage	Hearing and reproduction of musical stimuli	Effects of listening to musical stimuli on subjective

							time
48	Trichter *et al.*	2009	Observational study	Human volunteers (54)	Ayahuasca beverage	Peak Experience Profile; Spiritual Well-being Scale; Mysticism Scale	Influence on spirituality
49	Bussmann *et al.*	2010	*In vitro*	*Escherichia coli* and *Staphylococcus aureus*	141 plant species	Minimal inhibitory concentration	Minimum inhibitory concentration and antibacterial properties determination
50	Liu *et al.*	2017	*In vitro* and *in vivo*	HEK-293T cells and mice (not specified)	*P. harmala* beverage	Gene reporter assay; Determination of nitric oxide production; Enzyme-linked immunosorbent assay; Quantitative real-time PCR; Immunofluorescence staining and microscopic imaging; H&E staining	Anti-inflamató Anti-inflammatory effects
51	Galvão-Coelho *et al.*	2020	Clinical trial	Human volunteers (73)	Ayahuasca beverage	Blood inflammatory biomarkers measure; Montgomery-Åsberg Depression Rating Scale	Blood inflammatory biomarkers
52	Katchborian-Neto *et al.*	2020	*In vitro*	SH-SY5Y neuroblastoma cells	*P. viridis*, *B. caapi* extracts and harmine and DMT	MTT assay; Ki-67 staining (cell proliferation); calcein-AM/PI staining	Neuroprotector potential
53	Morales-Garcia *et al.*	2020	*In vitro* and *in vivo*	neural stem cells and C57/BL6 mice (24)	DMT	Growth and proliferation measurements; differentiation of cultures; western blot analysis; immunocytochemistry; immunohistochemistry; cell count analysis; behavioral studies	Neurogenic effects
54	Samoylenko *et al.*	2010	*In vitro*	Recombinant human brain monoamine oxidase -A and -B	*B. caapi* extracts	Inhibition kinetics assay; cytotoxicity assay; Determination of ROS assay; Ensaio de inibição da atividade das enzimas acetilcolinesterase, butirilcolinesterase e catecol-O-metil transferase	Prevention of neurological disorders through the antioxidant and inhibitory activity of Monoamine Oxidase
55	Schwarz *et al.*	2003	*In vitro*	Wistar rats liver	*B. caapi* extract, harmine and harmaline	MAO inhibition assay; *In vitro* release of [3H] dopamine ([3H]DA)	Investigation of *in vitro* activity relevant to Parkinson's Disease
56	Bouso *et al.*	2013	Observational study	Human volunteers (24)	Ayahuasca beverage	Tower of London task; Stroop color and word test; Sternberg working memory task	Neuropsychological performance (working memory and executive function) effects
57	Lafrance *et al.*	2017	Observational study	Human volunteers (16)	Ayahuasca beverage	Interview with questions of etiological, clinical, and treatment histories	Potential therapeutic in eating disorders
58	Santos *et al.*	2012	Clinical trial	Human volunteers (17)	Ayahuasca beverage	visual analog scales; Hallucinogen Rating Scale; Addiction Research Center Inventory; Neurophysiological measures; Cardiovascular measures; Autonomic measures; Neuroendocrine measures	Tolerance or sensitization effects
59	Halpern *et al.*	2008	Observational study	Human volunteers (32)	Ayahuasca beverage	Structured Clinical Interview for DSM-IV Disorders; 14-item Hamilton Anxiety Rating Scale; 21-item Hamilton Depression Rating Scale; Symptom Check List 90 Revised; Uplifts, Hassles, Stresses, and Cognitive Failures questionnaire; Wender Utah Rating Scale for attention-deficit hyperactivity Disorder; Structured Clinical Interview for DSM-IV Axis II	Beneficial effects

						Personality Disorders	
60	Mello *et al.*	2018	Clinical trial	Human volunteers (22)	Ayahuasca beverage	Evaluation of biochemical parameters	Hepatic biochemical parameters
61	Madrid-Gambin *et al.*	2022	Clinical trial	Human volunteers (23)	Ayahuasca beverage	5-Dimension Altered States of Consciousness Rating Scale	Human metabolomics signature investigation
62	Riba *et al.*	2002	Clinical trial	Human volunteers (18)	Ayahuasca beverage	Topographic quantitative-electroencephalography; Hallucinogen Rating Scale	Cerebral bioavailability and time-course
63	Schenberg *et al.*	2015	Clinical trial	Human volunteers (30)	Ayahuasca beverage	Hallucinogen Rating Scale-Brazilian Version; Electroencephalography	Effects of oscillatory activity of different brain regions
64	Brierley *et al.*	2013	*In vitro*	Rat brain slices	Harmine	Fast cyclic voltammetry; Carbon fibre microelectrodes	Acute effects and pharmacological mechanism on electrically evoked dopamine efflux parameters
65	Santos *et al.*	2011	Clinical trial	Human volunteers (10)	Ayahuasca beverage	Hallucinogen Rating Scale; Addiction Research Center Inventory; autonomic measures; neuroendocrine measures; lymphocyte subpopulations measures; electroencephalographic measures	Autonomic, neuroendocrine, and immunomodulatory effects
66	Dakic *et al.*	2016	*In vitro*	Human embryonic stem cells	Harmine	Cell proliferation (Immunocytochemistry); cell death; DNA damage	proliferation of human neural progenitor effects
67	Riba *et al.*	2004	Clinical trial	Human volunteers (18)	Ayahuasca beverage	Hallucinogen Rating Scale; self-report questionnaire measuring psychedelicinduced subjective effects; topographic pharmaco-electroencephalography; low-resolution electromagnetic tomography	Changes in brain electrical activity
68	Viol *et al.*	2017	Clinical trial	Human volunteers (10)	Ayahuasca beverage	Complex networks measures	Differences in complex networks with and without ayahuasca influence
69	Alvarenga *et al.*	2014	*In vivo*	Rats (80)	Ayahuasca beverage	Sexual behavior evaluation; paradoxical sleep deprivation; hormone concentrations evaluation	Sexual performance (sleep deprived)
70	Barbanoj *et al.*	2008	Clinical trial	Human volunteers (22)	Ayahuasca beverage	Subjective sleep quality; polysomnography; spectral analysis	Sleep parameters effects
71	Riba *et al.*	2002	Clinical trial	Human volunteers (18)	Ayahuasca beverage	P50 elicitation and recording; Startle reflex elicitation and recording; Hallucinogen Rating Scale; Spanish version of the Altered States of Consciousness Questionnaire	P50 suppression and PPI effects
72	Oliveira-Lima *et al.*	2015	*In vivo*	Mice (146)	Ayahuasca beverage	Open-field test	Ethanol treatment
73	Pitol *et al.*	2015	*In vivo*	Rats (40)	Ayahuasca beverage	Morphometric Analysis (qualitative and quantitative)	Structural parameters in aorta effects
74	Serra *et al.*	2022	*In vivo*	Mice (90)	Ayahuasca beverage	behavioral observation	Role of 5-HT2A receptors in the treatment of ethanol withdrawal
75	Frecska *et al.*	2004	Clinical trial	Human volunteers (10)	Ayahuasca beverage	Binocular rivalry test	Influence of binocular rivalry on high rates of dichotic stimulus alternation

							investigation
76	Frecska *et al.*	2003	Ayahuasca ritual	Human volunteers (10)	Ayahuasca beverage	Binocular rivalry test	Influence of binocular rivalry in revealing the temporal characteristics of brain dominance in an altered state of consciousness investigation
77	Kummrow *et al.*	2019	*In vitro*	TA98 and TA100 strains of *Salmonella enterica* serovar Typhimurium	*P. viridis* and *B. caapi* beverages; harmine and harmaline	Salmonella/MicrosomeAssay	Mutagenicity
78	Colaço *et al.*	2020	*In vivo*	Wistar rats (85)	Ayahuasca beverage	Open field test; elevated-plus-maze apparatus test; hematological and biochemical evaluation	Toxic effects
79	Pic-Taylor *et al.*	2015	*In vivo*	Wistar rats (6/condition)	Ayahuasca beverage (*P. viridis* and *B. caapi*)	Open field, elevated plus maze, and forced swimming tests; neuronal activation (c-fos marked neurons); toxicity (Fluoro-Jade B and Nissl/Cresyl staining)	Behavioural and neurotoxic effects
80	Motta *et al.*	2018	*In vivo*	Rats (130)	Ayahuasca beverage	Histological evaluation	Maternal and developmental toxicity
81	Simão *et al.*	2020	*In vitro*	Dopaminergic cells	*P. viridis*, *B. caapi*, *M. hostilis*, *P. harmala* and a commercial mixture beverages	Cellular viability assay; protein quantification;	Cytotoxicity

1.2.5. References

1. Gonçalves, J.; Luís, Â.; Gallardo, E.; Duarte, A.P. Evaluation of the *In Vitro* Wound-Healing Potential of Ayahuasca. *Molecules* **2022**, *27*, doi:10.3390/MOLECULES27185760.
2. Camejo-Rodrigues, J.; Ascensão, L.; Bonet, M.À.; Vallès, J. An ethnobotanical study of medicinal and aromatic plants in the Natural Park of “Serra de São Mamede” (Portugal). *J. Ethnopharmacol.* **2003**, *89*, 199–209, doi:10.1016/S0378-8741(03)00270-8.
3. Santos, E.S.; Luís, Â.; Gonçalves, J.; Rosado, T.; Pereira, L.; Gallardo, E.; Duarte, A.P. *Julbernardia paniculata* and *Pterocarpus angolensis*: From Ethnobotanical Surveys to Phytochemical Characterization and Bioactivities Evaluation. *Molecules* **2020**, *25*, doi:10.3390/MOLECULES25081828.
4. Gonçalves, J.; Luís, Â.; Gallardo, E.; Duarte, A.P. Psychoactive Substances of Natural Origin: Toxicological Aspects, Therapeutic Properties and Analysis in Biological Samples. *Molecules* **2021**, *26*, doi:10.3390/MOLECULES26051397.
5. Lo Faro, A.F.; Di Trana, A.; La Maida, N.; Tagliabracci, A.; Giorgetti, R.; Busardò, F.P. Biomedical analysis of New Psychoactive Substances (NPS) of natural origin. *J. Pharm. Biomed. Anal.* **2020**, *179*, 112945, doi:10.1016/J.JPBA.2019.112945.
6. Simão, A.Y.; Gonçalves, J.; Duarte, A.P.; Barroso, M.; Cristóvão, A.C.; Gallardo, E. Toxicological Aspects and Determination of the Main Components of Ayahuasca: A Critical Review. *Med. 2019, Vol. 6, Page 106* **2019**, *6*, 106, doi:10.3390/MEDICINES6040106.
7. Gonçalves, J.; Luís, Â.; Gradillas, A.; García, A.; Restolho, J.; Fernández, N.; Domingues, F.; Gallardo, E.; Duarte, A.P. Ayahuasca Beverages: Phytochemical Analysis and Biological Properties. *Antibiot. 2020, Vol. 9, Page 731* **2020**, *9*, 731, doi:10.3390/ANTIBIOTICS9110731.
8. Gaujac, A.; Dempster, N.; Navickiene, S.; Brandt, S.D.; De Andrade, J.B. Determination of *N,N*-dimethyltryptamine in beverages consumed in religious practices by headspace solid-phase microextraction followed by gas chromatography ion trap mass spectrometry. *Talanta* **2013**, *106*, 394–398, doi:10.1016/J.TALANTA.2013.01.017.
9. Domínguez-Clavé, E.; Soler, J.; Elices, M.; Pascual, J.C.; Álvarez, E.; de la Fuente Revenga, M.; Friedlander, P.; Feilding, A.; Riba, J. Ayahuasca: Pharmacology, neuroscience and therapeutic potential. *Brain Res. Bull.* **2016**, *126*, 89–101, doi:10.1016/J.BRAINRESBULL.2016.03.002.
10. dos Santos, R.G.; Bouso, J.C.; Hallak, J.E.C. Ayahuasca, dimethyltryptamine, and psychosis: a systematic review of human studies. *Ther. Adv. Psychopharmacol.* **2017**, *7*, 141–157, doi:10.1177/2045125316689030.
11. Gonçalves, J.; Castilho, M.; Rosado, T.; Luís, Â.; Restolho, J.; Fernández, N.; Gallardo, E.; Duarte, A.P. *In Vitro* Study of the Bioavailability and Bioaccessibility of the Main Compounds Present in Ayahuasca Beverages. *Mol. 2021, Vol. 26, Page 5555* **2021**, *26*, 5555, doi:10.3390/MOLECULES26185555.
12. dos Santos, R.G.; Hallak, J.E.C. Ayahuasca, an ancient substance with traditional and contemporary use in neuropsychiatry and neuroscience. *Epilepsy Behav.* **2021**, *121*, doi:10.1016/J.YEBEH.2019.04.053.
13. Brabec de Mori, B. The Power of Social Attribution: Perspectives on the Healing Efficacy of Ayahuasca. *Front. Psychol.* **2021**, *12*, doi:10.3389/FPSYG.2021.748131.
14. Andrade, T.S.; de Oliveira, R.; da Silva, M.L.; Von Zuben, M.V.; Grisolia, C.K.; Domingues, I.; Caldas, E.D.; Pic-Taylor, A. Exposure to ayahuasca induces developmental and behavioral alterations on early life stages of zebrafish.

Chem. Biol. Interact. **2018**, *293*, 133–140, doi:10.1016/J.CBI.2018.08.001.
15. Gable, R.S. Risk assessment of ritual use of oral dimethyltryptamine (DMT) and harmala alkaloids. *Addiction* **2007**, *102*, 24–34, doi:10.1111/J.1360-0443.2006.01652.X.
16. Sklerov, J.; Levine, B.; Moore, K.A.; King, T.; Fowler, D. A fatal intoxication following the ingestion of 5-methoxy-*N,N*-dimethyltryptamine in an ayahuasca preparation. *J. Anal. Toxicol.* **2005**, *29*, 838–841, doi:10.1093/JAT/29.8.838.
17. Frison, G.; Favretto, D.; Zancanaro, F.; Fazzin, G.; Ferrara, S.D. A case of β-carboline alkaloid intoxication following ingestion of Peganum harmala seed extract. *Forensic Sci. Int.* **2008**, *179*, e37–e43, doi:10.1016/J.FORSCIINT.2008.05.003.
18. Riba, J.; Valle, M.; Urbano, G.; Yritia, M.; Morte, A.; Barbanoj, M.J. Human Pharmacology of Ayahuasca: Subjective and Cardiovascular Effects, Monoamine Metabolite Excretion, and Pharmacokinetics. *J. Pharmacol. Exp. Ther.* **2003**, *306*, 73–83, doi:10.1124/JPET.103.049882.
19. Dos Santos, R.G.; Balthazar, F.M.; Bouso, J.C.; Hallak, J.E.C. The current state of research on ayahuasca: A systematic review of human studies assessing psychiatric symptoms, neuropsychological functioning, and neuroimaging. *J. Psychopharmacol* **2016**, *30*, 1230–1247, doi:10.1177/0269881116652578.
20. Riba, J.; Romero, S.; Grasa, E.; Mena, E.; Carrió, I.; Barbanoj, M.J. Increased frontal and paralimbic activation following ayahuasca, the pan-amazonian inebriant. *Psychopharmacology (Berl).* **2006**, *186*, 93–98, doi:10.1007/S00213-006-0358-7/TABLES/1.
21. Durante, Í.; Dos Santos, R.G.; Bouso, J.C.; Hallak, J.E. Risk assessment of ayahuasca use in a religious context: self-reported risk factors and adverse effects. *Rev. Bras. Psiquiatr.* **2021**, *43*, 362–369, doi:10.1590/1516-4446-2020-0913.
22. Hassan, Z.; Bosch, O.G.; Singh, D.; Narayanan, S.; Kasinather, B.V.; Seifritz, E.; Kornhuber, J.; Quednow, B.B.; Müller, C.P. Novel Psychoactive Substances-Recent Progress on Neuropharmacological Mechanisms of Action for Selected Drugs. *Front. psychiatry* **2017**, *8*, doi:10.3389/FPSYT.2017.00152.
23. Ruffell, S.G.D.; Netzband, N.; Tsang, W.; Davies, M.; Butler, M.; Rucker, J.J.H.; Tófoli, L.F.; Dempster, E.L.; Young, A.H.; Morgan, C.J.A. Ceremonial Ayahuasca in Amazonian Retreats-Mental Health and Epigenetic Outcomes From a Six-Month Naturalistic Study. *Front. psychiatry* **2021**, *12*, doi:10.3389/FPSYT.2021.687615.
24. Da Silva, F.S.; Silva, E.A.S.; De Sousa, G.M.; Maia-De-oliveira, J.P.; De Soares-Rachetti, V.P.; De Araujo, D.B.; Sousa, M.B.C.; Lobão-Soares, B.; Hallak, J.; Galvão-Coelho, N.L. Acute effects of ayahuasca in a juvenile non-human primate model of depression. *Rev. Bras. Psiquiatr.* **2019**, *41*, 280–288, doi:10.1590/1516-4446-2018-0140.
25. Barbosa, P.C.R.; Cazorla, I.M.; Giglio, J.S.; Strassman, R.S. A six-month prospective evaluation of personality traits, psychiatric symptoms and quality of life in ayahuasca-naïve subjects. *J. Psychoactive Drugs* **2009**, *41*, 205–212, doi:10.1080/02791072.2009.10400530.
26. Kuypers, K.P.C.; Riba, J.; de la Fuente Revenga, M.; Barker, S.; Theunissen, E.L.; Ramaekers, J.G. Ayahuasca enhances creative divergent thinking while decreasing conventional convergent thinking. *Psychopharmacology (Berl).* **2016**, *233*, 3395–3403, doi:10.1007/S00213-016-4377-8/FIGURES/2.
27. Goulart da Silva, M.; Daros, G.C.; Santos, F.P.; Yonamine, M.; de Bitencourt, R.M. Antidepressant and anxiolytic-like effects of ayahuasca in rats subjected to LPS-induced neuroinflammation. *Behav. Brain Res.* **2022**, *434*, 114007, doi:10.1016/J.BBR.2022.114007.
28. Correa-Netto, N.F.; Masukawa, M.Y.; Nishide, F.; Galfano, G.S.; Tamura, F.; Shimizo, M.K.; Marcato, M.P.; Santos-Junior, J.G.; Linardi, A. An ontogenic study of the behavioral effects of chronic intermittent exposure to ayahuasca in mice. *Brazilian J. Med. Biol. Res. = Rev. Bras. Pesqui. medicas e Biol.* **2017**, *50*, *1414-1443* doi:10.1590/1414-431X20176036.
29. Sanches, R.F.; De Lima Osório, F.; Santos, R.G.D.; Macedo, L.R.H.; Maia-De-Oliveira, J.P.; Wichert-Ana, L.; De Araujo, D.B.; Riba, J.; Crippa, J.A.S.; Hallak, J.E.C. Antidepressant effects of a single dose of ayahuasca in patients with recurrent depression a SPECT study. *J. Clin. Psychopharmacol.* **2016**, *36*, 77–81, doi:10.1097/JCP.0000000000000436.
30. Santos, R.G.; Landeira-Fernandez, J.; Strassman, R.J.; Motta, V.; Cruz, A.P.M. Effects of ayahuasca on psychometric measures of anxiety, panic-like and hopelessness in Santo Daime members. *J. Ethnopharmacol.* **2007**, *112*, 507–513, doi:10.1016/j.jep.2007.04.012.
31. Mian, M.N.; Altman, B.R.; Earleywine, M. Ayahuasca's Antidepressant Effects Covary with Behavioral Activation as Well as Mindfulness. *J. Psychoactive Drugs* **2020**, *52*, 130–137, doi:10.1080/02791072.2019.1674428.
32. Nolli, L.M.; de Oliveira, D.G.R.; Alves, S.S.; von Zuben, M.V.; Pic-Taylor, A.; Mortari, M.R.; Caldas, E.D. Effects of the hallucinogenic beverage ayahuasca on voluntary ethanol intake by rats and on cFos expression in brain areas relevant to drug addiction. *Alcohol* **2020**, *84*, 67–75, doi:10.1016/J.ALCOHOL.2019.10.005.
33. Talin, P.; Sanabria, E. Ayahuasca's entwined efficacy: An ethnographic study of ritual healing from "addiction." *Int. J. Drug Policy* **2017**, *44*, 23–30, doi:10.1016/J.DRUGPO.2017.02.017.
34. Loizaga-Velder, A.; Verres, R. Therapeutic effects of ritual ayahuasca use in the treatment of substance dependence--qualitative results. *J. Psychoactive Drugs* **2014**, *46*, 63–72, doi:10.1080/02791072.2013.873157.
35. Apud Peláez, I.E. Personality Traits in Former Spanish Substance Users Recovered with Ayahuasca. *J. Psychoactive Drugs* **2020**, *52*, 264–272, doi:10.1080/02791072.2020.1752960.
36. González, D.; Carvalho, M.; Cantillo, J.; Aixalá, M.; Farré, M. Potential Use of Ayahuasca in Grief Therapy. *Omega* **2019**, *79*, 260–285, doi:10.1177/0030222817710879.
37. Uthaug, M. V.; van Oorsouw, K.; Kuypers, K.P.C.; van Boxtel, M.; Broers, N.J.; Mason, N.L.; Toennes, S.W.; Riba, J.; Ramaekers, J.G. Sub-acute and long-term effects of ayahuasca on affect and cognitive thinking style and their association with ego dissolution. *Psychopharmacology (Berl).* **2018**, *235*, 2979–2989, doi:10.1007/S00213-018-4988-3/FIGURES/4.
38. Uthaug, M. V.; Mason, N.L.; Toennes, S.W.; Reckweg, J.T.; de Sousa Fernandes Perna, E.B.; Kuypers, K.P.C.; van Oorsouw, K.; Riba, J.; Ramaekers, J.G. A placebo-controlled study of the effects of ayahuasca, set and setting on mental health of participants in ayahuasca group retreats. *Psychopharmacology (Berl).* **2021**, *238*, 1899, doi:10.1007/S00213-021-05817-8.
39. Soler, J.; Elices, M.; Franquesa, A.; Barker, S.; Friedlander, P.; Feilding, A.; Pascual, J.C.; Riba, J. Exploring the therapeutic potential of Ayahuasca: acute intake increases mindfulness-related capacities. *Psychopharmacology (Berl).* **2016**, *233*, 823–829, doi:10.1007/S00213-015-4162-0.
40. Harris, R.; Gurel, L. A Study of Ayahuasca Use in North America. *http://dx.doi.org/10.1080/02791072.2012.703100* **2012**, *44*, 209–215, doi:10.1080/02791072.2012.703100.
41. Barbosa, P.C.R.; Giglio, J.S.; Dalgalarrondo, P. Altered states of consciousness and short-term psychological after-

effects induced by the first time ritual use of ayahuasca in an urban context in Brazil. *J. Psychoactive Drugs* **2005**, *37*, 193–201, doi:10.1080/02791072.2005.10399801.
42. Riba, J.; Rodríguez-Fornells, A.; Urbano, G.; Morte, A.; Antonijoan, R.; Montero, M.; Callaway, J.C.; Barbanoj, M.J. Subjective effects and tolerability of the South American psychoactive beverage Ayahuasca in healthy volunteers. *Psychopharmacology (Berl).* **2001**, *154*, 85–95, doi:10.1007/S002130000606.
43. Domínguez-Clavé, E.; Soler, J.; Pascual, J.C.; Elices, M.; Franquesa, A.; Valle, M.; Alvarez, E.; Riba, J. Ayahuasca improves emotion dysregulation in a community sample and in individuals with borderline-like traits. *Psychopharmacology (Berl).* **2019**, *236*, 573–580, doi:10.1007/S00213-018-5085-3/TABLES/2.
44. Franquesa, A.; Sainz-Cort, A.; Gandy, S.; Soler, J.; Alcázar-Córcoles, M.Á.; Bouso, J.C. Psychological variables implied in the therapeutic effect of ayahuasca: A contextual approach. *Psychiatry Res.* **2018**, *264*, 334–339, doi:10.1016/J.PSYCHRES.2018.04.012.
45. Frecska, E.; Móré, C.E.; Vargha, A.; Luna, L.E. Enhancement of creative expression and entoptic phenomena as after-effects of repeated ayahuasca ceremonies. *J. Psychoactive Drugs* **2012**, *44*, 191–199, doi:10.1080/02791072.2012.703099.
46. Weiss, B.; Miller, J.D.; Carter, N.T.; Keith Campbell, W. Examining changes in personality following shamanic ceremonial use of ayahuasca. *Sci. Rep.* **2021**, *11*, doi:10.1038/S41598-021-84746-0.
47. Campagnoli, A.P.S.; Pereira, L.A.S.; Bueno, J.L.O. Subjective time under altered states of consciousness in ayahuasca users in shamanistic rituals involving music. *Brazilian J. Med. Biol. Res. = Rev. Bras. Pesqui. medicas e Biol.* **2020**, *53*, 1–8, doi:10.1590/1414-431X20209278.
48. Trichter, S.; Klimo, J.; Krippner, S. Changes in spirituality among ayahuasca ceremony novice participants. *J. Psychoactive Drugs* **2009**, *41*, 121–134, doi:10.1080/02791072.2009.10399905.
49. Bussmann, R.W.; Malca-García, G.; Glenn, A.; Sharon, D.; Chait, G.; Díaz, D.; Pourmand, K.; Jonat, B.; Somogy, S.; Guardado, G.; et al. Minimum inhibitory concentrations of medicinal plants used in Northern Peru as antibacterial remedies. *J. Ethnopharmacol.* **2010**, *132*, 101–108, doi:10.1016/J.JEP.2010.07.048.
50. Liu, X.; Li, M.; Tan, S.; Wang, C.; Fan, S.; Huang, C. Harmine is an inflammatory inhibitor through the suppression of NF-κB signaling. *Biochem. Biophys. Res. Commun.* **2017**, *489*, 332–338, doi:10.1016/J.BBRC.2017.05.126.
51. Galvão-Coelho, N.L.; de Menezes Galvão, A.C.; de Almeida, R.N.; Palhano-Fontes, F.; Campos Braga, I.; Lobão Soares, B.; Maia-de-Oliveira, J.P.; Perkins, D.; Sarris, J.; de Araujo, D.B. Changes in inflammatory biomarkers are related to the antidepressant effects of Ayahuasca. *https://doi.org/10.1177/0269881120936486* **2020**, *34*, 1125–1133, doi:10.1177/0269881120936486.
52. Katchborian-Neto, A.; Santos, W.T.; Nicácio, K.J.; Corrêa, J.O.A.; Murgu, M.; Martins, T.M.M.; Gomes, D.A.; Goes, A.M.; Soares, M.G.; Dias, D.F.; et al. Neuroprotective potential of Ayahuasca and untargeted metabolomics analyses: applicability to Parkinson's disease. *J. Ethnopharmacol.* **2020**, *255*, 112743, doi:10.1016/J.JEP.2020.112743.
53. Morales-Garcia, J.A.; Calleja-Conde, J.; Lopez-Moreno, J.A.; Alonso-Gil, S.; Sanz-SanCristobal, M.; Riba, J.; Perez-Castillo, A. N,N-dimethyltryptamine compound found in the hallucinogenic tea ayahuasca, regulates adult neurogenesis in vitro and in vivo. *Transl. Psychiatry* **2020**, *10*, doi:10.1038/S41398-020-01011-0.
54. Samoylenko, V.; Rahman, M.M.; Tekwani, B.L.; Tripathi, L.M.; Wang, Y.H.; Khan, S.I.; Khan, I.A.; Miller, L.S.; Joshi, V.C.; Muhammad, I. Banisteriopsis caapi, a unique combination of MAO inhibitory and antioxidative constituents for the activities relevant to neurodegenerative disorders and Parkinson's disease. *J. Ethnopharmacol.* **2010**, *127*, 357–367, doi:10.1016/J.JEP.2009.10.030.
55. Schwarz, M.J.; Houghton, P.J.; Rose, S.; Jenner, P.; Lees, A.D. Activities of extract and constituents of *Banisteriopsis caapi* relevant to parkinsonism. *Pharmacol. Biochem. Behav.* **2003**, *75*, 627–633, doi:10.1016/S0091-3057(03)00129-1.
56. Bouso, J.C.; Fábregas, J.M.; Antonijoan, R.M.; Rodríguez-Fornells, A.; Riba, J. Acute effects of ayahuasca on neuropsychological performance: Differences in executive function between experienced and occasional users. *Psychopharmacology (Berl).* **2013**, *230*, 415–424, doi:10.1007/S00213-013-3167-9/TABLES/3.
57. Lafrance, A.; Loizaga-Velder, A.; Fletcher, J.; Renelli, M.; Files, N.; Tupper, K.W. Nourishing the Spirit: Exploratory Research on Ayahuasca Experiences along the Continuum of Recovery from Eating Disorders. *J. Psychoactive Drugs* **2017**, *49*, 427–435, doi:10.1080/02791072.2017.1361559.
58. Dos Santos, R.G.; Grasa, E.; Valle, M.; Ballester, M.R.; Bouso, J.C.; Nomdedéu, J.F.; Homs, R.; Barbanoj, M.J.; Riba, J. Pharmacology of ayahuasca administered in two repeated doses. *Psychopharmacology (Berl).* **2012**, *219*, 1039–1053, doi:10.1007/S00213-011-2434-X/FIGURES/4.
59. Halpern, J.H.; Sherwood, A.R.; Passie, T.; Blackwell, K.C.; Ruttenber, A.J. Evidence of health and safety in American members of a religion who use a hallucinogenic sacrament. *Med. Sci. Monit.* **2008**, *14*, 15–22.
60. Mello, S.M.; Soubhia, P.C.; Silveira, G.; Corrêa-Neto, N.F.; Lanaro, R.; Costa, J.L.; Linardi, A. Effect of Ritualistic Consumption of Ayahuasca on Hepatic Function in Chronic Users. *https://doi.org/10.1080/02791072.2018.1557355* **2018**, *51*, 3–11, doi:10.1080/02791072.2018.1557355.
61. Madrid-Gambin, F.; Gomez-Gomez, A.; Busquets-Garcia, A.; Haro, N.; Marco, S.; Mason, N.L.; Reckweg, J.T.; Mallaroni, P.; Kloft, L.; van Oorsouw, K.; et al. Metabolomics and integrated network analysis reveal roles of endocannabinoids and large neutral amino acid balance in the ayahuasca experience. *Biomed. Pharmacother.* **2022**, *149*, 112845, doi:10.1016/J.BIOPHA.2022.112845.
62. Riba, J.; Anderer, P.; Morte, A.; Urbano, G.; Jané, F.; Saletu, B.; Barbanoj, M.J. Topographic pharmaco-EEG mapping of the effects of the South American psychoactive beverage ayahuasca in healthy volunteers. *Br. J. Clin. Pharmacol.* **2002**, *53*, 613–628, doi:10.1046/J.1365-2125.2002.01609.X.
63. Schenberg, E.E.; Alexandre, J.F.M.; Filev, R.; Cravo, A.M.; Sato, J.R.; Muthukumaraswamy, S.D.; Yonamine, M.; Waguespack, M.; Lomnicka, I.; Barker, S.A.; et al. Acute Biphasic Effects of Ayahuasca. *PLoS One* **2015**, *10*, doi:10.1371/JOURNAL.PONE.0137202.
64. Brierley, D.I.; Davidson, C. Harmine augments electrically evoked dopamine efflux in the nucleus accumbens shell. *J. Psychopharmacol.* **2013**, *27*, 98–108, doi:10.1177/0269881112463125.
65. Dos Santos, R.G.; Valle, M.; Bouso, J.C.; Nomdedéu, J.F.; Rodríguez-Espinosa, J.; McIlhenny, E.H.; Barker, S.A.; Barbanoj, M.J.; Riba, J. Autonomic, neuroendocrine, and immunological effects of ayahuasca: A comparative study with d-amphetamine. *J. Clin. Psychopharmacol.* **2011**, *31*, 717–726, doi:10.1097/JCP.0B013E31823607F6.
66. Dakic, V.; de Moraes Maciel, R.; Drummond, H.; Nascimento, J.M.; Trindade, P.; Rehen, S.K. Harmine stimulates proliferation of human neural progenitors. *PeerJ* **2016**, *4*, doi:10.7717/PEERJ.2727.
67. Riba, J.; Anderer, P.; Jané, F.; Saletu, B.; Barbanoj, M.J. Effects of the South American psychoactive beverage ayahuasca on regional brain electrical activity in humans: a functional neuroimaging study using low-resolution

electromagnetic tomography. *Neuropsychobiology* **2004**, *50*, 89–101, doi:10.1159/000077946.
68. Viol, A.; Palhano-Fontes, F.; Onias, H.; De Araujo, D.B.; Viswanathan, G.M. Shannon entropy of brain functional complex networks under the influence of the psychedelic Ayahuasca. *Sci. Rep.* **2017**, *7*, doi:10.1038/S41598-017-06854-0.
69. Alvarenga, T.A.; Polesel, D.N.; Matos, G.; Garcia, V.A.; Costa, J.L.; Tufik, S.; Andersen, M.L. Can Ayahuasca and sleep loss change sexual performance in male rats? *Behav. Processes* **2014**, *108*, 110–116, doi:10.1016/J.BEPROC.2014.09.024.
70. Barbanoj, M.J.; Riba, J.; Clos, S.; Giménez, S.; Grasa, E.; Romero, S. Daytime Ayahuasca administration modulates REM and slow-wave sleep in healthy volunteers. *Psychopharmacology (Berl).* **2008**, *196*, 315–326, doi:10.1007/S00213-007-0963-0/TABLES/5.
71. Riba, J.; Rodríguez-Fornells, A.; Barbanoj, M.J. Effects of ayahuasca on sensory and sensorimotor gating in humans as measured by P50 suppression and prepulse inhibition of the startle reflex, respectively. *Psychopharmacology (Berl).* **2002**, *165*, 18–28, doi:10.1007/S00213-002-1237-5/METRICS.
72. Oliveira-Lima, A.J.; Santos, R.; Hollais, A.W.; Gerardi-Junior, C.A.; Baldaia, M.A.; Wuo-Silva, R.; Yokoyama, T.S.; Costa, J.L.; Malpezzi-Marinho, E.L.A.; Ribeiro-Barbosa, P.C.; et al. Effects of ayahuasca on the development of ethanol-induced behavioral sensitization and on a post-sensitization treatment in mice. *Physiol. Behav.* **2015**, *142*, 28–36, doi:10.1016/J.PHYSBEH.2015.01.032.
73. Pitol, D.L.; Siéssere, S.; Dos Santos, R.G.; Rosa, M.L.N.M.; Hallak, J.E.C.; Scalize, P.H.; Pereira, B.F.; Iyomasa, M.M.; Semprini, M.; Riba, J.; et al. Ayahuasca Alters Structural Parameters of the Rat Aorta. *J. Cardiovasc. Pharmacol.* **2015**, *66*, 58–62, doi:10.1097/FJC.0000000000000243.
74. Serra, Y.A.; Barros-Santos, T.; Anjos-Santos, A.; Kisaki, N.D.; Jovita-Farias, C.; Leite, J.P.C.; Santana, M.C.E.; Coimbra, J.P.S.A.; de Jesus, N.M.S.; Sulima, A.; et al. Role of 5-HT2A receptors in the effects of ayahuasca on ethanol self-administration using a two-bottle choice paradigm in male mice. *Psychopharmacology (Berl).* **2022**, *239*, 1679–1687, doi:10.1007/S00213-022-06104-W.
75. Frecska, E.; White, K.D.; Luna, L.E. Effects of ayahuasca on binocular rivalry with dichoptic stimulus alternation. *Psychopharmacology (Berl).* **2004**, *173*, 79–87, doi:10.1007/S00213-003-1701-X.
76. Frecska, E.; White, K.D.; Luna, L.E. Effects of the Amazonian psychoactive beverage Ayahuasca on binocular rivalry: interhemispheric switching or interhemispheric fusion? *J. Psychoactive Drugs* **2003**, *35*, 367–374, doi:10.1080/02791072.2003.10400019.
77. Kummrow, F.; Maselli, B.S.; Lanaro, R.; Costa, J.L.; Umbuzeiro, G.A.; Linardi, A. Mutagenicity of Ayahuasca and Their Constituents to the Salmonella/Microsome Assay. *Environ. Mol. Mutagen.* **2019**, *60*, 269–276, doi:10.1002/EM.22263.
78. Colaço, C.S.; Alves, S.S.; Nolli, L.M.; Pinheiro, W.O.; de Oliveira, D.G.R.; Santos, B.W.L.; Pic-Taylor, A.; Mortari, M.R.; Caldas, E.D. Toxicity of ayahuasca after 28 days daily exposure and effects on monoamines and brain-derived neurotrophic factor (BDNF) in brain of Wistar rats. *Metab. Brain Dis.* **2020**, *35*, 739–751, doi:10.1007/S11011-020-00547-W/FIGURES/6.
79. Pic-Taylor, A.; da Motta, L.G.; de Morais, J.A.; Junior, W.M.; Santos, A. de F.A.; Campos, L.A.; Mortari, M.R.; von Zuben, M.V.; Caldas, E.D. Behavioural and neurotoxic effects of ayahuasca infusion (*Banisteriopsis caapi* and *Psychotria viridis*) in female Wistar rat. *Behav. Processes* **2015**, *118*, 102–110, doi:10.1016/J.BEPROC.2015.05.004.
80. da Motta, L.G.; de Morais, J.A.; Tavares, A.C.A.M.; Vianna, L.M.S.; Mortari, M.R.; Amorim, R.F.B.; Carvalho, R.R.; Paumgartten, F.J.R.; Pic-Taylor, A.; Caldas, E.D. Maternal and developmental toxicity of the hallucinogenic plant-based beverage ayahuasca in rats. *Reprod. Toxicol.* **2018**, *77*, 143–153, doi:10.1016/J.REPROTOX.2018.03.002.
81. Simão, A.Y.; Gonçalves, J.; Gradillas, A.; García, A.; Restolho, J.; Fernández, N.; Rodilla, J.M.; Barroso, M.; Duarte, A.P.; Cristóvão, A.C.; et al. Evaluation of the Cytotoxicity of Ayahuasca Beverages. *Molecules* **2020**, *25*, doi:10.3390/MOLECULES25235594.
82. Page, M.J.; McKenzie, J.E.; Bossuyt, P.M.; Boutron, I.; Hoffmann, T.C.; Mulrow, C.D.; Shamseer, L.; Tetzlaff, J.M.; Akl, E.A.; Brennan, S.E.; et al. The PRISMA 2020 statement: An updated guideline for reporting systematic reviews. *PLOS Med.* **2021**, *18*, e1003583, doi:10.1371/JOURNAL.PMED.1003583.
83. Moher, D.; Liberati, A.; Tetzlaff, J.; Altman, D.G.; Altman, D.; Antes, G.; Atkins, D.; Barbour, V.; Barrowman, N.; Berlin, J.A.; et al. Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. *PLOS Med.* **2009**, *6*, e1000097, doi:10.1371/JOURNAL.PMED.1000097.
84. Moher, D.; Shamseer, L.; Clarke, M.; Ghersi, D.; Liberati, A.; Petticrew, M.; Shekelle, P.; Stewart, L.A.; Estarli, M.; Barrera, E.S.A.; et al. Preferred reporting items for systematic review and meta-analysis protocols (PRISMA-P) 2015 statement. *Syst. Rev.* **2015**, *4*, 148–160, doi:10.1186/2046-4053-4-1.

Chapter 2

AIMS OF THE THESIS

2.1 Aims and justification of the thesis theme

The uncontrolled use of substances around the world is a major concern. In general, the use of illicit substances is often associated with high rates of mortality and morbidity. In recent years, there has been a great trend in the demand for NPS, which basically consist of substances that somehow manage to escape legislation and, therefore, end up not being fully controlled. However, countries like Portugal have made efforts to establish legislation, particularly regarding the sale (Decree-Law 54/2013 of April 17th).

Although there are reports of its use since antiquity, the demand for NPS of natural origin has been also increasing. However, the demand for some NPS of natural origin is due not only to their potential for recreational use, but also to their healing and medicinal properties. It should be noted that religious motivations sometimes also boost the demand for some NPS.

In recent years, the concept of traditional medicine using natural compounds and plant species has been rethought, although this practice existed since ancient times. In fact, although medicine is currently in constant evolution, there is still a large part of the world's population that does not have access to primary health care. Thus, part of the world's population is still dependent on the use of medicinal plants to obtain treatments. The use of plants with medicinal properties is in fact less expensive, but associated with this demand are also cultural reasons and cases where conventional treatments do not guarantee success. Additionally, nowadays, there is a great demand for everything that is considered "natural", which also enhances the use of medicinal plants.

The use of plants with reported medicinal properties, which may often produce other effects simultaneously, cannot always be considered safe. Currently, there is still a great lack of knowledge, especially at the molecular level. Additionally, there is also a great lack of studies on toxicity and the therapeutic properties associated with some products of natural origin, which are also not fully understood. This is the case of ayahuasca, which despite being used for hundreds of years, namely for healing purposes, has not been fully studied yet. In this particular case, most of the existing studies were carried out on religious rituals in individuals with religious beliefs in this drink, which may present a bias. Interestingly, there are also several clinical trials, but studies at the molecular level *in vitro* and *in vivo* studies are limited. In this way, it becomes crucial to develop analytical methodologies that allow the detection and quantification of the compounds present in plant species, as well as the evaluation of their potential toxic effects and the study of their behaviour at the molecular level. Clarifying these issues will lead to a safer use of ayahuasca and a better understanding of its therapeutic potential. Thus, this doctoral thesis aims to:

1. Develop an analytical methodology to determine and quantify the main compounds present samples in ayahuasca decoctions;
2. Understand the behavior of the main constituents of ayahuasca throughout the digestive process *in vitro*;
3. Determine the phytochemical profile of ayahuasca decoction;
4. Evaluate *in vitro* the potential bioactive and therapeutic properties associated with ayahuasca and its consumption:

a. Determination of antioxidant properties;
b. Evaluation of anti-inflammatory activity;
c. Assessment of antimicrobial properties;
d. Evaluation of wound healing potential;
e. Evaluation of anticancer properties.

Chapter 3

RESULTS

This chapter was published in:

Joana Gonçalves, Tiago Rosado, Mário Barroso, José Restolho, Nicolás Fernández, Ângelo Luís, Eugenia Gallardo and Ana Paula Duarte. "*Comparative study of sample preparation procedures to determine the main compounds in ayahuasca beverages by QuEChERS and high-performance liquid chromatography analysis*" Phytochemical Analysis 2024, 1-12

Joana Gonçalves, Miguel Castilho, Tiago Rosado, Ângelo Luís, José Restolho, Nicolás Fernández, Eugenia Gallardo and Ana Paula Duarte. "*In Vitro Study of the Bioavailability and Bioaccessibility of the Main Compounds Present in Ayahuasca Beverages*" Molecules 2021, 26, 5555

Joana Gonçalves, Ângelo Luís, Ana Gradillas, Antonia García, José Restolho, Nicolás Fernández, Fernanda Domingues, Eugenia Gallardo and Ana Paula Duarte. "*Ayahuasca Beverages: Phytochemical Analysis and Biological Properties*" Antibiotics 2020, 9, 731

Joana Gonçalves, Ângelo Luís, Eugenia Gallardo and Ana Paula Duarte. "*Evaluation of the In Vitro Wound-Healing Potential of Ayahuasca*" Molecules 2022, 27, 5760

Joana Gonçalves, Mariana Feijó, Sílvia Socorro, Ângelo Luís, Eugenia Gallardo and Ana Paula Duarte. "*The Role of Ayahuasca in Colorectal Adenocarcinoma Cell Survival, Proliferation and Oxidative StressEvaluation of the anticancer properties of Ayahuasca in colorectal adenocarcinoma cells: Cell survival and proliferation and oxidative stress*" Pharmaceuticals 2024, 17, 719

Joana Gonçalves, José Francisco Cascalheira, Patrícia Valentão, Ângelo Luís, Eugenia Gallardo and Ana Paula Duarte. "*The role of ayahuasca in cell viability and oxidative stress in gastric adenocarcinoma cell line*" Natural Product Research 2024

3.1. Paper III- Comparative Study of Sample Preparation Procedures to Determine the Main Compounds in Ayahuasca Beverages by Quechers and High-Performance Liquid Chromatography Analysis

Abstract

Introduction: Ayahuasca is a psychoactive drink originally consumed by indigenous people of the Amazon. The lack of regulation of this drink leads to uncontrolled consumption, and it is often consumed in religious contexts.
Objective: The aim of this work is to compare three miniaturised extraction techniques for extracting the main ayahuasca compounds from beverages.
Methodology: Three sample pretreatment techniques were evaluated (dispersive liquid–liquid microextraction [DLLME], microextraction by packed sorbent [MEPS] and QuEChERS [Quick, Easy, Cheap, Effective, Rugged and Safe]) for the simultaneous extraction of N,N-dimethyltryptamine (DMT), tetrahydroharmine (THH), harmine, harmaline, harmol and harmalol from ayahuasca beverage samples. Then, the most promising technique (QuEChERS) was chosen to pre-concentrate the analytes, subsequently detected by high-performance liquid chromatography coupled to a diode array detector (HPLC-DAD).
Results: The procedure was optimised, with the final conditions being 500 µL of extractor solvent, 85 mg of primary secondary amine (PSA) and 4 s of vortexing. The analytical method was validated, showing to be linear between 0.16 and 10 µg/mL for β-carbolines and between 0.016 and 1 µg/mL for DMT, with coefficients of determination (R2) between 0.9968 and 0.9993. The limit of detection (LOD) and lower limit of quantification (LLOQ) were 0.16 µg/mL for all compounds, except for
DMT (0.016 µg/mL) and extraction efficiencies varied between 60.2% and 88.0%. Conclusion: The analytical methodology proved to be accurate and precise, with good linearity, LODs and LLOQs.
This method has been fully validated and successfully applied to ayahuasca beverage samples.
Keywords: Ayahuasca, QuEChERS, MEPS, DLLME

3.1.1. Introdução

Ayahuasca is a decoction prepared from leaves of *Psychotria viridis* Ruiz & Pav. and *Banisteriopsis caapi* (Spruce ex Griseb.) C. V. Morton, being a thick and brown oily liquid[1,2].

The active ingredient of this mixture is the alkaloid *N,N*-dimethyltryptamine (DMT), a hallucinogenic compound that acts on serotonergic receptors (5-HT1A, 5-HT2A, and 5-HT2C) [2,3]. The actions of DMT are made possible through the actions of the β-carbolines harmaline, harmine, and tetrahydroharmine (THH), derived from *B. caapi* [2,4], since these components

inhibit monoamine oxidase-A (MAO-A), an enzyme that degrades DMT in the digestive tract. The synergy between DMT and β-carbolines results in hallucinogenic and visionary effects associated with this decoction[2,5,6]. The effects of ayahuasca can last for about 4 hours, starting 30 to 60 minutes after ingestion and reaching maximum intensity between 60 and 120 minutes after[4]. This psychoactive beverage was originally used by indigenous people as a sacred drink for healing purposes (spiritual, mental, and social) [1,4]. Later, the consumption of ayahuasca began to be used ritualistically as a religious sacrament and to facilitate self-knowledge, giving rise to the religions of the Barquinha, União do Vegetal and Santo Daime [1,2]. Currently, its consumption occurs all over the world (Europe, United States and Oceania), being known by the most varied names: *yage*, *caapi*, *natem*, *mihi*, *dapa*, *daime* and *hoasca*, among others [1,4].

The effects of taking ayahuasca may include physical symptoms as vomiting, diarrhea and nausea [2]. However, this drink leads to changes in auditory, visual and somatosensory perception, causing an altered state of consciousness with transpersonal experiences, visions, autobiographical memories and introspective effects [2,3]. The expansion of consumption of this psychoactive drink worldwide, as well as the report of its therapeutic potential, aroused much interest [7]. Thus, the reported effects have led to a constant increase in demand for ayahuasca for psychotherapeutic, self-realization and spiritual enlightenment purposes [7]. Nevertheless, the consumption of *B. caapi* or *P. viridis* is not regulated, and it is inclusively legal practice for religious purposes in Brazil and the United States of America [2,8]. The lack of regulation leads to uncontrolled consumption, occurring in a religious context from one year to a lifetime, with a monthly consumption of two or more times [2]. Therefore, it is important to control the safety regarding the regular consumption of this drink [2].

Among the compounds present in ayahuasca, indole alkaloids are the most investigated, not only for their structural diversity, but also for their potential beneficial effects [1]. A number of analytical methodologies has been developed aiming the detection and quantification of DMT and β-carboline alkaloids in samples of plasma [9–11], urine [12], hair [13]and blood [14], or in ayahuasca preparations [14–20]. However, sample pre-treatment methodologies are not always applied, and dilution and direct injection into the chromatographic equipment [12,14,17,18] is usually the practice, especially for the analysis of ayahuasca preparations. Additionally, the use of miniaturized techniques is also reduced in this type of samples, and, as far as we know, methodologies for detecting ayahuasca constituents in preparations of the drink have not yet been developed, namely by the Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) approach, microextraction by packed sorbent (MEPS) or dispersive liquid-liquid microextraction (DLLME). Adequate sample preparation is important for chromatographic analysis, having an important role on analyte isolation [21].

MEPS functions as a miniaturized version of solid-phase extraction (SPE) [21]. Unlike SPE, where the sorbent material is contained in a separate column, MEPS employs the syringe itself for sorbent placement, and can be reused several times. Moreover, MEPS significantly reduces solvent volume and allows for online coupling, enabling automation [22–25]. As for DLLME, it involves introducing an organic solvent with an extracting function and a dispersing solvent into an aqueous sample [26]. Following vigorous agitation, fine droplets form and disperse within the sample.

Subsequent centrifugation allows the collected drop in the tube to be injected into the chromatographic equipment [26]. The formation of droplets allows for a large contact area, making extraction immediate [27,28]. Moreover, DLLME is a low-cost, ecological technique that uses very simple and common instrumentation [28]. Concerning to QuEChERS, two phases are involved:: an initial partitioning and an extract cleanup phase using dispersive solid phase extraction (d-SPE) [29]. This methodology presents several advantages, since small volumes of sample and solvent are required, being a fast, simple and direct technique [29]. Additionally, QuEChERS generally presents a higher recovery rate, with better analytical performance, and a great versatility in the application to the most varied analytes and samples [29].

In this study, three miniaturized extraction techniques (DLLME, MEPS, and QuEChERS) were employed on four plant mixtures utilized in the preparation of ayahuasca decoctions, four individual plants, and a commercial mixture. Among these techniques, QuEChERS demonstrated superior efficacy in analyte extraction following a preliminary study and was therefore chosen for subsequent optimization and validation for the quantification of harmine, DMT, harmaline, harmol, harmalol, and THH in ayahuasca decoctions. This technique is usually not considered a miniaturized approach; however, in the present study, it suits this classification, due to the low amounts of solvents that were used (microliters).

3.1.2. Experimental

3.1.2.1. Reagents and Standards

The analytical standards of harmine, harmaline, THH, harmol, harmalol and DMT were kindly provided by Nal von Minden, GmbH (Regensburg, Germany). Methanol (HPLC grade), Isopropanol, Chloroform, Acetic acid, and Acetonitrile were obtained from Fischer Scientific (Loughborough, UK). Formic acid and Internal Standard (IS) 3,4-dihydroxybenzylamine (DHBA) were purchased from Sigma-Aldrich (Sintra, Portugal) and Ammonium hydroxide from Enzymatic (Santo Antão do Tojal, Portugal). Deionized water was obtained from a Milli-Q System (Millipore, Billerica, MA, USA). Primary Secondary Amine (PSA), Magnesium sulfate, and Sodium acetate were purchased from Laborspirit (Sintra, Portugal).

Working solutions of harmol, harmalol, THH, harmine and harmaline were prepared by dilution of the stock solutions (1 mg/mL) with methanol to the final concentration of 100 µg/mL and 10 µg/mL. Concerning DMT, the concentration of the stock solution was 100 µg/mL, and then serial dilutions were prepared, to the final concentrations of 10 µg/mL and 1 µg/mL (working solutions). The concentration of IS was 100 µg/mL. All solutions were stored protected from light at 4 °C.

3.1.2.2. Sample preparation

Plant samples were purchased from the Shayana Shop (https://www.shayanashop.com, Amsterdam, The Netherlands) (accessed May 25, 2019). The decoctions were prepared based on a

recipe provided by an ayahuasca consumer who was admitted to the emergency room with symptoms of intoxication. In this way, 0.210 g of each of the plant samples (*P. viridis*, *B. caapi*, *M. hostilis*, *P. harmala* and a commercial mixture) were weighed, and then ground in a mortar with a few drops of water.

Afterwards, the contents were transferred to a Schott flask together with250 mL of ultrapure water and then the flask was heated at 100 °C for 4 h. Four decoctions of mixtures of two plants were also prepared (*P. viridis* and *P. harmala*; *P. viridis* and *B. caapi*; *M. hostilis* and *P. harmala*; *M. hostilis* and *B. caapi*). After this time, the decoctions were cooled, filtered, frozen at – 80°C and lyophilized.

3.1.2.3. Sample pre-treatment

3.1.2.3.1. MEPS

The procedure was performed according to the study by Malaca *et al.* [30], with some modifications. The MEPS C_{18} sorbent was initially conditioned with 250 µL of methanol (1 cycle) followed by 250 µL of water (1 cycle). Subsequently, the sample, comprising 100 µL (including 50 µL of IS at 100 µg/mL), was loaded (10 cycles). A washing step was performed using 150 µL of water (1 cycle) and 150 µL of a 5% methanol solution (1 cycle). Next, the elution of the sample occurred with 100 µL of a solution containing acetonitrile with 2% ammonium hydroxide (4 cycles). The resulting extract was evaporated to dryness using a stream of nitrogen, reconstituted in 150 µL methanol, filtered through a 0.2 µm filter, and then injected into the HPLC-DAD system. To reconstitute the sorbent, 100 µL of two solutions was used (1% formic acid in isopropanol:water (10:90) and ammonium hydroxide in acetonitrile:methanol (1:1)) (4 cycles).

3.1.2.3.2. DLLME

The extraction procedure was performed by modifying the technique of Fernández *et al.* [30]. The sample (1 mL) was mixed with 1 mL of acetonitrile, 125 µL of chloroform and 50 µL of IS at 100 µg/mL. Then, the mixture was submerged in a ultrasonic bath for 3 minutes, and then centrifuged for 5 minutes at 4000 rpm. Finally, the drop deposited at the bottom of the tube was aspirated and evaporated to dryness under a stream of nitrogen. The extract was reconstituted in 150 µL methanol, filtered with a 0.2 µm filter and injected into the HPLC-DAD system.

3.1.2.3.3. QuEChERS

The QuEChERS procedure was performed as follows: 50 µL of IS (100 µg/mL) was mixed with 1 mL of sample, 0.4 g of MgSO4, 0.1 g of NaC2H3O2 and 1% acetic acid in acetonitrile (1.5 mL). The mixture was vortexed for 10 seconds and then centrifuged for 2 minutes at 4400 rpm. The resulting supernatant was collected, followed by the addition of 150 mg MgSO4 and 25 mg of PSA. After another 10-second vortex, the solution underwent centrifugation at 4400 rpm for 3 minutes.

The supernatant was once again collected, subjected to evaporation under a nitrogen stream until dry, reconstituted in 150 µL of methanol, filtered through a 0.2 µm filter, and subsequently injected into the HPLC-DAD system.

3.1.2.4. Instrumental and Chromatographic Conditions

The compounds present in the ayahuasca decoctions were quantified using a HPLC system coupled to a diode array detector (DAD) (Agilent technologies Soquímica, Lisbon, Portugal). Samples were kept in the sampler at 4°C and injected onto a YMC-Triart PFP analytical column (5 µm, 4.6 i.d. × 150 mm) coupled to a Guard-c holder (4 × 10 mm) and a Triart PFP (5 µm, 3 × 10 mm) pre-column, all from YMC Europe GMBH (Solitica, Lisbon, Portugal), maintained at 25 °C. The mobile phase was composed of 0.1% formic acid in methanol in line A and 0.1% formic acid in water in line B, at a flow rate of 1 mL/min. Fifty µL was injected, and the elution was performed in gradient mode: 5% A (0–2 min), 50% A (2–50 min) and again, 5% A (50–60 min). harmol and harmine were detected at 246 nm, harmalol and harmaline at 360 nm, THH and DMT at 278 nm.

3.1.2.5. Validation procedure

In order to validate the described method, guiding principles of the ANSI/ASB Standard 036 were followed [32], and the studied parameters included selectivity; linearity and limits; intermediate, intraday and inter-day precision and accuracy, and extraction efficiency. The linearity (n =5) was determined between 0.016 and 1 µg/mL for DMT and 0.16 and 10 µg/mL for the remaining analytes. The calibration curves were obtained by plotting the ratio of the peak area between each analyte and the peak area of the IS against the analyte concentration. Accuracy results within 15% (20% at the LLOQ), with a coefficient of determination (R2) equal to or higher than 0.99, and coefficients of variation (CVs) not exceeding 15% (20% at the LLOQ) were accepted. The LLOQ was defined as the minimum concentration that could be measured precisely and accurately, i.e. presenting a relative error (RE) of less than 20% of the nominal concentration and coefficient of variation of less than 20%. In order to define the limits of detection (LOD), three replicates of enriched samples were analysed. The LOD was defined as the lowest concentrations where it was possible to visualize a distinct peak, clearly discernible from the blank and with a signal-to-noise ratio of at least 3. To evaluate the intraday precision and accuracy, on the same day, blank samples spiked with the target analytes (minimum of three different concentration levels) were analysed in triplicate. Within the same period, inter-day precision and accuracy were evaluated at seven concentrations. Intermediate precision and accuracy were calculated with three quality controls (QC) samples in triplicate at the concentrations 0.16, 0.63 and 5 µg/mL (0.016, 0.063 and 0.5 µg/mL for DMT) over the 3-day protocol. Two sets of samples in triplicate were prepared at the concentrations 0.16, 0.63 and 5 µg/mL, in order to analyse extraction efficiency. Set one represented peaks obtained by analysis of samples spiked before extraction, while set 2 consisted of peaks obtained by after extraction spikes (representing 100% efficiency), with IS being added to

both sets after extraction. The efficiency results were calculated by the ratio between the relative peak areas of sample set 1 and sample set 2.

3.1.3. Results and Discussion

3.1.3.1. Extraction Procedure Selection

Initially, the best sample pre-treatment method was chosen. Thus, the three extraction methods (QuEChERS, DLLME and MEPS) were tested on a sample of ayahuasca beverage, in order to verify for which better areas were obtained. Analysing Figure III.1.1, it is possible to verify that for harmol and harmalol, there are high variations in the areas of the compounds after applying the different extraction techniques, whereas for the remaining compounds, the QuEChERS technique clearly showed superior areas. Thus, this technique was chosen to proceed to the following stages of the work.

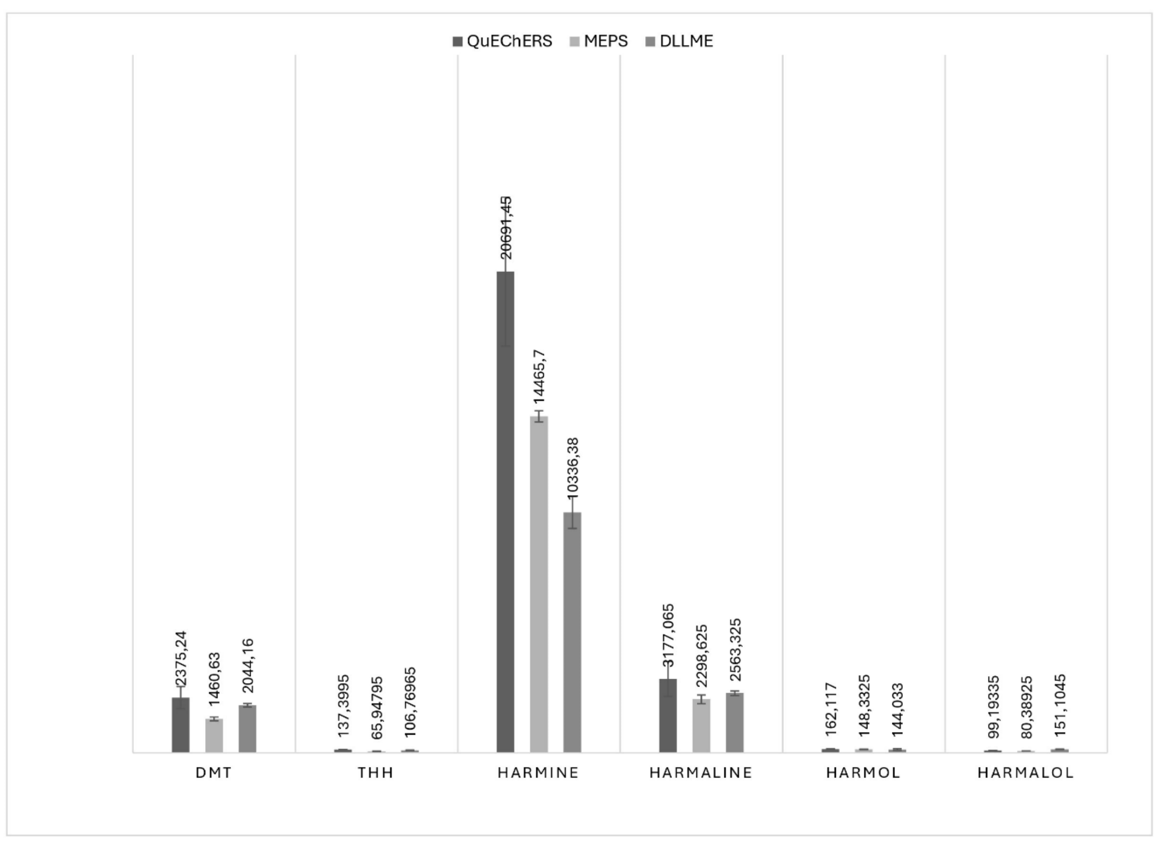


Figure III.1.1. Analyte peak areas obtained in each extraction procedure (n=3).

DLLME has been used to quantify DMT and β-carboline alkaloids in human plasma in a paper by Silveira *et al.* [33], however none of the described sample treatment approaches was applied to ayahuasca beverage samples.

3.1.3.2. Optimization of the QuEChERS extraction

In an initial phase of the optimization process, the DOE statistical tool MINITAB® was used. This tool allows multivariate analysis of parameters that can significantly influence the extraction procedure. Multifactor analysis is crucial in an optimization process, since its use allows obtaining the best analyte recoveries, while maintaining the number of experiments at a minimum [21]. Thus, a two-level, three-component (23) factorial design was performed to analyse the relevant variables to the recovery of analytes and their main effects. The independent variables consisted of extractor solvent volume, amount of PSA and vortex time. A total of eleven runs were generated from this factorial design, covering all potential combinations of factor values. The independent variables were systematically tested at high and low levels: the values chosen for the extractor solvent volume were 0.5 mL and 2.5 mL, for the amount of PSA were 15 and 35 mg, and for the vortex time 10 and 30 seconds. Each experiment was randomly conducted with a central point in triplicate, aiming to minimize the impact of noise factors and systematic errors. The obtained results are shown in Figures 2 and 3, which represent the main effects and Pareto charts generated for each target analyte through the Design of Experiments (DOE) analysis.

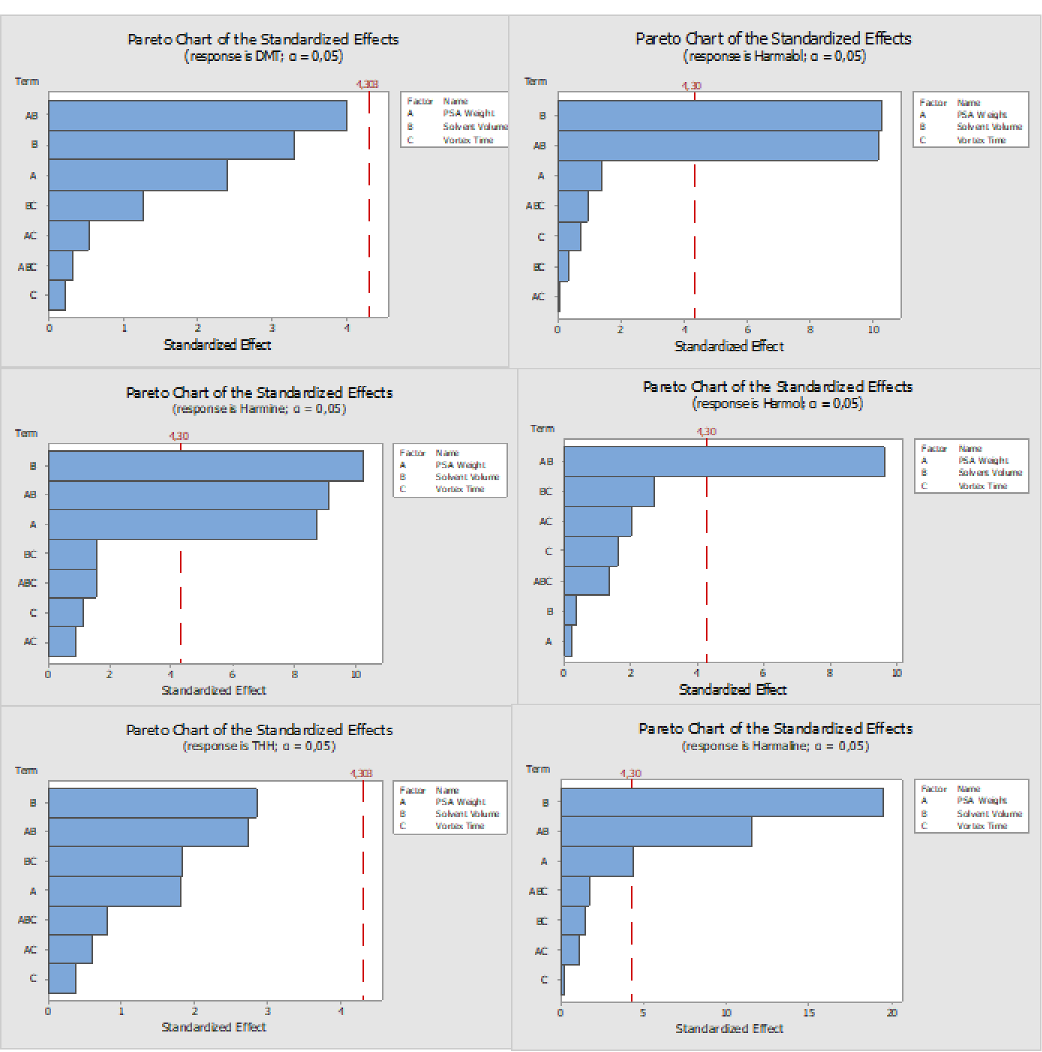


Figure III.1.2. Pareto diagrams of the compounds under study.

Looking at these Pareto charts, one can see that while DMT and THH were not affected significantly by none of the chosen parameters, harmine and harmaline were affected by the volume of solvent and amount of PSA, and by an interaction of these two factors. Regarding harmol, only one interaction of the solvent volume and amount of PSA was statistically significant, while for harmalol the same interaction and solvent volume also significantly influenced the recovery.

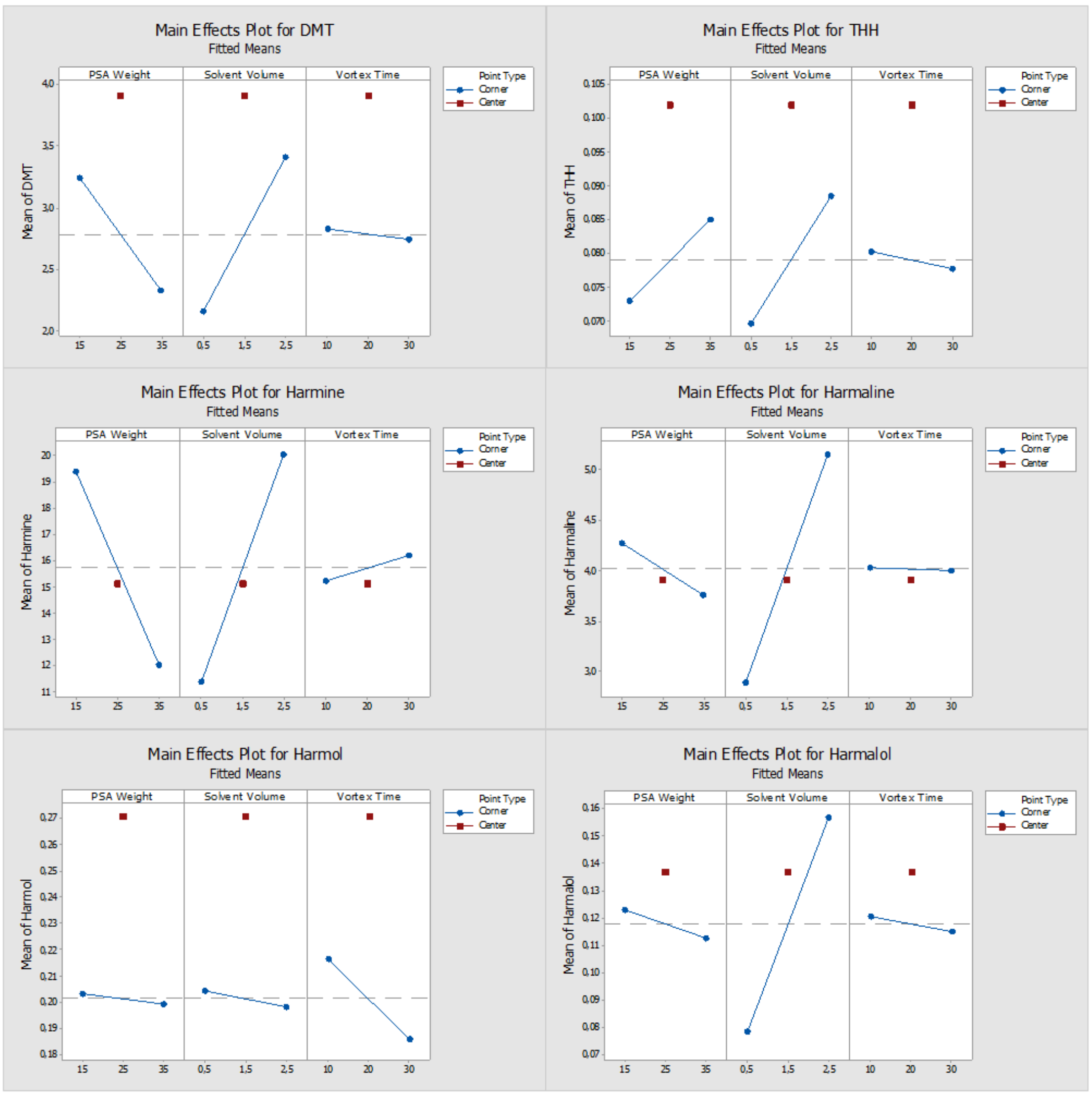


Figure III.1.3. Plots of main effects of the factors tested on each parameter of each analyte.

Figure III.1.3 shows the graphs of main effects where the effect of each factor studied for each studied analyte can be observed. Except for THH, a greater recovery was obtained using a lower amount of PSA. Regarding the volume of solvent, a greater amount of analyte is obtained with a greater volume of solvent, except for harmol whose recovery is favoured with a smaller volume of extractor solvent. Finally, a shorter vortex time favoured the recovery of all analytes,

except for harmine. Despite these results, it must be considered that none of these factors significantly influenced the response obtained for DMT and THH. Likewise, the vortex time factor also did not significantly influence the behaviour of the analytes. However, harmaline, harmol, harmine and harmalol were significantly influenced by the volume of extractor solvent, by the amount of PSA, or even by an interaction between these two variables. Thus, since four of the analytes were significantly influenced by at least one of the variables tested, it was deemed necessary to further optimize the method.

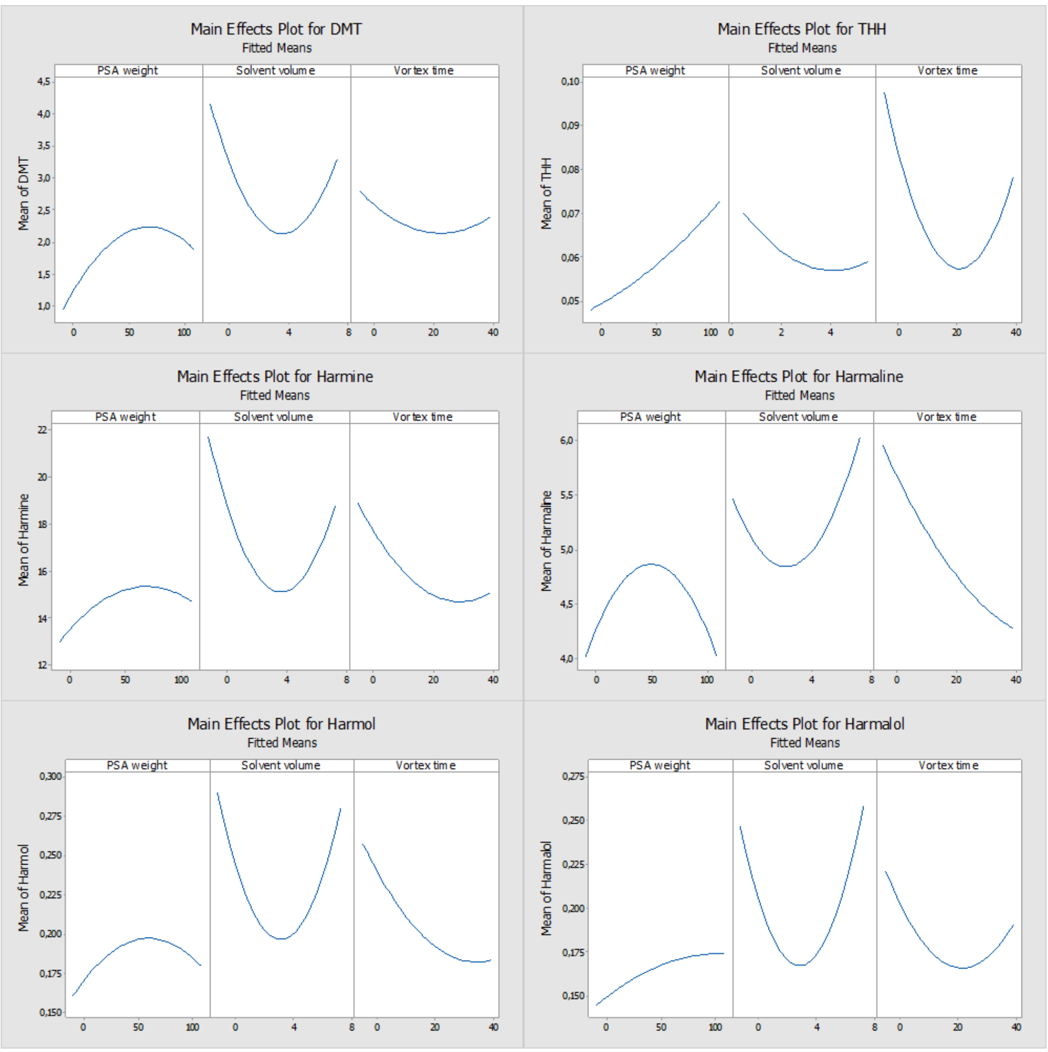


Figure III.1.4. Results from RSM response optimizer for analytes.

With DOE optimization it was not possible to draw an adequate conclusion, and therefore an experimental response surface (RSM) methodology was applied to all analytes. A new matrix was constructed with the same variables. However, the number of experiments increased, and the testing range was wider (volume of solvent varying between 0.5 mL and 7.2 mL, amount of PSA between 5 mg and 108.8 mg and vortex time between 4 and 39 seconds). The response to the main effects (Figure III.1.4.) exhibited that the analytes showed a better response for the minimum vortex time (4 seconds), although this variable was not significant. In addition, the recovery of the analytes was maximum for the minimum volume of extractor solvent (500 µL) for all analytes,

except for harmaline and harmalol. Finally, the amount of analyte was maximum when using 85 mg of PSA for DMT, harmine and harmol. Conversely, a better response was obtained with an amount of PSA between 85 and 100 mg for THH and harmalol. Regarding harmaline, the response was more favourable with only 50 mg of PSA.

The following sample pre-treatment procedure was established: 85 mg of PSA, 500 µL of extracting solvent, and vortex for 4 seconds.

3.1.3.3. Method Validation Parameters

3.1.3.3.1. Linearity and Calibration Model

The analytical method was considered linear between 0.16 and 10 µg/mL for all β-carbolines, for DMT it was linear in the range of 0.016 to 1 µg/mL, with the R^2 between 0.9968 and 0.9993. The accuracy of the calibrators used (mean relative error (BIAS) between measured and spiked values) remained within the range of ± 15% (± 20% for LLOQ) for all concentrations and coefficients of variation (CV) below 15% indicating an acceptable precision. The data are shown in Table III.1.1.

Table III.1.1. Linearity data (n = 5).

Compound	Linear Range (µg/mL)	Linearity (mean ± SD)		R2 (meane ± SD)	LLOQ and LOD (µg/mL)
		Slope (m)	Intercept (b)		
DMT	0.016-1	0.2651 ± 0.0065	0.0035 ± 0.0005	0.9993 ± 0.0004	0.016
Harmalol	0.16 - 10	0,1743 ± 0.0159	0.0001 ± 0.0031	0.9982 ± 0.0025	0.16
Harmol	0.16 - 10	1.0293± 0.1758	-0.0261 ± 0.0206	0.9978 ± 0.0035	0.16
THH	0.16 - 10	0. 0680± 0.0067	-0.0062± 0.0036	0.9985 ± 0.0016	0.16
Harmaline	0.16 - 10	0.3941 ± 0.0575	-0.0010± 0.0223	0.9968 ± 0.0034	0.16
Harmine	0.16 - 10	2.2773 ± 0.1989	-0.0515 ± 0.0806	0.9978 ± 0.0025	0.16

3.1.3.3.2. Limits of Detection and Quantification

Except for DMT, where the value was 0.016 µg/mL, the LLOQ and LOD obtained was 0.16 µg/mL for all compounds (Table III.1.1.). As a result, the LLOQs are satisfactory compared to other published works where the same analytes were analysed. Thus, comparable or better results were obtained for DMT [15,34–39], THH [15,35,37], harmine [15,35,37], harmaline [15,35,37] and harmalol [37]. However, Eller *et al.* [38] reported lower LLOQs for harmine, harmaline and THH,

but using however a much more sensitive equipment (UHPLC-MS/MS). Other works that used liquid chromatography associated with tandem mass spectrometry, reported LLOQs of the same order of magnitude or higher than those of the present study [17,40]. Analytical methods for the detection of DMT and β-carbolines in biological samples were also described, namely for plasma [9–11,33,41], hair [13], blood [14], sweat [42] and urine [43]. In these works, LOD and LLOQ values are presented in the order of ng/mL or ng/mg, but once again the equipment used is much more sensitive and almost always associated with mass spectrometry [11,13,14,33,41–44].

This technique proves to be very advantageous when comparing the limits obtained and volume of solvent (microliters), amount of reagents and reduced preparation time. This methodology is the first to quickly detect and quantify DMT and β-carboline alkaloids in ayahuasca decoctions, using QuEChERS and liquid chromatography, presenting adequate LLOQs, perfectly comparable to those published elsewhere.

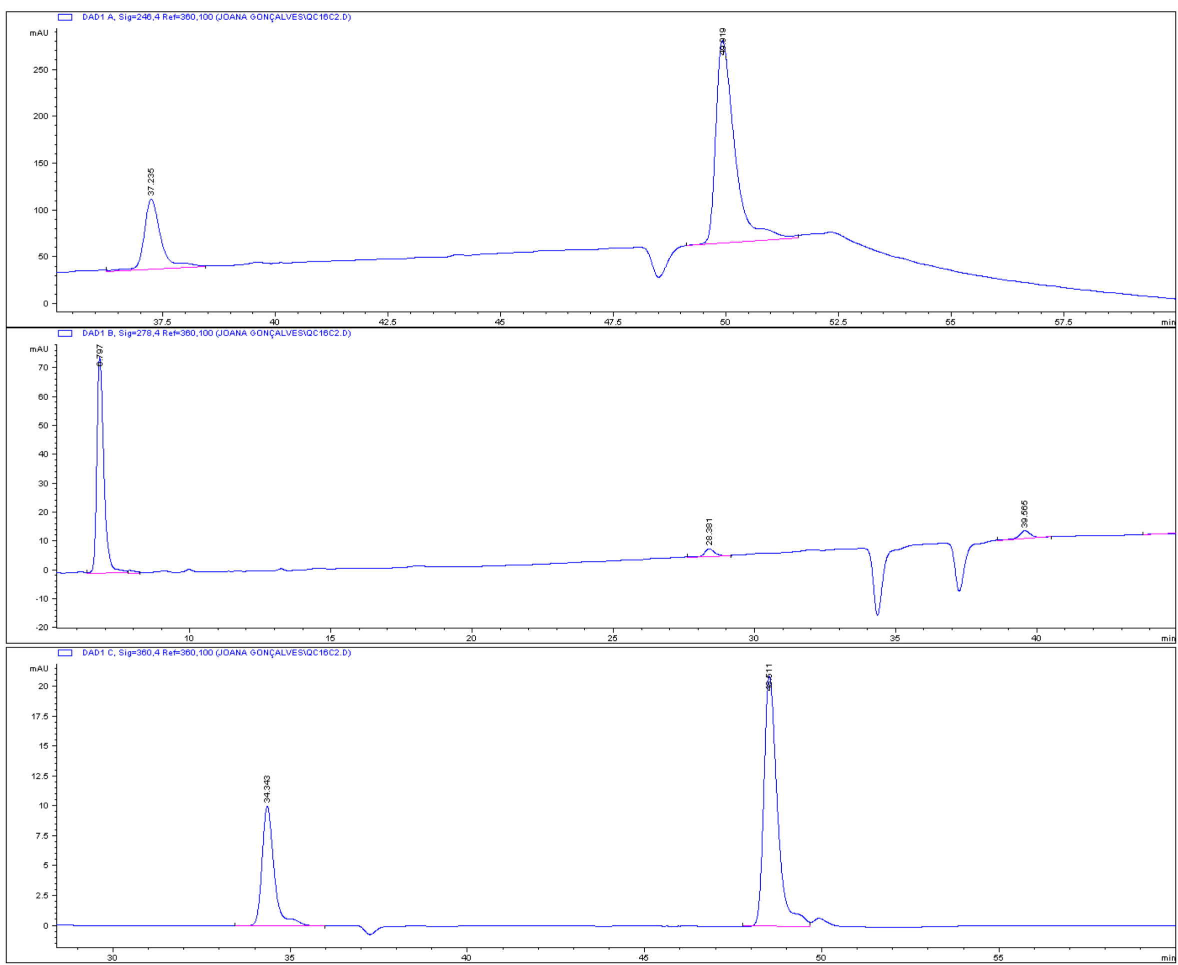


Figure III.1.5. Chromatograms of compounds at the LLOQ. 246 nm: harmol (retention time 37.2 min) and harmine (retention time 49.9 min); 278 nm: IS (retention time 6.8 min), DMT (retention time 28.4 min) and THH (retention time 39.6 min) and 360 nm: harmalol (retention time 31.3) and harmaline (retention time 48.5 min).

3.1.3.3.3. Intra-Day, Inter-Day, and Intermediate Precision and Accuracy

The evaluation of intra-day accuracy and precision was performed through the analysis of three calibrators of known concentrations, within the linear range (n=7). The results obtained are shown in Table III.1.2, where CVs for all studied concentrations were less than 15%, with a mean relative error of ± 13.2%.

Table III.1.2. Intra-day precision and accuracy.

	Intra-day			
Compound	**Concentration (μg/mL)**	**Measured Concentration (mean± SD) (μg/mL)**	**CV (%)**	**RE (%)**
DMT	0.016	0.01 ± 0.00	2.44	-11.94
	0.063	0.07 ±0.00	2.04	13.22
	0.5	0.53 ± 0.02	3.80	5.43
Harmalol	0.16	0.16 ± 0.02	9.48	-0.52
	0.63	0.69 ± 0.04	5.62	9.25
	5	5.13 ± 0.50	9.72	2.62
Harmol	0.16	0.16 ± 0.01	5.33	1.65
	0.63	0.64 ± 0.04	5.97	0.85
	5	4.97 ± 0.12	2.51	-0.56
THH	0.16	0.14 ± 0.01	4.47	-10.51
	0.63	0.59 ± 0.06	9.93	-6.82
	5	4.74 ± 0.12	2.54	-5.22
Harmaline	0.16	0.16 ± 0.00	3.01	3.09
	0.63	0.61 ± 0.06	10.14	-3.41
	5	4.98 ± 0.15	3.09	-0.46
Harmine	0.16	0.17 ± 0.00	2.68	8.97
	0.63	0.71 ± 0.02	2.59	12.40
	5	5.15 ± 0.28	5.39	2.94

Inter-day precision and accuracy were evaluated over five days for a total of seven calibrators. All concentrations had CVs of less than 15%, with a mean relative error of ± 16.3%. The results obtained are shown in Table III.1.3.

Table III.1.3. Inter-day precision and accuracy.

	Inter-day			
Compound	**Concentration (μg/mL)**	**Measured Concentration (mean ± SD) (μg/mL)**	**CV (%)**	**RE (%)**
DMT	0.016	0.02 ± 0.00	15.18	-4.51
	0.031	0.03 ± 0.00	8.62	1.71
	0.063	0.07 ± 0.00	4.74	9.53
	0.125	0.12 ± 0.01	6.61	-1.90
	0.25	0.25 ± 0.00	0.80	-1.34
	0.5	0.50 ± 0.02	4.48	-0.25
	1	1.00 ± 0.01	1.02	0.13
Harmalol	0.16	0.16 ± 0.02	15.43	2.85
	0.31	0.32 ± 0.01	2.63	2.88
	0.63	0.63 ± 0.04	6.49	0.52
	1.25	1.30 ± 0.14	10.93	3.73
	2.5	2.46 ± 0.12	4.68	-1.51
	5	4.82 ± 0.32	6.70	-3.60

	10	10.07 ± 0.13	1.25	0.66
Harmol	0.16	0.15 ± 0.02	10.16	-4.00
	0.31	0.29 ± 0.02	7.50	-6.33
	0.63	0.60 ± 0.04	7.10	-4.36
	1.25	1.22 ± 0.11	9.11	-2.23
	2.5	2.44 ± 0.10	4.08	-2.52
	5	4.78 ± 0.41	8.62	-4.38
	10	10.04 ± 0.12	1.20	0.40
THH	0.16	0.17 ± 0.01	7.05	10.87
	0.31	0.33 ± 0.03	8.26	5.83
	0.63	0.66 ± 0.03	5.27	5.49
	1.25	1.17 ± 0.05	4.57	-6.53
	2.5	2.38 ± 0.15	6.19	-4.80
	5	5.20 ± 0.22	4.30	3.92
	10	9.94 ± 0.08	0.80	-0.56
Harmaline	0.16	0.18 ± 0.00	1.39	14.23
	0.31	0.31 ± 0.02	5.95	0.24
	0.63	0.66 ± 0.04	5.80	5.84
	1.25	1.27 ± 0.14	10.76	1.78
	2.5	2.47 ± 0.13	5.08	-1.29
	5	4.67 ± 0.38	8.05	-6.52
	10	10.12 ± 0.14	1.41	1.22
Harmine	0.16	0.18 ± 0.01	5.44	16.29
	0.31	0.34 ± 0.01	2.58	12.10
	0.63	0.70 ± 0.03	4.71	10.11
	1.25	1.28 ± 0.14	11.11	7.48
	2.5	2.45 ± 0.17	6.87	0.34
	5	4.77 ± 0.27	5.66	-3.25
	10	10.12 ± 0.12	1.16	-1.69

Table III.1.4. Intermediate precision and accuracy.

		Intermediate		
Compound	**Concentration (µg/mL)**	**Measured Concentration (mean ± SD) (µg/mL)**	**CV (%)**	**RE (%)**
DMT	0.016	0.02 ± 0.00	10.84	3.36
	0.063	0.07 ±0.00	1.03	14.06
	0.5	0.53 ± 0.02	3.68	6.46
Harmalol	0.16	0.17 ± 0.01	6.06	8.96
	0.63	0.72 ± 0.02	2.39	13.79
	5	5.49 ± 0.21	3.83	9.78
Harmol	0.16	0.18 ± 0.01	3.10	12.62
	0.63	0.70 ± 0.04	5.11	11.70
	5	5.51 ± 0.38	6.92	10.14
THH	0.16	0.17 ± 0.02	10.29	4.89
	0.63	0.68 ± 0.04	5.45	8.45
	5	5.28 ± 0.44	8.43	5.53
Harmaline	0.16	0.18 ± 0.01	3.40	9.45
	0.63	0.69 ± 0.04	5.31	10.05
	5	5.38 ± 0.49	9.07	7.59
Harmine	0.16	0.17 ± 0.02	13.36	0.5
	0.63	0.71 ± 0.01	2.07	12.80
	5	5.65 ± 0.10	1.70	12.97

Finally, for the evaluaion of the intermediate precision and accuracy, three QCs of known concentrations were used (n=3). Solutions of 0.16, 0.63 and 5 µg/mL (0.016, 0.063 and 0.5 µg/mL

for DMT) were analysed for five days, with CVs obtained being less than 13.4% with an imprecision of ± 14.1% (Table III.1.4).

3.1.3.3.4. Extraction Efficiency

The extraction efficiency of the technique was measured using two sets of three samples, prepared by adding analytes at concentrations of 0.16, 0.63 and 5 µg/mL (0.016, 0.06 and 0.5 µg/mL for DMT) to water. One of the sets was fortified before extraction and the other set was fortified after extraction, corresponding to 100% recovery. The extraction efficiency was calculated through the proportion between the relative peak areas of each sample of the first set to those of the second set. Extraction efficiencies are shown in Table III.1.5.

Table III.1.5. Extraction efficiency (%) of the target analytes (n = 3).

Compound	Concentration (µg/mL)	Efficiency (mean % ± SD)
DMT	0.016	70.1 ± 6.4
	0.032	88.0 ± 12.8
	0.5	78.5 ± 5.0
Harmalol	0.16	68.5 ± 7.6
	0.32	73.5 ± 7.5
	5	77.6 ± 3.5
Harmol	0.16	75.8 ± 7.1
	0.32	81.4 ± 10.3
	5	63.9 ± 3.4
THH	0.16	64.8 ± 3.8
	0.32	84.4 ± 5.9
	5	77.3 ± 3.5
Harmaline	0.16	63.8 ± 9.8
	0.32	79.3 ± 7.00
	5	67.4 ± 7.5
Harmine	0.16	60.2 ± 9.2
	0.32	79.1 ± 1.9
	5	64.5 ± 5.6

As far as we know, this is the only analytical methodology that uses the QuEChERS technique in ayahuasca samples. In other methods where the quantification of analytes in ayahuasca samples was performed either did not pre-treat the sample [17,34,35,38,40] or have used approaches such as solid phase extraction (SPE) [15,37], solid-phase microextraction (SPME) [36] and matrix solid phase dispersion (MSPD) [39]. The use of SPE as extraction technique showed higher recoveries for DMT [15,37], THH [15,37], harmine [15,37] and harmaline [15,37]. However, the present study demonstrated a better extraction efficiency for harmalol (68.9-75.8%) compared to SPE, where these values varied between 45 and 58.4% [37]. Gaujac *et al.* [36] obtained better recoveries for DMT (71-109%) with the application of SPME; however, it is important to mention that in that study only DMT was quantified. The same research group used MSPD as a technique for extracting DMT, again showing better recoveries for only one compound [39].It should be noted that despite presenting lower recoveries, the QuEChERS technique is more environmentally friendly compared to SPE, where larger volumes of organic solvents are used, and more economical, since it is not necessary to purchase single-use cartridges [29]. In contrast,

although the SPME technique is a miniaturized technique, and uses smaller volumes of solvents, it requires the use of fibers, which, although reusable, are quite expensive [29]. The QuEChERS technique is a quick, simple, easy and economical approach, which does not require the acquisition of specific material, making it very advantageous [29]. However, we must bear in mind that we are dealing with a sample prepared with plant products, which may present variability in compounds between samples of different origins. Additionally, it should be noted that extraction efficiency can also be influenced by parameters such as sample viscosity or analyte solubility, which determines the success of the sample filtration step.

3.1.3.4. Method Applicability

The developed methodology was successfully applied to four decoctions of individual plants used in the preparation of ayahuasca, one decoction of a commercial mixture and four decoctions of two mixtures of plants. The results are presented in Table III.1.6.

Table III.1.6. Concentrations of compounds found in authentic samples.

Sample	Compound	Concentration (μg/mL)
P. viridis	DMT	31.13 ± 4.78
B. caapi	Harmalol	0.02 ± 0.00
	Harmol	0.08 ± 0.00
	THH	1.22 ± 0.11
	Harmaline	0.11 ± 0.01
	Harmine	2.14 ± 0.33
P. harmala	Harmalol	1.20 ± 0.11
	Harmol	0.30 ± 0.03
	THH	1.98 ± 0.17
	Harmaline	17.30 ± 2.00
	Harmine	13.13 ± 1.66
M. hostilis	DMT	15.00 ± 1.78
Commercial Mixture	DMT	3.33 ± 0.05
	Harmalol	ND
	Harmol	ND
	THH	ND
	Harmaline	0.03 ± 0.00
	Harmine	0.16 ± 0.02
P. viridis + B. caapi	DMT	3.43 ± 0.08
	Harmalol	0.06 ± 0.01
	Harmol	0.07 ± 0.00
	THH	2.87 ± 0.32
	Harmaline	1.45 ±0.28
	Harmine	2.46 ± 0.05
	DMT	3.81 ± 0.61

P. viridis + P. harmala	Harmalol	0.21 ± 0.03
	Harmol	0.09 ± 0.00
	THH	1.08 ± 0.08
	Harmaline	3.44 ± 0.66
	Harmine	3.91 ± 0.43
M. hostilis + B. caapi	DMT	15.43 ± 0.48
	Harmalol	0.19 ± 0.00
	Harmol	0.41 ± 0.02
	THH	10.66 ± 0.32
	Harmaline	0.44 ± 0.01
	Harmine	7.26 ± 0.19
M. hostilis + P. harmala	DMT	5.32 ± 0.19
	Harmalol	0.05 ± 0.03
	Harmol	0.40 ± 0.06
	THH	1.43 ± 0.01
	Harmaline	7.03 ± 0.24
	Harmine	5.78 ± 0.23

Caption: ND: not detected

3.1.4. Conclusion

This study aims at the development and validation of a new analytical method to simultaneously determine six constituents of ayahuasca. The QuEChERS technique was used and fully optimized for sample pre-treatment. This procedure allowed the maximization of results, leading to the achievement of lower LODs and LLOQs with a quick, simple, economical method.

The analytical methodology proved to be accurate and precise, being linear in the range of 0.16 to 10 μg/mL (0.016 to 1 μg/mL for DMT). It was possible to obtain LODs and LLOQs of 0.16 μg/mL (0.016 μg/mL for DMT). This method was fully validated and successfully applied to ayahuasca beverage samples.

To the best of our knowledge, this is the first study where three sample pretreatment techniques were compared and where QuEChERS were used for extracting ayahuasca constituents. This analytical approach can be considered advantageous for analytical laboratories, since it is easy, has low costs and allows the use of a reduced amount of organic solvents.

3.1.5. References

1. Katchborian-Neto, A.; Santos, W.T.; Nicácio, K.J.; Corrêa, J.O.A.; Murgu, M.; Martins, T.M.M.; Gomes, D.A.; Goes, A.M.; Soares, M.G.; Dias, D.F.; et al. Neuroprotective potential of Ayahuasca and untargeted metabolomics analyses: applicability to Parkinson’s disease. J. Ethnopharmacol. 2020, 255, doi:10.1016/J.JEP.2020.112743.

2. Mello, S.M.; Soubhia, P.C.; Silveira, G.; Corrêa-Neto, N.F.; Lanaro, R.; Costa, J.L.; Linardi, A. Effect of Ritualistic Consumption of Ayahuasca on Hepatic Function in Chronic Users. J. Psychoactive Drugs 2019, 51, 3–11, doi:10.1080/02791072.2018.1557355.

3. Franquesa, A.; Sainz-Cort, A.; Gandy, S.; Soler, J.; Alcázar-Córcoles, M.Á.; Bouso, J.C. Psychological variables implied in the therapeutic effect of ayahuasca: A contextual approach. Psychiatry Res. 2018, 264, 334–339,

doi:10.1016/J.PSYCHRES.2018.04.012.

4. Uthaug, M. V.; van Oorsouw, K.; Kuypers, K.P.C.; van Boxtel, M.; Broers, N.J.; Mason, N.L.; Toennes, S.W.; Riba, J.; Ramaekers, J.G. Sub-acute and long-term effects of ayahuasca on affect and cognitive thinking style and their association with ego dissolution. Psychopharmacology (Berl). 2018, 235, 2979–2989, doi:10.1007/S00213-018-4988-3.

5. Madrid-Gambin, F.; Gomez-Gomez, A.; Busquets-Garcia, A.; Haro, N.; Marco, S.; Mason, N.L.; Reckweg, J.T.; Mallaroni, P.; Kloft, L.; van Oorsouw, K.; et al. Metabolomics and integrated network analysis reveal roles of endocannabinoids and large neutral amino acid balance in the ayahuasca experience. Biomed. Pharmacother. 2022, 149, doi:10.1016/J.BIOPHA.2022.112845.

6. Ruffell, S.; Netzband, N.; Bird, C.; Young, A.H.; Juruena, M.F. The pharmacological interaction of compounds in ayahuasca: a systematic review. Brazilian J. Psychiatry 2020, 42, 646, doi:10.1590/1516-4446-2020-0884.

7. Uthaug, M. V.; Mason, N.L.; Toennes, S.W.; Reckweg, J.T.; de Sousa Fernandes Perna, E.B.; Kuypers, K.P.C.; van Oorsouw, K.; Riba, J.; Ramaekers, J.G. A placebo-controlled study of the effects of ayahuasca, set and setting on mental health of participants in ayahuasca group retreats. Psychopharmacology (Berl). 2021, 238, 1899–1910, doi:10.1007/S00213-021-05817-8.

8. Gonçalves, J.; Luís, Â.; Gallardo, E.; Duarte, A.P. Psychoactive Substances of Natural Origin: Toxicological Aspects, Therapeutic Properties and Analysis in Biological Samples. Mol. 2021, Vol. 26, Page 1397 2021, 26, 1397, doi:10.3390/MOLECULES26051397.

9. Callaway, J.C.; Raymon, L.P.; Hearn, W.L.; McKenna, D.J.; Grob, C.S.; Brito, G.S.; Mash, D.C. Quantitation of *N,N*-dimethyltryptamine and harmala alkaloids in human plasma after oral dosing with ayahuasca. J. Anal. Toxicol. 1996, 20, 492–497, doi:10.1093/JAT/20.6.492.

10. Yritia, M.; Riba, J.; Ortuño, J.; Ramirez, A.; Castillo, A.; Alfaro, Y.; de la Torre, R.; Barbanoj, M.J. Determination of *N, N*-dimethyltryptamine and β-carboline alkaloids in human plasma following oral administration of Ayahuasca. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 2002, 779, 271–281, doi:10.1016/S1570-0232(02)00397-5.

11. Oliveira, C.D.R.; Okai, G.G.; Da Costa, J.L.; De Almeida, R.M.; Oliveira-Silva, D.; Yonamine, M. Determination of dimethyltryptamine and β-carbolines (ayahuasca alkaloids) in plasma samples by LC–MS/MS. http://dx.doi.org/10.4155/bio.12.124 2012, 4, 1731–1738, doi:10.4155/BIO.12.124.

12. Riba, J.; McIlhenny, E.H.; Valle, M.; Bouso, J.C.; Barker, S.A. Metabolism and disposition of *N,N*-dimethyltryptamine and harmala alkaloids after oral administration of ayahuasca. Drug Test. Anal. 2012, 4, 610–616, doi:10.1002/DTA.1344.

13. Pichini, S.; Marchei, E.; García-Algar, O.; Gomez, A.; Di Giovannandrea, R.; Pacifici, R. Ultra-high-pressure liquid chromatography tandem mass spectrometry determination of hallucinogenic drugs in hair of psychedelic plants and mushrooms consumers. J. Pharm. Biomed. Anal. 2014, 100, 284–289, doi:10.1016/J.JPBA.2014.08.006.

14. McIlhenny, E.H.; Riba, J.; Barbanoj, M.J.; Strassman, R.; Barker, S.A. Methodology for determining major constituents of ayahuasca and their metabolites in blood. Biomed. Chromatogr. 2012, 26, 301–313, doi:10.1002/BMC.1657.

15. Pires, A.P.S.; De Oliveira, C.D.R.; Moura, S.; Dörr, F.A.; Silva, W.A.E.; Yonamine, M. Gas chromatographic analysis of dimethyltryptamine and beta-carboline alkaloids in ayahuasca, an Amazonian psychoactive plant beverage. Phytochem. Anal. 2009, 20, 149–153, doi:10.1002/PCA.1110.

16. Gambelunghe, C.; Aroni, K.; Rossi, R.; Moretti, L.; Bacci, M. Identification of *N,N*-dimethyltryptamine and β-carbolines in psychotropic ayahuasca beverage. Biomed. Chromatogr. 2008, 22, 1056–1059, doi:10.1002/BMC.1023.

17. McIlhenny, E.H.; Pipkin, K.E.; Standish, L.J.; Wechkin, H.A.; Strassman, R.; Barker, S.A. Direct analysis of psychoactive tryptamine and harmala alkaloids in the Amazonian botanical medicine ayahuasca by liquid chromatography–electrospray ionization-tandem mass spectrometry. J. Chromatogr. A 2009, 1216, 8960–8968, doi:10.1016/J.CHROMA.2009.10.088.

18. Lesiak, A.D.; Musah, R.A. Application of ambient ionization high resolution mass spectrometry to determination of the botanical provenance of the constituents of psychoactive drug mixtures. Forensic Sci. Int. 2016, 266, 271–280, doi:10.1016/J.FORSCIINT.2016.06.009.

19. Huhn, C.; Neusüß, C.; Pelzing, M.; Pyell, U.; Mannhardt, J.; Pütz, M. Capillary electrophoresis-laser induced fluorescence-electrospray ionization-mass spectrometry: A case study. Electrophoresis 2005, 26, 1389–1397, doi:10.1002/ELPS.200410163.

20. Moura, S.; Carvalho, F.G.; de Oliveira, C.D.R.; Pinto, E.; Yonamine, M. qNMR: An applicable method for the determination of dimethyltryptamine in ayahuasca, a psychoactive plant preparation. Phytochem. Lett. 2010, 3,

79–83, doi:10.1016/J.PHYTOL.2009.12.004.

21. Rosendo, L.M.; Rosado, T.; Oliveira, P.; Simão, A.Y.; Margalho, C.; Costa, S.; Passarinha, L.A.; Barroso, M.; Gallardo, E. The Determination of Cannabinoids in Urine Samples Using Microextraction by Packed Sorbent and Gas Chromatography-Mass Spectrometry. Molecules 2022, 27, doi:10.3390/MOLECULES27175503.

22. Moein, M.M.; Abdel-Rehim, A.; Abdel-Rehim, M. Microextraction by packed sorbent (MEPS). TrAC Trends Anal. Chem. 2015, 67, 34–44, doi:10.1016/J.TRAC.2014.12.003.

23. Abdel-Rehim, M. Recent advances in microextraction by packed sorbent for bioanalysis. J. Chromatogr. A 2010, 1217, 2569–2580, doi:10.1016/J.CHROMA.2009.09.053.

24. Abdel-Rehim, M. New trend in sample preparation: on-line microextraction in packed syringe for liquid and gas chromatography applications: I. Determination of local anaesthetics in human plasma samples using gas chromatography–mass spectrometry. J. Chromatogr. B 2004, 801, 317–321, doi:10.1016/J.JCHROMB.2003.11.042.

25. Silva, C.; Cavaco, C.; Perestrelo, R.; Pereira, J.; Câmara, J.S. Microextraction by Packed Sorbent (MEPS) and Solid-Phase Microextraction (SPME) as Sample Preparation Procedures for the Metabolomic Profiling of Urine. Metabolites 2014, 4, 71–97, doi:10.3390/METABO4010071.

26. Viñas, P.; Campillo, N.; López-García, I.; Hernández-Córdoba, M. Dispersive liquid-liquid microextraction in food analysis. A critical review. Anal. Bioanal. Chem. 2014, 406, 2067–2099, doi:10.1007/S00216-013-7344-9.

27. Ban, T.; Kawaizumi, F.; Nii, S.; Takahashi, K. Study of drop coalescence behavior for liquid–liquid extraction operation. Chem. Eng. Sci. 2000, 55, 5385–5391, doi:10.1016/S0009-2509(00)00156-1.

28. Kissoudi, M.; Samanidou, V. Recent Advances in Applications of Ionic Liquids in Miniaturized Microextraction Techniques. Molecules 2018, 23, doi:10.3390/MOLECULES23061437.

29. Perestrelo, R.; Silva, P.; Porto-Figueira, P.; Pereira, J.A.M.; Silva, C.; Medina, S.; Câmara, J.S. QuEChERS - Fundamentals, relevant improvements, applications and future trends. Anal. Chim. Acta 2019, 1070, 1–28, doi:10.1016/J.ACA.2019.02.036.

30. Malaca, S.; Rosado, T.; Restolho, J.; Rodilla, J.M.; Rocha, P.M.M.; Silva, L.; Margalho, C.; Barroso, M.; Gallardo, E. Determination of amphetamine-type stimulants in urine samples using microextraction by packed sorbent and gas chromatography-mass spectrometry. J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci. 2019, 1120, 41–50, doi:10.1016/J.JCHROMB.2019.04.052.

31. Fernández, P.; Taboada, V.; Regenjo, M.; Morales, L.; Alvarez, I.; Carro, A.M.; Lorenzo, R.A. Optimization of ultrasound assisted dispersive liquid-liquid microextraction of six antidepressants in human plasma using experimental design. J. Pharm. Biomed. Anal. 2016, 124, 189–197, doi:10.1016/J.JPBA.2016.02.041.

32. ANSI/ASB Standard 036, Standard Practices for Method Validation in Forensic Toxicology.

33. Silveira, G. de O.; Lourenço, F.R.; Fonseca Pego, A.M.; Guimarães dos Santos, R.; Rossi, G.N.; Hallak, J.E.C.; Yonamine, M. Essential oil-based dispersive liquid-liquid microextraction for the determination of *N,N*-dimethyltryptamine and β-carbolines in human plasma: A novel solvent-free alternative. Talanta 2021, 225, doi:10.1016/J.TALANTA.2020.121976.

34. Chambers, M.I.; Appley, M.G.; Longo, C.M.; Musah, R.A. Detection and Quantification of Psychoactive *N, N*-Dimethyltryptamine in Ayahuasca Brews by Ambient Ionization High-Resolution Mass Spectrometry. ACS omega 2020, 5, 28547–28554, doi:10.1021/ACSOMEGA.0C03196.

35. Souza, R.C.Z.; Zandonadi, F.S.; Freitas, D.P.; Tófoli, L.F.F.; Sussulini, A. Validation of an analytical method for the determination of the main ayahuasca active compounds and application to real ayahuasca samples from Brazil. J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci. 2019, 1124, 197–203, doi:10.1016/J.JCHROMB.2019.06.014.

36. Gaujac, A.; Dempster, N.; Navickiene, S.; Brandt, S.D.; De Andrade, J.B. Determination of *N,N*-dimethyltryptamine in beverages consumed in religious practices by headspace solid-phase microextraction followed by gas chromatography ion trap mass spectrometry. Talanta 2013, 106, 394–398, doi:10.1016/J.TALANTA.2013.01.017.

37. Santos, M.C.; Navickiene, S.; Gaujac, A. Determination of Tryptamines and β-Carbolines in Ayahuasca Beverage Consumed During Brazilian Religious Ceremonies. J. AOAC Int. 2017, 100, 820–824, doi:10.5740/JAOACINT.16-0337.

38. Eller, S.; Borges, G.R.; Ossanes, D.S.; Birk, L.; Scheid, C.; Yonamine, M.; Grossi, P.; Merib, J. de O.; Oliveira, T.F. de A rapid analytical strategy for the determination of ayahuasca alkaloids in non-ritualistic approaches by UHPLC-MS/MS. Forensic Sci. Int. 2020, 312, 110298, doi:10.1016/J.FORSCIINT.2020.110298.

39. Gaujac, A.; Aquino, A.; Navickiene, S.; De Andrade, J.B. Determination of *N,N*-dimethyltryptamine in *Mimosa tenuiflora* inner barks by matrix solid-phase dispersion procedure and GC–MS. J. Chromatogr. B 2012, 881–882, 107–110, doi:10.1016/J.JCHROMB.2011.11.014.

40. Rodríguez, L.; López, A.; Moyna, G.; Seoane, G.A.; Davyt, D.; Vázquez, Á.; Hernández, G.; Carrera, I. New Insights into the Chemical Composition of Ayahuasca. ACS omega 2022, 7, 12307–12317, doi:10.1021/ACSOMEGA.2C00795.

41. Luethi, D.; Kolaczynska, K.E.; Vogt, S.B.; Ley, L.; Erne, L.; Liechti, M.E.; Duthaler, U. Liquid chromatography-tandem mass spectrometry method for the bioanalysis of *N,N*-dimethyltryptamine (DMT) and its metabolites DMT-*N*-oxide and indole-3-acetic acid in human plasma. J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci. 2022, 1213, doi:10.1016/J.JCHROMB.2022.123534.

42. Tavares, L.; Monedeiro, F.; Bordin, D.M.; de Martinis, B.S. Investigation of Ayahuasca β-Carboline Alkaloids and Tryptamine in Sweat Samples from Religious Community Participants by GC-MS. J. Anal. Toxicol. 2020, 44, 601–609, doi:10.1093/JAT/BKZ116.

43. Mcilhenny, E.H.; Riba, J.; Barbanoj, M.J.; Strassman, R.; Barker, S.A. Methodology for and the determination of the major constituents and metabolites of the Amazonian botanical medicine ayahuasca in human urine. Biomed. Chromatogr. 2011, 25, 970–984, doi:10.1002/BMC.1551.

44. Silveira, G. de O.; Lourenço, F.R.; Bruno, V.; Yonamine, M. Fast Hollow Fiber Liquid-Phase Microextraction as a Greener Alternative for the Determination of *N,N*-Dimethyltryptamine and Harmala Alkaloids in Human Urine. Front. Chem. 2020, 8, doi:10.3389/FCHEM.2020.558501.

3.2. Paper IV- *In Vitro* Study of the Bioavailability and Bioaccessibility of the Main Compounds Present in Ayahuasca Beverages

Abstract

Ayahuasca is a psychoactive beverage that contains the psychoactive compound *N,N*-dimethyltryptamine and β-carboline alkaloids. This study aims at determining *in vitro* the bioavailability and bioaccessibility of the main compounds present in decoctions of four individual plants, in a commercial mixture and in four mixtures of two individual plants used in the preparation of ayahuasca. The samples were subjected to an *in vitro* digestion process, and the Caco-2 cell line was used as an absorption model. The integrity and permeability of the cell monolayer were evaluated, as well as the cytotoxicity of the extracts. After digestion and cell incubation, the compounds absorbed by the cell monolayer were quantified by high-performance liquid chromatography coupled to a diode array detector. The results showed that compounds such as *N,N*-dimethyltryptamine, harmine, harmaline, harmol, harmalol and tetrahydroharmine were released from the matrix during the *in vitro* digestion process, becoming bioaccessible. Similarly, some of these compounds, after being incubated with the cell monolayer, were absorbed, becoming bioavailable. The extracts did not show cytotoxicity after cell incubation, and the integrity and permeability of the cell monolayer were not compromised.

Keywords: Ayahuasca; bioavailability; bioaccessibility; PAMPA; HPLC-DAD

3.2.1. Introduction

Ayahuasca is a psychoactive beverage traditionally consumed in the Amazon Basin of South America [1]. This word of Quechua origin, means "vine of the soul" or "vine of the dead" and is composed of the terms "*aya*" and "*wasca*", which means "spirit" and "vine", respectively [2,3]. This psychoactive beverage consists of a thick, oily and brownish decoction, which is prepared from the leaves of *Psychotria viridis* (*P. viridis*) and scraps from the stem of *Banisteriopsis caapi* (*B. caapi*) [4,5]. However, over the years, the preparation of ayahuasca has undergone variations [1,2,6]. Thus, some species of natural origin have been used in the preparation of the beverage, namely *Brunfelsia* spp., *Daturaolens*, *Malouetia tamarquina*, *Psychotria carthagenesis*, *Brugmansia suaveolens*, *Tabernaemontana* spp., or *Nicotiana tabacum*, replacing *P. viridis*, or *Peganum harmala*, harmine freebase/HCl, Moclobemide and tetrahydroharmine freebase/HCl, replacing *B. caapi* [6,7].

This psychoactive beverage was traditionally used by native healers for divine cults and in the cure of psychological disorders, stimulation of visual creativity and creative thinking [1,6]. Its hallucinogenic effects are due to the presence of *N,N*-dimethyltryptamine (DMT) (Figure III.2.1) from *P. viridis*, which behaves as an agonist of serotonin receptors (5-HT1A/2A/2C) [3]. When

ingested alone, this compound is inactive, as it is rapidly metabolized by peripheral monoamine oxidase A (MAO-A) [8]. However, in the presence of β-carbolinic alkaloids, such as harmaline, harmine and tetrahydroharmine (THH), from *B. caapi*, DMT can access the central nervous system, since these are temporary inhibitors of hepatic and intestinal MAO-A [2]. THH also acts as a serotonin reuptake inhibitor, enhancing the effects of DMT [2,3]. The knowledge about this synergy between compounds present in the two plants has been known by indigenous peoples for about 3000 years [9].

Figure III.2.1. Molecular structure of DMT.

Although ayahuasca has been consumed for centuries, in the last 25 years, its use has been increasing in different parts of the world [10,11]. The consumption of ayahuasca in controlled environments such as religious rituals and experimental procedures is not associated with psychotic episodes [10]. However, the expansion of ayahuasca has raised some concerns about the possible adverse effects associated with consumption, but also an interest in its potential therapeutic effects [6,10].

Bioavailability and bioaccessibility are important concepts that make it possible to understand the behaviour of some compounds in the body. Bioaccessibility consists of the amount of a compound that is released from a matrix, being available to be absorbed, after ingestion and consequent digestion [12]. On the other hand, bioavailability is defined as the fraction that reaches the bloodstream and that, after metabolization and distribution, produces an effect [13]. *In vitro* digestion is a procedure that has been used to determine the fraction of compounds that are released from the matrix and become bioaccessible [14]. *In vitro* digestion models aim to mimic the digestive process along the digestive tract (mouth, stomach and intestine), simulating physiological conditions such as pH, salt concentration, digestion time, among others [15,16]. Regarding the assessment of bioaccessibility, cell lines are frequently used, namely the line derived from a human colon carcinoma Caco-2, due to its similar morphology with the cells of the small intestine [17]. In addition, these cells have narrow intracellular junctions and express enzymes similar to those that are present in the small intestine, allowing to mimic the transport mechanisms that occur therein [18,19,20].

There are no studies concerning the fate of the active ingredients of ayahuasca formulations after ingestion, namely concerning their absorption to the general circulation for distribution. Therefore, with this study we aimed at evaluating the bioavailability and bioaccessibility of the active compounds present in four individual plants, a commercial mixture and four plant mixtures, used to prepare the Ayhauasca decoction. For that, an *in vitro* digestion process was used, as well

as the parallel artificial membrane permeability assay (PAMPA) using Caco-2 cells, in order to know better their path in the human body and part of the mechanisms regulating their passage to the blood stream.

3.2.2. Results and Discussion

Considering the several potential bioactive properties of ayahuasca, four individual decoctions of each plant used in the preparation of ayahuasca were prepared in this work, as well as four decoctions of a mixture of plants (with two different plant materials, one source of DMT and the other of β-carboline alkaloids). A decoction of a commercial mixture was also prepared. The bioavailability and bioaccessibility of the main compounds present in ayahuasca was evaluated in the nine samples.

3.2.2.1. Characterization of Main Compounds in Initial Samples and after Digestive Process

DMT is present in some plants used in the preparation of ayahuasca beverages. Given the use of plant samples containing this compound in religious rituals, it has received some attention over the years due to its psychoactive effects [21]. Besides that, the access of this compound to the bloodstream is dependent on the β-carboline alkaloids [1,8,22,23]. Thus, an analytical method by high performance liquid chromatography coupled to diode array detector (HPLC-DAD) was developed, which allowed the quantification of the main active compounds present in the samples of ayahuasca beverages (Table III.2.1). This analytical method was developed and validated in accordance with the standards of the Food and Drug Administration [24]. Thus, it was linear between 0.16 and 10.00 μg/mL for harmol, THH, harmaline and harmine, between 0.31 and 10.00 μg/mL for harmalol and between 0.031 and 1.00 μg/mL for DMT, with coefficients of determination (R^2) higher than 0.997. The intra- and inter-day precision revealed coefficients of variation below 15% and the accuracy was within the range of ±15%. The LOD and LLOQ obtained were 0.31 μg/mL for all compounds, except for DMT (0.031 μg/mL).

All samples from a mixture of two plants showed substantial concentrations of DMT, with the mixture of *M. hostilis* and *B. caapi* having the highest concentration, and the mixture of *P. viridis* and *B. caapi* having the lowest concentration. Regarding the individual samples, both the *P. viridis* and *M. hostilis* decoctions and the commercial mixture showed substantial amounts of DMT. In contrast, in the decoctions of *B. caapi* and *P. harmala*, this compound was not detected. Moreover, all mixtures presented considerable concentrations of β-carboline alkaloids, with the mixture of M. *hostilis* and *P. harmala* presenting the highest amount. Regarding the individual samples, these compounds were not detected in the decoctions of *P. viridis* and *M. hostilis*. On the other hand, in the decoctions of *B. caapi* and *P. harmala*, all β-carboline alkaloids were detected, with THH and harmol being in greater quantity in *B. caapi* and harmine, harmalol and harmaline in greater quantity in *P. harmala*. Regarding the commercial mixture, it was possible to detect all

β-carbolines, except for harmalol. Bensalem *et al.* [25] carried out the quantification of harmine, harmaline, harmol and harmalol in samples of *P. harmala*, having verified that, similarly to what was observed in the present study, the compound with the highest concentration was harmaline, followed by harmine, harmalol and, the least concentrated, harmol. In addition, Avula *et al.* [26] carried out the quantification of harmol, harmine, harmaline, among other compounds, using ultra-performance liquid chromatography coupled to mass spectrometry and ultraviolet detector (UPLC-UV-MS) and high-performance thin-layer chromatography (HPTLC). It was found that, similarly to the results now obtained, harmine was found in a higher quantity than harmol, being harmaline not detected [26]. Several studies were also carried out, with the aim of determining the concentration of DMT and β-carbolines in ayahuasca samples. Pires *et al.* [27] used gas chromatography equipment with nitrogen/phosphorous detector to quantify DMT, harmine, harmaline and THH in eight samples of ayahuasca. Similar to what was observed in the present work, the four compounds were detected in all samples [27]. Moreover, Souza *et al.* [28] analysed 38 ayahuasca samples using liquid chromatography coupled mass spectrometry in tandem (LC-MS/MS) verified the presence of THH, DMT, harmine and harmaline. Recently, Chambers *et al.* [29] quantified the DMT present in 6 samples of ayahuasca, obtaining values between 45.7 and 230.5 mg/L. It is important to point out that the concentrations of each compound in the ayahuasca samples can be very variable. This fact can be due to a number of factors, namely the variability of the proportion used by each user, as well as the different preparation methods [27,28]. Additionally, the concentration of the compounds in each plant can also be very variable [27]. According to Kaasik *et al.* [30], the average variations of concentrations of DMT, THH, harmine and harmaline, can be, respectively, 26.2%, 29.8%, 41.5% and 2.5%. The samples used in this study were acquired online, making it difficult to know their degree of purity.

Table III.2.1. Concentration of the main compounds of ayahuasca in different vegetal samples. The values are expressed as mean (μg/mg extract) ± SD.

Samples	Compound	Initial concentration
P. viridis	DMT	6.50 ± 0.01
B. caapi	THH	5.00 ± 0.10
	Harmol	0.14 ± 0.00
	Harmine	10.00 ± 0.28
	Harmalol	0.05 ± 0.00
	Harmaline	4.68 ± 0.14
P. harmala	THH	3.05 ± 0.04
	Harmol	0.02 ± 0.00
	Harmine	12.00 ± 0.00
	Harmalol	0.66 ± 0.01
	Harmaline	17.00 ± 0.00
M. hostilis	DMT	10.50 ± 0.02
Commercial mixture	DMT	10.40 ± 0.01
	THH	2.09 ± 0.07

	Harmol	0.01 ± 0.00
	Harmine	0.02 ± 0.00
	Harmalol	ND
	Harmaline	0.37 ± 0.02
P. viridis* + *B. caapi	DMT	4.50 ± 0.01
	THH	2.50 ± 0.07
	Harmol	0.01 ± 0.00
	Harmine	0.48 ± 0.00
	Harmalol	ND
	Harmaline	0.07 ± 0.00
P. viridis* + *P. harmala	DMT	6.50 ± 0.09
	THH	0.63 ± 0.05
	Harmol	0.02 ± 0.00
	Harmine	0.30 ± 0.01
	Harmalol	0.08 ± 0.00
	Harmaline	0.48 ± 0.01
M. hostilis* + *B. caapi	DMT	8.00 ± 0.02
	THH	1.90 ± 0.06
	Harmol	0.03 ± 0.00
	Harmine	0.82 ± 0.02
	Harmalol	0.04 ± 0.00
	Harmaline	0.12 ± 0.00
M. hostilis* + *P. harmala	DMT	8.50 ± 0.01
	THH	3.44 ± 0.05
	Harmol	0.06 ± 0.00
	Harmine	9.00 ± 0.00
	Harmalol	0.36 ± 0.00
	Harmaline	13.5 ± 0.06

Caption: ND: not detected

After quantifying the main compounds present in samples of ayahuasca beverages, the same compounds were quantified over the three stages of the *in vitro* digestion process (salivary, gastric and duodenal). By observing the aliquots collected in each step, it is possible to verify that there were colour variations throughout the process. Likewise, the concentrations of DMT and β-carboline alkaloids also varied between samples and, within the same sample, between digestion steps (Table III.2.2).

Table III.2.2. Concentration of the main compounds of ayahuasca in different digestion steps. The values are expressed as mean (μg/mL) ± SD.

Samples	Compound	Salivary	Gastric	Duodenal
P. viridis	DMT	0.84 ± 0.60	7.77 ± 0.08	7.49 ± 0.19
B. caapi	THH	0.83 ± 0.00	0.78 ± 0.13	1.05 ± 0.09
	Harmol	ND	ND	ND

	Harmine	1.56 ± 0.00	4.13 ± 0.03	1.98 ± 0.03
	Harmalol	ND	ND	ND
	Harmaline	0.19 ± 0.00	0.33 ± 0.00	0.21 ± 0.00
P. harmala	THH	1.52 ± 0.09	1.66 ± 0.12	1.32 ± 0.01
	Harmol	ND	ND	ND
	Harmine	18.38 ± 0.18	19.52 ± 0.05	10.02 ± 0.01
	Harmalol	1.47 ± 0.04	1.54 ± 0.02	1.03 ± 0.07
	Harmaline	29.66 ± 0.10	26.18 ± 0.14	22.88 ± 0.26
M. hostilis	DMT	9.55 ± 0.03	8.96 ± 0.17	8.33 ± 0.00
Commercial mixture	DMT	4.28 ± 0.05	4.09 ± 0.02	3.38 ± 0.08
	THH	0.95 ± 0.05	1.42 ± 0.00	0.50 ± 0.03
	Harmol	ND	ND	ND
	Harmine	ND	ND	ND
	Harmalol	1.21 ± 0.00	1.02 ± 0.01	0.81 ± 0.01
	Harmaline	1.29 ± 0.03	1.12 ± 0.01	0.85 ± 0.00
P. viridis* + *B. caapi	DMT	2.37 ± 0.01	1.61 ± 0.05	2.00 ± 0.02
	THH	3.33 ± 0.05	4.05 ± 0.11	2.82 ± 0.02
	Harmol	0.29 ± 0.00	0.34 ± 0.00	0.26 ± 0.01
	Harmine	1.23 ± 0.03	3.09 ± 0.22	1.80 ± 0.05
	Harmalol	0.26 ± 0.00	0.27 ± 0.02	0.25 ± 0.00
	Harmaline	0.19 ± 0.01	0.40 ± 0.00	0.26 ± 0.01
P. viridis* + *P. harmala	DMT	4.30 ± 0.08	3.94 ± 1.33	4.56 ± 0.15
	THH	ND	ND	ND
	Harmol	ND	ND	ND
	Harmine	1.64 ± 0.01	6.70 ± 0.12	3.05 ± 0.10
	Harmalol	0.37 ± 0.01	0.38 ± 0.01	0.34 ± 0.04
	Harmaline	4.62 ± 0.02	8.93 ± 0.04	4.05 ± 0.05
M. hostilis* + *B. caapi	DMT	9.07 ± 0.04	5.52 ± 0.09	7.36 ± 0.05
	THH	2.89 ± 0.21	2.37 ± 0.11	2.45 ± 0.04
	Harmol	0.28 ± 0.02	0.24 ± 0.01	0.22 ± 0.00
	Harmine	4.02 ± 0.04	10.34 ± 0.07	7.69 ± 0.27
	Harmalol	ND	ND	ND
	Harmaline	0.28 ± 0.01	0.42 ± 0.01	0.29 ± 0.01
M. hostilis* + *P. harmala	DMT	9.65 ± 0.12	4.76 ± 0.07	6.68 ± 0.16
	THH	0.89 ± 0.01	ND	ND
	Harmol	ND	ND	ND
	Harmine	4.41 ± 0.07	10.92 ± 0.23	9.38 ± 0.05
	Harmalol	0.60 ± 0.03	0.87 ± 0.01	0.63 ± 0.04
	Harmaline	11.45 ± 0.20	16.96 ± 0.11	12.08 ± 0.02

Caption: ND: not detected

Analysing the results, it was possible to verify that the amount of DMT varies throughout the *in vitro* digestion process. In general, the concentration of DMT at the end of the entire process decreased in samples of *M. hostilis*, in the commercial mixture and in the mixtures of *M. hostilis*

and *B. caapi* and *M. hostilis* and *P. harmala*. Conversely, there was an increase in DMT in the sample of *P. viridis*, while in the mixtures of *P. viridis* and *B. caapi* and *P. viridis* and *P. harmala* there were no noticeable changes. With respect to β-carbolines, there was a variation from compound to compound. The concentration of harmol remained constant throughout the digestion process of the sample of *M. hostilis* and *B. caapi*, increased in the mixture of *P. viridis* and *B. caapi* and was not detected in the other samples. It was also not possible to detect harmalol during the digestion of the samples of *B. caapi* and in the mixture of *M. hostilis* and *B. caapi*. In the other samples where this compound was initially detected, its concentration decreased slightly. Regarding THH, it was verified that its concentration increased in the samples of *B. caapi* and decreased in the commercial mixture and in mixtures of *P. viridis* and *B. caapi* and *M. hostilis* and *P. harmala*. A slight decrease of this compound was also observed in the sample of *P. harmala* and in the mixture of *M. hostilis* and *B. caapi*. In the mixture of *P. viridis* and *P. harmala* this compound was not detected. It was verified that the concentration of harmine increased, except in the commercial mixture (not detected) and in *P. harmala* (decreased). The concentration of harmaline remained constant in the mixture of *M. hostilis* and *B. caapi* and decreased in the sample of *P. harmala*, in the commercial mixture and in the mixture of *P. viridis* and *P. harmala*. In the samples of *B. caapi*, and in the mixtures of *P. viridis* and *B. caapi* and *M. hostilis* and *P. harmala*, there was a slight increase in the concentration of harmaline. These variations in the concentrations of β-carboline alkaloids may be due to the fact that these compounds degrade and easily give rise to another β-carboline (Figure III.2.2) [31].

Figure III.2.2. Reactions between β-carboline alkaloids.

So far, no bioaccessibility studies have been carried out on ayahuasca or plants used in its preparation. Digestion studies including this type of samples have not been carried out so far, so it is not possible to make a comparison.

3.2.2.2. Cell Culture

3.2.2.2.1. Evaluation of Cell Viability

The cytotoxicity of each sample was assessed using the MTT assay. In analysing the results, it was verified that there was a slight decrease in cell viability in the samples of the digested

commercial mixture and in the crude extract of *B. caapi*. The other samples showed no decrease in cell viability (Table III.2.3). These results are in agreement with those obtained by Katchborian-Neto *et al.* [32], which evaluated the cytotoxicity of ayahuasca samples in SH-SY5Y cells. Additionally, three of the samples intensely increased cell viability within the first 48 h [32]. In addition, Samoylenko *et al.* [33] evaluated the cytotoxicity of *B. caapi* extracts in six cell lines, verifying that the extracts did not show cytotoxicity.

Table III.2.3. Cell viability after exposure to extracts. The values are expressed as mean ± SD.

Samples	Cell viability (%)
P. viridis **Crude**	156.01 ± 27.31
P. viridis **Digested**	128.85 ± 9.03
B. caapi **Crude**	95.92 ± 1.83
B. caapi **Digested**	113.66 ± 11.59
P. harmala **Crude**	171.46 ± 28.88
P. harmala **Digested**	117.62 ± 3.59
M. hostilis **Crude**	148.28 ± 14.18
M. hostilis **Digested**	96.04 ± 12.23
Commercial mixture Crude	101.50 ± 13.25
Commercial mixture Digested	79.52 ± 0.93
P. viridis **+** ***B. caapi*** **Crude**	148.07 ± 26.83
P. viridis **+** ***B. caapi*** **Digested**	103.74 ± 3.43
P. viridis **+** ***P. harmala*** **Crude**	162.55 ± 15.63
P. viridis **+** ***P. harmala*** **Digested**	127.75 ± 9.97
M. hostilis **+** ***B. caapi*** **Crude**	126.61 ± 16.39
M. hostilis **+** ***B. caapi*** **Digested**	118.50 ± 1.59
M. hostilis **+** ***P. harmala*** **Crude**	138.41 ± 17.63
M. hostilis **+** ***P. harmala*** **Digested**	120.70 ± 3.12

3.2.2.2.2. Evaluation of the Electrical Resistance of the Cell Transendothelial Membrane

The integrity of the cell monolayer was evaluated by the TEER assay, before and after cell incubation with the extracts (Table III.2.4). The TEER assay allows monitoring the integrity of cell layers in *in vitro* assays, as well as possible changes in intercellular junctions, by evaluating transendothelial electrical resistance [34]. Analysing the results of the TEER measurements before incubation with the extracts, it was observed that the monolayer was intact, since the values were above the 150–200 Ω cm^2 range, minimum acceptable limit [35]. After incubation with the extracts, a new TEER measurement was performed, with no significant differences between the values of the first and second measurements. Additionally, the values of the second measurement were also above the minimum acceptable limit. Therefore, the integrity of the cell monolayer was

confirmed [35]. So far, there are no studies with ayahuasca samples where the TEER assay has been performed.

Table III.2.4. TEER values obtained before and after incubation with the extracts. The values are expressed as mean ± SD. Statistically significant values were considered if $p < 0.05$ (*).

Samples	TEER (Ω cm²)		
	Before	**After**	***p*-Value**
Control	990 ± 31.11	1034 ± 31.11	0.293
***P. viridis* Crude**	1298 ± 155.56	1628 ± 207.94	0.239
***P. viridis* Digested**	1518 ± 93.34	2046 ± 155.56	0.054
***B. caapi* Crude**	1166 ± 155.56	1408 ± 110.73	0.146
***B. caapi* Digested**	1232 ± 134.42	1276 ± 116.41	0.317
***P. harmala* Crude**	1386 ± 217.79	1408 ± 124.45	0.913
***P. harmala* Digested**	1188 ± 186.68	1496 ± 110.73	0.107
***M. hostilis* Crude**	1254 ± 155.56	1298 ± 31.11	0.733
***M. hostilis* Digested**	1584 ± 177.82	1496 ± 116.41	0.112
Commercial mixture Crude	1694 ± 155.56	1716 ± 232.83	0.754
Commercial mixture Digested	1232 ± 44.00	1232 ± 25.40	0.643
***P. viridis* + *B. caapi* Crude**	1364 ± 186.68	1496 ± 0.00	0.423
***P. viridis* + *B. caapi* Digested**	1166 ± 93.34	1386 ± 31.11	0.087
***P. viridis* + *P. harmala* Crude**	1415 ± 93.34	1408 ± 91.59	0.936
***P. viridis* + *P. harmala* Digested**	1100 ± 141.44	1144 ± 116.41	0.795
***M. hostilis* + *B. caapi* Crude**	1232 ± 76.21	1276 ± 25.40	0.189
***M. hostilis* + *B. caapi* Digested**	1188 ± 248.90	1408 ± 127.02	0.619
***M. hostilis* + *P. harmala* Crude**	1254 ± 93.34	1430 ± 31.11	0.127
***M. hostilis* + *P. harmala* Digested**	1232 ± 177.82	1452 ± 0.00	0.246

3.2.2.2.3. Evaluation of Cell Monolayer Permeability

Cell monolayer permeability was assessed by Lucifer Yellow permeability assay (Table III.2.5). The Lucifer Yellow permeability assay allows evaluating the permeability characteristics of a cell monolayer, by measuring the passive diffusion of different molecules through it [36]. This assay was performed after exposing the cells to extracts. Analysing the results, it was shown that there were no significant changes in cell permeability, when compared to the control. These results are in agreement with those obtained in the TEER assay, suggesting that there were no changes in intracellular spaces, nor in cell barrier function and in membrane permeability [37,38]. Previous studies also suggest that both TEER measurement and permeability are related, and that a significant increase in the permeability is accompanied by a decrease in TEER values [37,38].

Similarly to what was observed in the TEER assay, no studies were found where the Lucifer Yellow permeability assay was performed with ayahuasca samples.

Table III.2.5. Percentage of permeability of Caco-2 cells after incubation with the extracts. The values are expressed as mean ± SD. Statistically significant values were considered if $p < 0.05$ (*).

Samples	Permeability (%)	*p*-Value
Control	16.94 ± 2.35	-
***P. viridis* Crude**	19.59 ± 3.00	0.281
***P. viridis* Digested**	13.49 ± 1.03	0.165
***B. caapi* Crude**	16.79 ± 0.14	0.879
***B. caapi* Digested**	16.11 ± 0.49	0.823
***P. harmala* Crude**	15.01 ± 0.46	0.462
***P. harmala* Digested**	17.97 ± 1.37	0.523
***M. hostilis* Crude**	14.38 ± 0.72	0.322
***M. hostilis* Digested**	14.10 ± 0.41	0.267
Commercial mixture Crude	19.88 ± 2.84	0.383
Commercial mixture Digested	16.13 ± 1.83	0.865
***P. viridis* + *B. caapi* Crude**	16.42 ± 0.40	0.959
***P. viridis* + *B. caapi* Digested**	16.03 ± 1.50	0.463
***P. viridis* + *P. harmala* Crude**	13.81 ± 0.49	0.225
***P. viridis* + *P. harmala* Digested**	13.47 ± 1.85	0.283
***M. hostilis* + *B. caapi* Crude**	13.07 ± 1.89	0.139
***M. hostilis* + *B. caapi* Digested**	13.18 ± 0.16	0.074
***M. hostilis* + *P. harmala* Crude**	12.40 ± 1.64	0.069
***M. hostilis* + *P. harmala* Digested**	11.65 ± 1.79	0.058

3.2.2.2.4. Characterization of the Main Compounds after Cell Incubation

The amount of compounds present in the collected aliquots after cell incubation of crude and digested extracts were also quantified by HPLC-DAD (Table III.2.6 and Table III.2.7). It was verified that DMT, harmine and harmaline are the compounds, from those present in the digested extract, which cross the cell monolayer the most. Similarly, in the crude extract the same results were observed. The concentration of these three compounds in all samples and for both extracts, increased gradually in the basolateral compartment throughout the incubation period, except in the digested extract in the mixture of *P. viridis* and *B. caapi*, where DMT increases after 2 h of incubation, remaining approximately constant until 4 h of cell incubation. In general, in the digested extract, all the compounds gradually increased during cell incubation, except for harmol and harmalol, which were not detected during the entire process. Similarly, in the crude extract, harmalol was not detected, but harmol was detected after 2 h of incubation in the *P. harmala*

sample. Moreover, as in what was observed in the digested extract, in the crude extract all compounds gradually increased during cell incubation, except for the THH present in the mixture of *M. hostilis* and *B. caapi*, which decreases slightly after 2 h of incubation, increasing again after 4 h.

Table III.2.6. Concentration of the main compounds of ayahuasca in the aliquots collected after the different incubation times with the crude extract (Mean µg/mL Extract) ± SD.

Samples	Compound	Time		
		1h	2h	4h
P. viridis	DMT	0.50 ± 0.01	0.89 ± 0.21	1.22 ± 0.05
B. caapi	THH	ND	0.66 ± 0.02	0.59 ± 0.06
	Harmol	ND	ND	ND
	Harmine	0.33 ± 0.03	1.00 ± 0.01	1.35 ± 0.03
	Harmalol	ND	ND	ND
	Harmaline	ND	ND	ND
P. harmala	THH	ND	ND	0.72 ± 0.12
	Harmol	ND	0.19 ± 0.02	ND
	Harmine	2.09 ± 0.02	4.41 ± 0.23	5.90 ± 0.04
	Harmalol	ND	ND	ND
	Harmaline	3.65 ± 0.06	5.71 ± 0.60	8.55 ± 0.17
M. hostilis	DMT	ND	1.18 ± 0.10	1.42 ± 0.06
Commercial mixture	DMT	ND	0.55 ± 0.04	0.73 ± 0.04
	THH	ND	ND	ND
	Harmol	ND	ND	ND
	Harmine	ND	ND	ND
	Harmalol	ND	ND	ND
	Harmaline	ND	ND	ND
P. viridis* + *B. caapi	DMT	0.16 ± 0.01	0.47 ± 0.06	0.63 ± 0.07
	THH	0.65 ± 0.02	0.78 ± 0.06	0.93 ± 0.04
	Harmol	ND	ND	ND
	Harmine	ND	0.42 ± 0.06	0.76 ± 0.04
	Harmalol	ND	ND	ND
	Harmaline	ND	ND	ND
P. viridis* + *P. harmala	DMT	0.33 ± 0.03	1.01 ± 0.16	1.10 ± 0.07
	THH	ND	ND	ND
	Harmol	ND	ND	ND
	Harmine	0.35 ± 0.02	0.39 ± 0.01	0.78 ± 0.04
	Harmalol	ND	ND	ND
	Harmaline	0.31 ± 0.00	0.67 ± 0.09	0.76 ± 0.03
M. hostilis* + *B. caapi	DMT	0.69 ± 0.07	1.67 ± 0.11	1.87 ± 0.04
	THH	0.74 ± 0.08	0.53 ± 0.03	0.65 ± 0.07
	Harmol	ND	ND	ND
	Harmine	0.98 ± 0.11	1.64 ± 0.11	2.42 ± 0.08

	Compound	1h	2h	4h
	Harmalol	ND	ND	ND
	Harmaline	ND	ND	ND
M. hostilis* + *P. harmala	DMT	0.29 ± 0.01	0.72 ± 0.04	1.24 ± 0.18
	THH	ND	ND	ND
	Harmol	ND	ND	ND
	Harmine	0.47 ± 0.02	1.07 ± 0.10	1.63 ± 0.27
	Harmalol	ND	ND	ND
	Harmaline	0.55 ± 0.08	1.07 ± 0.14	1.42 ± 0.22

Caption: ND: not detected

Table III.2.7. Concentration of the main compounds of ayahuasca in the aliquots collected after the different incubation times with the digested extract (Mean µg/mL) ± SD.

Samples	**Compound**	**Time**		
		1h	**2h**	**4h**
P. viridis	DMT	0.73 ± 0.00	1.48 ± 0.02	1.99 ± 0.03
B. caapi	THH	ND	ND	ND
	Harmol	ND	ND	ND
	Harmine	ND	ND	1.14 ± 0.03
	Harmalol	ND	ND	ND
	Harmaline	ND	ND	ND
P. harmala	THH	ND	ND	ND
	Harmol	ND	ND	ND
	Harmine	1.83 ± 0.01	3.08 ± 0.09	4.19 ± 0.03
	Harmalol	ND	ND	ND
	Harmaline	3.81 ± 0.13	4.75 ± 0.12	5.63 ± 0.08
M. hostilis	DMT	ND	1.61 ± 0.07	1.90 ± 0.02
Commercial mixture	DMT	ND	0.61 ± 0.02	0.67 ± 0.02
	THH	ND	ND	ND
	Harmol	ND	ND	ND
	Harmine	ND	ND	ND
	Harmalol	ND	ND	ND
	Harmaline	ND	ND	ND
P. viridis* + *B. caapi	DMT	ND	0.50 ± 0.07	0.48 ± 0.01
	THH	ND	ND	ND
	Harmol	ND	ND	ND
	Harmine	ND	ND	0.70 ± 0.02
	Harmalol	ND	ND	ND
	Harmaline	ND	ND	ND
P. viridis* + *P. harmala	DMT	ND	0.65 ± 0.00	0.86 ± 0.03
	THH	ND	ND	ND
	Harmol	ND	ND	ND
	Harmine	ND	1.02 ± 0.01	1.26 ± 0.03
	Harmalol	ND	ND	ND

	Harmaline	0.74 ± 0.00	0.99 ± 0.01	1.26 ± 0.01
M. hostilis* + *B. caapi	DMT	0.78 ± 0.01	1.74 ± 0.01	2.11 ± 0.02
	THH	ND	ND	ND
	Harmol	ND	ND	ND
	Harmine	0.77 ± 0.00	1.96 ± 0.01	2.54 ± 0.64
	Harmalol	ND	ND	ND
	Harmaline	ND	ND	ND
M. hostilis* + *P. harmala	DMT	0.60 ± 0.03	1.57 ± 0.06	1.86 ± 0.02
	THH	ND	ND	ND
	Harmol	ND	ND	ND
	Harmine	0.85 ± 0.03	2.38 ± 0.09	3.34 ± 0.04
	Harmalol	ND	ND	ND
	Harmaline	1.10 ± 0.02	2.21 ± 0.01	3.02 ± 0.03

Caption: ND: not detected

In general, it was possible to observe that all the analysed compounds managed to cross the cell monolayer, except harmalol and harmol. In the digested samples the bioavailability percentages ranged between 8.30–28.9% for DMT, 0–29.63% for harmaline and 33.03–57.58% for harmine. So far, no studies have been carried out on the bioavailability of ayahuasca, so it is not possible to make comparisons with the present study. However, differences in β-carboline concentrations can be explained by the rapid mutual conversion of these compounds (Figure III.2.2) [31]. Additionally, DMT easily crosses the barriers of the body, since it is a small and hydrophobic molecule with a low molecular weight [21]. It was also observed that the amount of the compounds decreased after crossing the cell monolayer, when compared to the values obtained after *in vitro* digestion. This fact has already been verified in bioavailability studies with other compounds of natural origin [39,40]. In a study that evaluated the bioavailability and bioaccessibility of *Prunus avium* L., carried out by our research group, this same decrease in the amount of compounds after crossing the Caco-2 cell monolayer was also verified [14].

3.2.3. Materials and Methods

3.2.3.1. Chemicals and Materials

The analytical standards DMT, harmine, harmaline, THH, harmol and harmalol were kindly provided by Nal von Minden, GmbH (Regensburg, Germany). Lucifer Yellow, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and Roswell Park Memorial Institute (RPMI) medium were obtained from Sigma-Aldrich (Sintra, Portugal). Methanol (HPLC grade) was obtained from Fischer Chemical (Loughborough, UK). Formic acid and dimethyl sulfoxide (99.9% of purity) were purchased from Sigma-Aldrich (Sintra, Portugal). Deionized water was obtained from a Milli-Q System (Millipore, Billerica, MA, USA).

3.2.3.2. Sample and Working Solutions Preparation

All vegetal samples were acquired online from Shayana Shop (https://www.shayanashop.com, Amsterdam, The Netherlands) (accessed on 25 May 2019). The decoctions of ayahuasca were prepared according to a traditional recipe kindly provided by Dr. Nicolás Fernández. Thus, 0.210 g of each of the five vegetal samples were weighed (*P. viridis* (leaves), *P. harmala* (seeds), *B. caapi* (scraps from the stem), *M. hostilis* (root bark) and commercial mixture) and were then milled in a mortar with a few drops of water. After that, 250 mL of ultra-pure water was added, and the mixture was transferred to a Schott flask. This preparation was boiled at 100 °C for 4 h. Similarly, four decoctions were prepared where two of the above vegetal samples were mixed (*P. viridis* and *P. harmala*; *P. viridis* and *B. caapi*; *M. hostilis* and *P. harmala*; *M. hostilis* and *B. caapi*). After boiling, the samples were cooled, filtered, frozen at −80 °C and freeze-dried. Individual stock solutions of DMT, hamine, harmaline, harmol and harmalol were prepared at 1 mg/mL in methanol. Working solutions were prepared by serial dilutions in methanol.

3.2.3.3. *In Vitro* Simulation of Human Digestion Process

The *in vitro* digestion assay was carried out as described in a previous work [14]. Initially, salivary fluid (potassium chloride, monosodium phosphate, sodium sulphate, sodium chloride, sodium bicarbonate, urea, α-amylase, mucin and uric acid), gastric fluid (sodium chloride, monosodium phosphate, potassium chloride, calcium chloride, ammonium chloride, hydrochloric acid, glucose, urea, pepsin, mucin and bovine serum albumin), duodenal fluid (sodium chloride, sodium bicarbonate, potassium dihydrogen phosphate, potassium chloride, magnesium chloride, hydrochloric acid, urea, calcium chloride dihydrate, bovine serum albumin, pancreatin and lipase) and bile fluid (sodium chloride, sodium bicarbonate, potassium chloride, hydrochloric acid, urea, calcium chloride dihydrate, bile and bovine serum albumin) were prepared. For the assay, each freeze-dried decoction was dissolved in 100 mL of deionized water. To each of the nine samples, 6 mL of simulated salivary fluid (pH 6.8) was added, being this mixture was incubated at 37 °C for 5 min with orbital shaking at 90 rpm. Then, 12 mL of simulated gastric fluid (pH 1.3) was added, followed by incubation in the same conditions for 2 h. After this time, 6 mL of simulated bile fluid (pH 8.2), 12 mL of simulated duodenal fluid (pH 8.1) and 2 mL of sodium bicarbonate solution (1M) was added. The solution was incubated again at 37 °C with orbital shaking at 90 rpm for 2 h.

Aliquots were collected at the end of each stage of the *in vitro* digestion process, which were immediately cooled, then frozen at −80 °C for 30 min and later sonicated at 4 °C in an ice bath for 30 min. Subsequently, the samples were filtered through a 0.22 µm cellulose acetate pore filter and subsequently analysed by high-performance liquid chromatography (HPLC). Furthermore, an additional aliquot of the last stage of the *in vitro* digestion of each sample was collected, which was also placed on ice, frozen at −80 °C and sonicated. Then, their pH was measured and corrected, when necessary, to physiological pH. Subsequently, these aliquots were used in the PAMPA assay.

3.2.3.4. Cell Culture

The Caco-2 cell line (Database name: American Type Culture Collection (ATCC) Accession numbers: HTB-37) [41] was cultured in RPMI medium supplemented with 1% antibiotic mixture and 10% foetal bovine serum, at passages between 33 and 37. Subsequently, the cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

For the MTT assay, the cells were seeded in 96 multi-well plates (cat. number 734-2802 avantor, VWR, Amadora Portugal) at a cell density of 0.5×10^4. For the PAMPA assay, the cells were seeded in culture inserts, placed in 12 multi-well plates (cat. number 734-2731 avantor, Laborspirit, Santo Antão de Tojal, Portugal) at a cell density of 6×10^4, remaining for a period of 21 days in order to form a confluent monolayer. After that time, 500 µL of each of the nine samples (digested and undigested) was added to the apical chamber, to be in contact with the cell monolayer (*P. viridis*—0.278 mg/mL; *B. caapi*—0.062 mg/mL; *P. harmala*—0.226 mg/mL; *M. hostilis*—0.382 mg/mL; commercial mixture—0.156 mg/mL; *P. viridis* + *B. caapi*—0.203 mg/mL; *P. viridis* + *P. harmala*—0.344 mg/mL; *M. hostilis* + *B. caapi*—0.555 mg/mL e *M. hostilis* + *P. harmala*—0.4 mg/mL). After 1, 2 and 4 h, 250 µL was collected in the basolateral chamber. The collected aliquots were analysed by HPLC. All tests were performed in triplicate.

3.2.3.4.1. MTT Cell Viability Assay

The cytotoxicity of the samples was assessed by the MTT assay. For that, after the cells became confluent, they were exposed to the samples (digested and undigested) 1, 2 and 4 h. RPMI medium was used as a negative control. After incubation, the medium was removed and an MTT solution was added. Then, the cells were incubated for 3 h. After that time, the MTT solution was removed, and the formazan crystals formed were dissolved in dimethyl sulfoxide (DMSO), being the absorbance measured using a microplate reader at 570 nm.

3.2.3.4.2. Transepithelial Electrical Resistance Assay

The integrity of the cell monolayer was evaluated by measuring the transepithelial electrical resistance (TEER). Before the incubation of the cells' monolayer with the extracts (digested and undigested), the TEER was measured. Initially, the electrode of the transepithelial resistance meter (EVOM2, World Precision Instrument, Sarasora, FL, USA) was equilibrated with RPMI medium and then was placed in each well to form an angle of 90°. The procedure was performed in triplicate and the TEER was determined according to the following equation:

$$\text{TEER value} = (\text{mean of the resistances of each well} - \text{mean of the resistance of blank}) \times \text{insert area} \quad (1)$$

3.2.3.4.3. Lucifer Yellow Permeability Assay

The Lucifer Yellow Permeability Assay allows evaluating changes in the permeability characteristics of the cell monolayer after passive passage of compounds. This test was performed as described in a previous work [14]. Briefly, the RPMI medium of the chambers delimited by the insert (apical and basolateral) was removed and replaced by 500 µL of the Lucifer Yellow solution in the apical chamber and 1.5 mL of Hank's balanced salt solution (HBSS) in the basolateral chamber. After that, the multi-well was incubated for 1 h, and then 200 µL of each basolateral chamber was pipetted to another culture plate, being the fluorescence measured at 485 nm (excitation) and 535 nm (emission) using a spectrofluorometer. HBSS was used as a blank and a Lucifer Yellow solution (0.1 mg/mL) was used as a positive control. The permeability percentage was calculated as follows:

$$\% \text{ permeability} = (\text{mean of fluorescence of each well} - \text{fluorescence of blank})/(\text{fluorescence of positive control} - \text{fluorescence of blank}) \times 100 \quad (2)$$

3.2.3.5. Instrumental and Chromatographic Conditions

The quantification of main compounds present in ayahuasca beverages was performed on an HPLC system coupled to a diode array detector (DAD) (Agilent technologies Soquímica, Lisbon, Portugal). The mobile phase was composed of 0.1% formic acid in methanol (A) and 0.1% formic acid in water (B). The elution was carried out in gradient mode and included 5% A (0–2 min), 50% A (2–32 min) and again, 5% A (32–40 min). The flow rate was 1.5 mL/min, and the injection volume was 50 µL. The stationary phase consisted of an YMC-Triart PFP (5 µm, 4.6 i.d. × 150 mm) analytical column coupled to a Guard-c holder (4 × 10 mm) and a Triart PFP (5 µm, 3 ×10 mm) pre-column, all from YMC Europe GMBH (Solítica, Lisbon, Portugal), being maintained at 25 °C. harmine and harmol were detected at 246 nm, DMT and THH at 278 nm and harmaline and harmalol at 360 nm. The temperature of the sampler was set at 4 °C.

3.2.3.6. Statistical Analysis

The results are expressed as mean values with standard deviations (SD). The Student's *t*-test was employed and statistically significant values were considered when $p < 0.05$ (*).

3.2.4. Conclusions

During the *in vitro* digestion, the compounds were released from the matrix, becoming bioaccessible. The concentration of β-carboline alkaloids shows an appreciable transformation, while the variation of DMT is smaller. After the *in vitro* digestion, the detected compounds could be absorbed by the cell monolayer, becoming bioavailable but in lower concentrations. Likewise, the compounds present in the extracts that did not undergo *in vitro* digestion also became bioavailable.

So, it can be inferred that digestion is not essential to occur absorption at the intestine level. After cell incubation with the extracts, it was verified that they were not cytotoxic, and the integrity and the permeability of the cell monolayer remained unchanged, suggesting that the compounds did not interfere with intercellular junctions. Further studies in which a more realistic approximation of the intestinal matrix is used should be carried out in order to overcome possible flaws of the used model.

3.2.5. References

1. Estrella-Parra, E.A.; Almanza-Pérez, J.C.; Alarcón-Aguilar, F.J. Ayahuasca: Uses, Phytochemical and Biological Activities. *Nat. Products Bioprospect.* 2019, *9*, 251–265.
2. Hamill, J.; Hallak, J.; Dursun, S.M.; Baker, G. Ayahuasca: Psychological and Physiologic Effects, Pharmacology and Potential Uses in Addiction and Mental Illness. *Curr. Neuropharmacol.* **2018**, *17*, 108–128, doi:10.2174/1570159x16666180125095902.
3. Gonçalves, J.; Luís, Â.; Gallardo, E.; Duarte, A.P. Psychoactive substances of natural origin: Toxicological aspects, therapeutic properties and analysis in biological samples. *Molecules* **2021**, *26*, doi:10.3390/molecules26051397.
4. Andrade, T.S.; de Oliveira, R.; da Silva, M.L.; Von Zuben, M.V.; Grisolia, C.K.; Domingues, I.; Caldas, E.D.; Pic-Taylor, A. Exposure to ayahuasca induces developmental and behavioral alterations on early life stages of zebrafish. *Chem. Biol. Interact.* **2018**, *293*, 133–140, doi:10.1016/j.cbi.2018.08.001.
5. Gable, R.S. Risk assessment of ritual use of oral dimethyltryptamine (DMT) and harmala alkaloids. *Addiction* **2007**, *102*, 24–34, doi:10.1111/j.1360-0443.2006.01652.x.
6. Gonçalves, J.; Luís, Â.; Gradillas, A.; García, A.; Restolho, J.; Fernández, N.; Domingues, F.; Gallardo, E.; Duarte, A.P. Ayahuasca beverages: Phytochemical analysis and biological properties. *Antibiotics* **2020**, *9*, 1–21, doi:10.3390/antibiotics9110731.
7. Simão, A.Y.; Gonçalves, J.; Duarte, A.P.; Barroso, M.; Cristóvão, A.C.; Gallardo, E. Toxicological Aspects and Determination of the Main Components of Ayahuasca: A Critical Review. *Medicines* **2019**, *6*, 106, doi:10.3390/medicines6040106.
8. Riba, J.; Valle, M.; Urbano, G.; Yritia, M.; Morte, A.; Barbanoj, M.J. Human pharmacology of ayahuasca: Subjective and cardiovascular effects, monoamine metabolite excretion, and pharmacokinetics. *J. Pharmacol. Exp. Ther.* **2003**, *306*, 73–83, doi:10.1124/jpet.103.049882.
9. Callaway, J.C.; McKenna, D.J.; Grob, C.S.; Brito, G.S.; Raymon, L.P.; Poland, R.E.; Andrade, E.N.; Andrade, E.O.; Mash, D.C. Pharmacokinetics of Hoasca alkaloids in healthy humans. *J. Ethnopharmacol.* **1999**, *65*, 243–256, doi:10.1016/S0378-8741(98)00168-8.
10. dos Santos, R.G.; Bouso, J.C.; Hallak, J.E.C. Ayahuasca, dimethyltryptamine, and psychosis: a systematic review of human studies. *Ther. Adv. Psychopharmacol.* **2017**, *7*, 141–157, doi:10.1177/2045125316689030.
11. Gaujac, A.; Dempster, N.; Navickiene, S.; Brandt, S.D.; De Andrade, J.B. Determination of *N,N*-dimethyltryptaminein beverages consumed in religious practices by headspace solid-phase microextraction followed by gas chromatography ion trap mass spectrometry. *Talanta* **2013**, *106*, 394–398, doi:10.1016/j.talanta.2013.01.017.
12. Li, Y.; Padoan, E.; Ajmone-Marsan, F. Soil particle size fraction and potentially toxic elements bioaccessibility: A review. *Ecotoxicol. Environ. Saf.* **2021**, *209*, 111806, doi:10.1016/j.ecoenv.2020.111806.
13. Carbonell-Capella, J.M.; Buniowska, M.; Barba, F.J.; Esteve, M.J.; Frígola, A. Analytical methods for determining bioavailability and bioaccessibility of bioactive compounds from fruits and vegetables: A review. *Compr. Rev. Food Sci. Food Saf.* **2014**, *13*, 155–171, doi:10.1111/1541-4337.12049.
14. Gonçalves, J.; Ramos, R.; Luís, Â.; Rocha, S.; Rosado, T.; Gallardo, E.; Duarte, A.P. Assessment of the Bioaccessibility and Bioavailability of the Phenolic Compounds of *Prunus avium* L. by in Vitro Digestion and Cell Model. *ACS Omega* **2019**, *4*, 7605–7613, doi:10.1021/acsomega.8b03499.
15. Versantvoort, C.H.M.; Oomen, A.G.; Van De Kamp, E.; Rompelberg, C.J.M.; Sips, A.J.A.M. Applicability of an *in vitro* digestion model in assessing the bioaccessibility of mycotoxins from food. *Food Chem. Toxicol.* **2005**, *43*, 31–40, doi:10.1016/j.fct.2004.08.007.
16. Minekus, M.; Alminger, M.; Alvito, P.; Ballance, S.; Bohn, T.; Bourlieu, C.; Carrière, F.; Boutrou, R.; Corredig, M.; Dupont, D.; et al. A standardised static *in vitro* digestion method suitable for food-an international consensus. *Food Funct.* **2014**, *5*, 1113–1124, doi:10.1039/c3fo60702j.
17. Sambuy, Y.; De Angelis, I.; Ranaldi, G.; Scarino, M.L.; Stammati, A.; Zucco, F. The Caco-2 cell line as a model of the intestinal barrier: Influence of cell and culture-related factors on Caco-2 cell functional characteristics. *Cell Biol. Toxicol.* **2005**, *21*, 1–26, doi:10.1007/s10565-005-0085-6.
18. Barthe, L.; Woodley, J.; Houin, G. Gastrointestinal absorption of drugs: Methods and studies. *Fundam. Clin. Pharmacol.* **1999**, *13*, 154–168, doi:10.1111/j.1472-8206.1999.tb00334.x.
19. Kosińska-Cagnazzo, A.; Diering, S.; Prim, D.; Andlauer, W. Identification of bioaccessible and uptaken phenolic compounds from strawberry fruits in *in vitro* digestion/Caco-2 absorption model. *Food Chem.* **2015**, *170*, 288–294, doi:10.1016/j.foodchem.2014.08.070.

20. Devkar, S.; Kandhare, A.; Sloley, B.; Jagtap, S.; Lin, J.; Tam, Y.; Katyare, S.; Bodhankar, S.; Hegde, M. Evaluation of the bioavailability of major withanolides of Withania somnifera using an *in vitro* absorption model system. *J. Adv. Pharm. Technol. Res.* **2015**, *6*, 159–164, doi:10.4103/2231-4040.165023.
21. Cameron, L.P.; Olson, D.E. Dark Classics in Chemical Neuroscience: *N, N*-Dimethyltryptamine (DMT). *ACS Chem. Neurosci.* **2018**, *9*, 2344–2357, doi:10.1021/acschemneuro.8b00101.
22. Riba, J.; Romero, S.; Grasa, E.; Mena, E.; Carrió, I.; Barbanoj, M.J. Increased frontal and paralimbic activation following ayahuasca, the pan-amazonian inebriant. *Psychopharmacology (Berl).* **2006**, *186*, 93–98, doi:10.1007/s00213-006-0358-7.
23. De Araujo, D.B.; Ribeiro, S.; Cecchi, G.A.; Carvalho, F.M.; Sanchez, T.A.; Pinto, J.P.; de Martinis, B.S.; Crippa, J.A.; Hallak, J.E.C.; Santos, A.C. Seeing with the eyes shut: Neural basis of enhanced imagery following ayahuasca ingestion. *Hum. Brain Mapp.* **2012**, *33*, 2550–2560, doi:10.1002/hbm.21381.
24. Food and Drug Administration Bioanalytical Method Validation Guidance for Industry Available online: https://www.fda.gov/drugs/guidance-compliance-regulatory-information/guidances-drugs (accessed on Jun 9, 2021).
25. Bensalem, S.; Soubhye, J.; Aldib, I.; Bournine, L.; Nguyen, A.T.; Vanhaeverbeek, M.; Rousseau, A.; Boudjeltia, K.Z.; Sarakbi, A.; Kauffmann, J.M.; et al. Inhibition of myeloperoxidase activity by the alkaloids of *Peganum harmala* L. (Zygophyllaceae). *J. Ethnopharmacol.* **2014**, *154*, 361–369, doi:10.1016/j.jep.2014.03.070.
26. Avula, B.; Wang, Y.H.; Rumalla, C.S.; Smillie, T.J.; Khan, I.A. Simultaneous determination of alkaloids and flavonoids from aerial parts of Passiflora species and dietary supplements using UPLC-UV-MS and HPTLC. *Nat. Prod. Commun.* **2012**, *7*, 1177–1180, doi:10.1177/1934578x1200700918.
27. Pires, A.P.S.; De Oliveira, C.D.R.; Moura, S.; Dörr, F.A.; Silva, W.A.E.; Yonamine, M. Gas chromatographic analysis of dimethyltryptamine and β-carboline alkaloids in Ayahuasca, an amazonian psychoactive plant beverage. *Phytochem. Anal.* **2009**, *20*, 149–153, doi:10.1002/pca.1110.
28. Souza, R.C.Z.; Zandonadi, F.S.; Freitas, D.P.; Tófoli, L.F.F.; Sussulini, A. Validation of an analytical method for the determination of the main ayahuasca active compounds and application to real ayahuasca samples from Brazil. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2019**, *1124*, 197–203, doi:10.1016/j.jchromb.2019.06.014.
29. Chambers, M.I.; Appley, M.G.; Longo, C.M.; Musah, R.A. Detection and Quantification of Psychoactive *N, N*-Dimethyltryptamine in Ayahuasca Brews by Ambient Ionization High-Resolution Mass Spectrometry. *ACS Omega* **2020**, *5*, 28547–28554, doi:10.1021/acsomega.0c03196.
30. Kaasik, H.; Souza, R.C.Z.; Zandonadi, F.S.; Tófoli, L.F.; Sussulini, A. Chemical Composition of Traditional and Analog Ayahuasca. *J. Psychoactive Drugs* **2021**, *53*, 65–75, doi:10.1080/02791072.2020.1815911.
31. Taylor, W.I. Simple Derivatives Of Tryptophan. In *Indole Alkaloids*; TAYLOR, W.I., Ed.; Elsevier: Pergamon, 1966; Vol. 1, pp. 29–51 ISBN 9781483196718.
32. Katchborian-Neto, A.; Santos, W.T.; Nicácio, K.J.; Corrêa, J.O.A.; Murgu, M.; Martins, T.M.M.; Gomes, D.A.; Goes, A.M.; Soares, M.G.; Dias, D.F.; et al. Neuroprotective potential of Ayahuasca and untargeted metabolomics analyses: applicability to Parkinson's disease. *J. Ethnopharmacol.* **2020**, *255*, doi:10.1016/j.jep.2020.112743.
33. Samoylenko, V.; Rahman, M.M.; Tekwani, B.L.; Tripathi, L.M.; Wang, Y.H.; Khan, S.I.; Khan, I.A.; Miller, L.S.; Joshi, V.C.; Muhammad, I. Banisteriopsis caapi, a unique combination of MAO inhibitory and antioxidative constituents for the activities relevant to neurodegenerative disorders and Parkinson's disease. *J. Ethnopharmacol.* **2010**, *127*, 357–367, doi:10.1016/j.jep.2009.10.030.
34. Elbrecht, D.H.; Long, C.J.; Hickman, J.J. Transepithelial/endothelial Electrical Resistance (TEER) theory and applications for microfluidic body-on-a-chip devices Keywords TEER Body-on-a-chip Barrier tissue Blood-brain barrier Organ Endothelial cells Epithelial cells Human-on-a-chip. *J Rare Dis Res Treat* **2016**, *1*, 46–52.
35. Hellinger, É.; Veszelka, S.; Tóth, A.E.; Walter, F.; Kittel, Á.; Bakk, M.L.; Tihanyi, K.; Háda, V.; Nakagawa, S.; Dinh Ha Duy, T.; et al. Comparison of brain capillary endothelial cell-based and epithelial (MDCK-MDR1, Caco-2, and VB-Caco-2) cell-based surrogate blood-brain barrier penetration models. *Eur. J. Pharm. Biopharm.* **2012**, *82*, 340–351, doi:10.1016/j.ejpb.2012.07.020.
36. Colombini, A.; Perego, S.; Ardoino, I.; Marasco, E.; Lombardi, G.; Fiorilli, A.; Biganzoli, E.; Tettamanti, G.; Ferraretto, A. Evaluation of a possible direct effect by casein phosphopeptides on paracellular and vitamin D controlled transcellular calcium transport mechanisms in intestinal human HT-29 and Caco2 cell lines. *Food Funct.* **2013**, *4*, 1195–1203, doi:10.1039/c3fo60099h.
37. Satsu, H.; Yokoyama, T.; Ogawa, N.; Fujiwara-Hatano, Y.; Shimizu, M. The changes in the neuronal PC12 and the intestinal epithelial Caco-2 cells during the coculture. The functional analysis using an *in vitro* coculture system. *Cytotechnology* **2001**, *35*, 73–79, doi:10.1023/A:1008136103927.
38. Piccolino, M.; Neyton, J.; Gerschenfeld, H.M. Decrease of gap junction permeability induced by dopamine and cyclic adenosine 3':5' -monophosphate in horizontal cells of turtle retina. *J. Neurosci.* **1984**, *4*, 2477–2488, doi:10.1523/jneurosci.04-10-02477.1984.
39. Fazzari, M.; Fukumoto, L.; Mazza, G.; Livrea, M.A.; Tesoriere, L.; Di Marco, L. *In vitro* bioavailability of phenolic compounds from five cultivars of frozen sweet cherries (*Prunus avium* L.). *J. Agric. Food Chem.* **2008**, *56*, 3561–3568, doi:10.1021/jf073506a.
40. Toydemir, G.; Boyacioglu, D.; Capanoglu, E.; Van Der Meer, I.M.; Tomassen, M.M.M.; Hall, R.D.; Mes, J.J.; Beekwilder, J. Investigating the transport dynamics of anthocyanins from unprocessed fruit and processed fruit juice from sour cherry (*Prunus cerasus* L.) across intestinal epithelial cells. *J. Agric. Food Chem.* **2013**, *61*, 11434–11441, doi:10.1021/jf4032519.
41. ATCC Caco-2 [Caco2] | ATCC Available online: https://www.atcc.org/products/htb-37 (accessed on Sep 2, 2021).

3.3. Paper V- Ayahuasca Beverages: Phytochemical Analysis and Biological Properties

Abstract

Ayahuasca is a psychoactive beverage, originally consumed by indigenous Amazon tribes, of which consumption has been increasing worldwide. The aim of this study was to evaluate the phytochemical profile, as well as the antioxidant, anti-inflammatory and antimicrobial properties of decoctions of four individual plants, a commercial mixture and four mixtures of two individual plants used in the ayahuasca preparation. For this purpose, a phytochemical characterization was performed, determining the content of flavonoids, total phenolic compounds, and analyzing the phenolic profile. Besides, 48 secondary metabolites were investigated by ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC-Q/TOF-MS) and their concentration estimated with real standards when present. The antioxidant activity was evaluated by both the β-carotene bleaching test and DPPH free radical scavenging assay, and the anti-inflammatory activity was determined by a protein denaturation method. Finally, the antimicrobial properties were evaluated using the disc diffusion assay, resazurin microtiter method, anti-quorum sensing and anti-biofilm activity assays. The obtained results showed that, in general, the samples have a high content of phenolic compounds and flavonoids with noticeable differences, reflecting on remarkable antioxidant and anti-inflammatory activities. Significant antimicrobial properties were also observed, with emphasis on the effect of *B. caapi* and *P. harmala* on planktonic and biofilm cells of *A. baumannii*, inhibiting both the biofilm formation and the production of violacein pigment.

Keywords: Ayahuasca; phytochemical characterization; antioxidant activity; anti-inflammatory activity; antimicrobial properties

3.3.1. Introduction

Ayahuasca is a psychoactive beverage that has been consumed for centuries [1,2]. Originally, it was used by indigenous tribes in the Northwest Amazon for therapeutic purposes and divine rituals; however, it has now expanded, being consumed worldwide [3,4,5]. The term “ayahuasca” has Quechua origin and derives from the words “aya”, which means “spirit”, and “huasca”, meaning “vine” [1,2,4,5,6]. The significance of this beverage, also known as *yajé*, *daime*, *vegetal*, *capi*, *nate*, *hoasca*, *natema*, can be translated as “vine of the dead” or “vine of the soul” [2,4,7,8]. Traditionally, ayahuasca consisted of a decoction prepared with the leaves of the *Psychotria viridis* bush and scraps of the stem of the *Banisteriopsis caapi* vine. However, over the years, several variations of this decoction have been developed [4,5]. Several analogues that can

replace *P. viridis* are also known, namely *Psychotria carthagenesis*, *Brugmansia suaveolens*, *Nicotiana tabacum*, *Brunfelsia*, spp., *Daturaolens*, *Malouetia tamarquina*, *Tabernaemontana* spp., among others [1,9]. In the case of *B. caapi*, besides to analogs of natural origin (*Peganum harmala*), there are also synthetic analogs (tetrahydroharmine freebase/HCl, moclobemide and harmine freebase/HCl) that can replace the use of the traditional plant [1,3,10,11].

The ayahuasca decoction contains β-carboline alkaloids such as harmine, tetrahydroharmine (THH) and harmaline, which come from *B. caapi*, and the hallucinogenic compound *N,N*-dimethyltryptamine (DMT) from *P. viridis* [12,13]. DMT is a serotonin 5-HT1A/2A/2C receptors agonist that, when ingested alone, is metabolized by peripheral monoamine oxidase-A (MAO-A), becoming harmless [14]. Nonetheless, when combined with the β-carboline alkaloids, can access the circulation and the central nervous system, since they temporarily inhibit MAO-A [12,14,15,16]. Additionally, THH also inhibits the reuptake of serotonin by increasing the effects of DMT [17]. This compound is hydrophobic and presents a low molecular weight, allowing it to quickly cross the blood-brain barrier. Furthermore, it is structurally similar to melatonin and serotonin, with a tryptamine moiety which provides high affinity for neurological receptors and triggers more robust behavioral responses [18].

In the last twenty-five years, the ayahuasca consumption has expanded worldwide (United States of America, Europe, Africa and Asia) raising concerns about the possible negative effects of its consumption, but also curiosity about the potential therapeutic effects described [13,17,19,20].

The increase of the antimicrobial resistance by pathogens is of major concern, since it leads to an increase in morbidity and mortality, endangering public health [21,22]. Thus, antibiotics that were used against pathogens are no longer effective, namely against Gram-positive bacteria such as *Staphylococcus aureus* that developed resistance against methicillin [23,24]. Additionally, studies performed on Gram-negative bacteria, such as *Acinetobacter baumannii*, *Escherichia coli* and *Pseudomonas aeruginosa*, have shown the emergence of antibiotic resistance [25,26]. Thus, it is crucial to search for new compounds with antimicrobial properties that allow the treatment of infections by resistant pathogens [27]. Higher plants have classes of compounds that can be used as sources of antibiotics, so it is important to evaluate the antimicrobial effects of those compounds in order to identify new molecules with potential inhibitory properties of pathogenic microorganisms [27,28].

The present work describes the phytochemical characterization of four individual plants, a commercial mixture and four plant mixtures used in the preparation of the ayahuasca decoctions. The antioxidant, anti-inflammatory and antimicrobial properties of those plant samples were also evaluated.

3.3.2. Results and Discussion

The search for new plant-derived compounds with bioactive properties is crucial. Thus, and considering the potential effects of ayahuasca, in this work, the biological activities of the plant materials used in the preparation of this beverage, namely antioxidant, anti-inflammatory and antimicrobial properties, were evaluated. In this sense, four decoctions of ayahuasca were prepared

(with two different plant materials, one source of DMT and another of β-carboline alkaloids). Besides, individual decoctions of each plant used in the preparation of ayahuasca were also prepared. In addition, a commercial mixture was also purchased and evaluated.

3.3.2.1. Phytochemical Characterization and Phenolic Profile

Ayahuasca has been shown to have important beneficial health effects [12,17,29,30,31,32,33,34,35]. Considering that there is a small number of phytochemical studies of the decoctions prepared from the plants *P. viridis*, *B. caapi*, *P. harmala* and *M. hostilis*, in the present study the determination of the content of flavonoids and total phenolics in these samples was performed. Additionally, the phenolic profile of the samples was determined by ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC/ESI-QTOF-MS).

Phenolic compounds are among the main secondary metabolites of plants, and it is possible to find some of them in all plants [36,37,38]. These compounds have some interesting properties from a clinical point of view [37,39]. In the present study, the total phenolics were determined using the Folin-Ciocalteu colorimetric method (Table III.3.1). Although some studies describe limitations of the Folin-Ciocalteu colorimetric method, it remains widely used for the determination of total phenolics [40]. All samples showed substantial concentrations of total phenolics, with *M. hostilis*, commercial mixture and the mixture of *M. hostilis* and *B. caapi* showing the highest concentrations, and *P. harmala* the lowest content of total phenolics. Recently, Hadadi *et al.* [41], developed a study where determined the content of total phenols, using the same method, having also verified the presence of these compounds in extracts of *P. harmala*.

Table III.3.1. Total phenolic compounds and flavonoids content (mean ± standard deviation)

Samples	Phenolic Compounds (mg GAE/g sample)	Flavonoids (mg QE/g sample)
Commercial mixture	364.67 ± 7.66	10.29 ± 0.68
P. viridis	210.67 ± 7.27	8.76 ± 0.94
B. caapi	114.67 ± 2.44	3.91 ± 0.28
M. hostilis	376.80 ± 15.84	14.63 ± 1.36
P. harmala	78.27 ± 5.75	25.92 ± 2.56
P. viridis* + *B. caapi	150.60 ± 10.47	9.81 ± 0.67
P. viridis* + *P. harmala	132.13 ± 3.84	19.93 ± 0.95
M. hostilis* + *B. caapi	327.47 ± 9.94	6.85 ± 0.23
M. hostilis* + *P. harmala	196.13 ± 1.67	16.25 ± 0.97

GAE—gallic acid equivalents; QE—quercetin equivalents.

Flavonoids have beneficial biological activities, namely anti-inflammatory, antimicrobial, antioxidant, cytotoxic and anti-tumor activities [42,43]. In the present study, flavonoids were determined by the aluminium chloride colorimetric method (Table III.3.1). All samples were found

to have flavonoids in their composition, with *P. harmala* and the mixture of *P. viridis* and *P. harmala* the samples having the highest flavonoid content. Contrariwise, the samples of *B. caapi* and the mixture of *B. caapi* and *M. hostilis* showed the lowest levels of flavonoids.

The samples were then analyzed by liquid chromatography with high resolution mass spectrometric detection in order to identify the compounds and to further complement the initial phytochemical characterization. Thus, the identification of compounds was carried out by comparing their retention times and accurate mass spectra provided by the UHPLC-QTOF-MS with those of authentic standards when available. The phytochemical library of 48 standards (Appendix A Figure III.3.A1) was used to characterize the metabolites present in the methanolic extracts. Thus, it was possible to verify that two groups of compounds were mainly present: hydroxybenzoic acids and flavonoids. The concentration of the identified compounds was estimated by comparing their peak areas in the chromatograms from the plant extracts with those of the corresponding standard solutions freshly prepared and analyzed by duplicate in the same batch as samples. The results are shown in Table III.3.2. In the sample of *P. viridis* it was possible to quantify two hydroxybenzoic acids (protocatechuic acid, 4-hydroxybenzoic acid), two hydroxycinnamic acids (chlorogenic acid and neochlorogenic acid) and six flavonoids ((+)-catechin, (-)-epicatechin, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-rutinoside and kaempferol-3-*O*-rutinoside). These results are similar to those obtained by Ma *et al.* [44], where the phenolic compounds of a sample of *P. viridis* were analyzed by liquid chromatography coupled to mass spectrometry, being (+)-catechin and (-)-epicatechin detected. Additionally, five other compounds were also detected (gallic acid, (+)-gallocatechin, dihydromyricetin, (+)-catechin-3-*O*-gallate and myricitrin) that are not part of the library used in this work [44]. In the sample of *P. harmala* it was only possible to quantify the hydroxybenzoic acids, protocatechuic, gentisic and salicylic acids. The remaining compounds were either not detected, or are below the limit of quantification. Regarding the sample of *M. hostilis*, protocatechuic, 4-hydroxybenzoic and salicylic acids, and the flavonoids (+)-catechin and (-)-epicatechin were quantified. Regarding the *B. caapi* sample, the hydroxybenzoic acids, protocatechuic and salicylic, and the flavonoids (+)-catechin, (-)-epicatechin, quercetin-3-*O*-glucoside, quercetin-3-*O*-rutinoside and phlorizin, were quantified. Finally, the commercial mixture was also analyzed by the same analytical method, allowing the quantification of three hydroxybenzoic acids (protocatechuic, 4-hydroxybenzoic and salicylic acids) and five flavonoids ((+)-catechin, (-)-epicatechin, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside and quercetin-3-*O*-rutinoside). The chemical composition and the proportion of the identified compounds are variable in the different analyzed samples. It is difficult to compare the results obtained for the samples of *B. caapi*, *M. hostilis, P. harmala* and for the commercial mixture, since most chromatographic studies focus on the detection of psychoactive compounds, such as DMT, or β-carboline alkaloids [3,45,46,47,48,49,50]. Therefore, future research on these plant samples should be focused on the phytochemical characterization and potential bioactive effects associated with these compounds.

Table III.3.2. Concentration of phenolic compounds (μg/g) in the methanol extracts from decoctions samples.

Compound	Commercial mixture	*P. viridis*	*B. caapi*	*M. hostilis*	*P. harmala*
Hydroxybenzoic acids					
protocatechuic acid	417.1	272.8	122.6	109.2	4.8
4-hydroxybenzoic acid	39.6	53.2	N.D.	4.3	<LOQ
gentisic acid	<LOQ	N.D.	<LOQ	N.D.	2.8
salicylic acid	1.5	<LOQ	9.1	0.25	3.8
Hydroxycinnamic acids					
chlorogenic acid	N.D.	42.1	N.D.	N.D.	N.D.
neochlorogenic acid	N.D.	6.0	N.D.	N.D.	N.D.
Flavonoids-Flavanols					
(+)-catechin	240.4	10.7	465.4	1.35	<LOQ
(-)-epicatechin	570.5	22.6	1112.0	4.6	<LOQ
quercetin-3-*O*-galactoside	7.1*	3.0*	N.D.	N.D.	N.D.
quercetin-3-*O*-glucoside			14.4	N.D.	N.D.
quercetin-3-*O*-rutinoside	18.7	47.4	6.3	N.D.	<LOQ
kaempferol-3-*O*-glucoside	N.D.	N.D.	<LOQ	N.D.	N.D.
kaempferol-3-*O*-rutinoside	N.D.	26.6	N.D.	N.D.	N.D.
Flavonoids-dihydrochalcone					
phlorizin	<LOQ	N.D.	1.0	<LOQ	N.D.

N.D.—not detected; LOQ—limit of quantitation; LOQ—0.006 μg/g (salicylic acid; quercetin-3-*O*-rutinoside); 0.010 μg/g (4-hydroxybenzoic acid; gentisic acid, (-)-epicatechin); 0.018 μg/g (kaempferol-3-*O*-rutinoside); 0.034 μg/g (phlorizin); * mixture of both metabolites.

3.3.2.2. Antioxidant Activity

In this study, the antioxidant activity of the ayahuasca decoctions was evaluated in order to identify new sources of antioxidants. Initially, the antioxidant activity of the extracts was evaluated by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay and the results are presented in Table III.3.3. This colorimetric assay is widely used because it is quick and easy, consisting in the evaluation of the potential for free radical scavenging of the samples [51]. Observing the results, it is possible to verify that the samples of *M. hostilis*, the mixtures of *M. hostilis* and *B. caapi* and *M. hostilis* and *P. harmala*, and the commercial mixture showed a "very strong" antioxidant activity, because their antioxidant activity index (AAI) values were higher than 2.0 [52]. Otherwise, the sample of *P. harmala* presented values of AAI below 0.5, and, therefore, "poor" antioxidant activity [52]. The remaining samples showed "strong" antioxidant activity [52]. These results may be related with the phenolic compounds present in the samples, given that the ones with the better antioxidant activity (mixture of *M. hostilis* and *B. caapi* and a commercial mixture) are also those with higher concentration of total phenolics (Table III.3.1). To the best of our knowledge, there are no previous studies where the antioxidant activity of *P. viridis*, *B. caapi*

and *M. hostilis* was evaluated. Regarding the results obtained for *P. harmala*, these are very similar to those obtained by other researchers, where the IC_{50} values are always greater than 100, resulting in reduced antioxidant activity [53,54].

Table III.3.3. Antioxidant properties of the samples (mean ± standard deviation).

Samples		DPPH Free Radical Scavenging Assay			β-Carotene Bleaching Test
		IC_{50} (mg/L)	**AAI**	**Antioxidant Activity**	**IC_{50} (mg/L)**
Commercial mixture		5.65 ± 1.05	6.36 ± 0.13	Very Strong	224.15 ± 31.72
P. viridis		18.89 ± 3.01	1.90 ± 0.13	Strong	225.51 ± 49.60
B. caapi		26.71 ± 3.77	1.51 ± 0.10	Strong	1497.88 ± 148.52
M. hostilis		7.17 ± 0.84	4.40 ± 0.13	Very Strong	243.35 ± 0.61
P. harmala		211.67 ± 20.41	0.14 ± 0.03	Poor	2713.16 ± 649.32
P. viridis* + *B. caapi		29.71 ± 3.46	1.98 ± 0.13	Strong	237.52 ± 31.94
P. viridis* + *P. harmala		29.74 ± 5.62	1.29 ± 0.04	Strong	322.55 ± 12.10
M. hostilis* + *B. caapi		7.74 ± 1.25	6.20 ± 1.39	Very Strong	1069.59 ± 65.74
M. hostilis* + *P. harmala		12.14 ± 2.42	3.27 ± 0.15	Very Strong	257.83 ± 50.28
Positive controls	gallic acid	2.23 ± 0.02	22.77 ± 0.25	Very Strong	-
	BHT	-	-	-	29.33 ± 0.34

IC_{50}—half maximal inhibitory concentration; AAI—antioxidant activity index; BHT—butylated hydroxytoluene.

The comparison of the results of the antioxidant activity of a determined sample is not linear, since the mechanism of action is very complex and varies within matrices. Additionally, the measurement of this activity depends on the method employed [27]. For these reasons, and in order to better understand the antioxidant mechanism of ayahuasca decoctions, their antioxidant activity was also evaluated by the β-carotene bleaching test (Table III.3.3). This assay is widely used in the evaluation of the antioxidant activity of samples of natural origin, since it allows the evaluation of the ability of the samples to inhibit the lipid peroxidation [40,55]. In this test, the antioxidant activity evaluation is undertaken by comparing two competitive chemical reactions involving the potentially antioxidant compounds present in the samples and the antioxidant model β-carotene [56]. As it was observed in the DPPH assay, the *P. harmala* sample showed the highest IC_{50} value and, consequently, the lowest antioxidant activity. Contrariwise, the samples with lower IC_{50}, and, therefore, greater antioxidant activity, were the commercial mixture and *P. viridis*.

In general, the results obtained allowed concluding that the ayahuasca is a good source of bioactive compounds with the ability to scavenge free radicals and to inhibit the lipid peroxidation.

3.3.2.3. Anti-Inflammatory Activity

Protein denaturation is an important indicator of an inflammation process, since it occurs during tissue damage, leading to the production of auto-antigens [57,58]. Thus, in this work, the anti-inflammatory activity of the samples was studied, by assessing its capacity to inhibit protein denaturation. Although this method is not a direct test, it is often used to assess the anti-inflammatory potential of plant samples [59]. Observing the results (Table III.3.4) it is possible to verify that the samples with the highest IC_{50} and, consequently, with the lowest anti-inflammatory activity are *P. viridis* and *B. caapi*. The samples with the best anti-inflammatory activity are *P. harmala*, *M. hostilis*, the mixture of *M. hostilis* and *P. harmala* and the commercial mixture. The

results obtained for *P. harmala* are in agreement with the literature, since previous studies corroborate its anti-inflammatory potential [60,61]. However, for the remaining samples, no studies were found to compare the results.

Table III.3.4. Anti-inflammatory activity results (mean ± standard deviation).

Sample		Anti-Inflammatory Activity - IC_{50} (mg/L)
Commercial mixture		30.89 ± 1.24
P. viridis		168.92 ± 25.49
B. caapi		163.75 ± 23.84
M. hostilis		43.84 ± 0.46
P. harmala		37.38 ± 2.78
P. viridis + *B. caapi*		117.88 ± 15.91
P. viridis + *P. harmala*		N.D.
M. hostilis + *B. caapi*		N.D.
M. hostilis + *P. harmala*		19.06 ± 0.87
Positive control	acetylsalicylic acid	0.80 ± 0.09

N.D.: Not detected; IC_{50}- half maximal inhibitory concentration

3.3.2.4. Antimicrobial Activity

Currently, the resistance to conventional drugs by pathogenic microorganisms is a major concern, and the search for alternatives of natural origin has been growing [62]. Thus, in this work, the potential antimicrobial activity of the ayahuasca decoctions was evaluated against four Gram-positive and four Gram-negative bacteria. The tested strains were chosen because they are human infective, being some of them well known for their pathogenicity and resistance to antibiotics. Namely, *L. monocytogenes* and *B. cereus*, known foodborne pathogens, and *E. faecalis*, *S. aureus*, *E. coli*, *A. baumannii*, *P. aeruginosa* and *S.* Typhimurium, responsible for several health-related infections [63,64,65,66,67,68,69,70].

Initially, the disc diffusion assay was performed, with some samples presenting antibacterial activity (Table III.3.5). Analyzing the results, it was verified that six samples inhibited the bacterial growth in all strains. However, the commercial mixture was unable to inhibit the growth of *E. faecalis*, and slightly inhibited the growth of *L. monocytogenes* and *S.* Typhimurium. Additionally, the mixture of *P. viridis* and *B. caapi* was not able to inhibit the growth of *L. monocytogenes*, *S.* Typhimurium, *E. coli* and *E. faecalis*. Regarding the mixture of *P. viridis* and *P. harmala*, there was also a reduced inhibition in the growth of *L. monocytogenes* and absence of growth inhibition of *E. faecalis*. The other samples showed remarkable antibacterial activity in all the tested strains. The strain that was less susceptible to the samples was *E. faecalis*, with a range of inhibition diameters between 6.00 mm and 10.13 mm. These results can be explained by the ability of this microorganism to adapt to severe situations, namely environmental changes, salt concentrations, extreme alkaline pH, or even to the deprivation of nutrition [71]. Otherwise, the most promising results were observed for *S. aureus* and *A. baumannii*, with inhibition diameters ranging between 20.39 mm and 13.27 mm and between 17.81 mm and 11.04 mm, respectively. It is

also important to note that in this study it was possible to observe antimicrobial activity against Gram-negative bacteria, which usually have greater resistance to samples of natural origin [72,73].

Table III.3.5. Diameter of inhibition zones (mm) in disc diffusion assay (mean ± standard deviation).

Samples (3 mg/disc)		Strains							
		S. aureus ATCC 25923	*L. monocytogenes* LMG 16779	*E. faecalis* ATCC 29212	*B. cereus* ATCC 11778	*E. coli* ATCC 25922	*S.* Typhimurium ATCC 13311	*P. aeruginosa* ATCC 27853	*A. baumannii* LMG 1025
P. viridis		19.85 ± 0.28	8.19 ± 1.68	9.20 ± 1.70	14.06 ± 2.06	12.04 ± 2.49	8.84 ± 0.88	12.73 ± 1.65	12.65 ± 1.32
B. caapi		17.52 ± 0.69	11.04 ± 1.75	9.83 ± 2.14	17.86 ± 0.16	13.56 ± 1.51	11.01 ± 0.29	8.14 ± 1.61	17.53 ± 0.33
P. harmala		14.34 ± 2.03	14.54 ± 1.87	9.86 ± 0.04	16.95 ± 0.37	18.19 ± 1.52	16.93 ± 0.88	9.09 ± 0.43	17.81 ± 0.34
M. hostilis		19.93 ± 3.41	11.18 ± 0.91	10.13 ± 1.33	14.68 ± 1.44	13.19 ± 1.30	11.58 ± 0.54	14.59 ± 0.25	14.66 ± 0.70
Commercial mixture		14.68 ± 1.58	6.49 ± 0.85	6.00 ± 0.00	13.92 ± 1.17	7.48 ± 1.33	6.56 ± 0.98	9.73 ± 0.30	11.8 ± 0.69
P. viridis* + *B. caapi		13.69 ± 2.87	6.00 ± 0.00	6.00 ± 0.00	11.55 ± 1.77	6.00 ± 0.00	6.00 ± 0.00	10.34 ± 1.00	11.04 ± 0.77
P. viridis* + *P. harmala		13.27 ± 1.73	6. 68 ± 1.18	6.00 ± 0.00	12.52 ± 2.27	11.56 ± 0.83	11.36 ± 2.81	10.57 ± 0.90	13.87 ± 0.36
M. hostilis* + *B. caapi		20.39 ± 0.94	10.82 ± 1.57	9.09 ± 0.89	14.12 ± 2.14	11.00 ± 1.84	11.71 ± 1.44	14.57 ± 1.18	15.09 ± 1.22
M. hostilis* + *P. harmala		17.94 ± 1.29	7.44 ± 1.26	8.55 ± 1.12	14.18 ± 1.77	12.68 ± 1.10	12.12 ± 0.37	12.29 ± 2.23	15.06 ± 0.53
Controls	DMSO (15 µL/disc)	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00
	tetracycline (30 µg/disc)	30.25 ± 0.50	18.25 ± 0.60	25.20 ± 0.58	30.00 ± 0.82	23.25 ± 0.50	28.45 ± 0.52	11.50 ± 0.58	25.63 ± 0.25

DMSO-dimethyl sulfoxide.

After the initial screening of the antimicrobial potential of the samples, the minimum inhibitory concentration (MIC) values were determined. For this, the resazurin microtiter assay was performed (Table III.3.6). The strains that presented the lowest MIC and, therefore, were more susceptible to the action of the samples, were *B. cereus* and *A. baumannii*, with values between 0.156 mg/mL and 5 mg/mL and between 0.625 mg/mL and 5 mg/mL, respectively. Similarly to what was verified in the disc diffusion assay, the mixtures of *P. viridis* and *B. caapi* and *P. viridis* and *P. harmala* showed the least promising results, with MIC values varying between 2.5 mg/mL and >10 mg/mL. However, these samples and the commercial mixture showed considerable MIC values against strains where no inhibition was observed in the disc diffusion assay. This result may be related to the poor diffusion of the extracts in the agar plates [27]. The samples that, in general, showed better MIC values, and consequently greater antimicrobial activity, were the ones of *B. caapi* and *P. harmala*. In previous studies, the antimicrobial action of *P. harmala* extracts against *E. coli* and *S.* Typhimurium was already reported [74]; however, the MIC values obtained in that study (0.625 mg/mL) were lower than those obtained now (1.25 mg/mL for *E. coli* and 2.5 mg/mL for *S.* Typhimurium). Bussmann *et al.* [75], also determined the MIC for *B. caapi* against *S. aureus* and *E. coli*, but the values presented in that study (0.0625 mg/mL for *E. coli* and 1 mg/mL for *S. aureus*) are lower than those now determined. Nevertheless, these comparisons must be made with caution, since in the present study the samples consists of a decoction, whereas in the previous studies, methanolic [74], and ethanolic [75], extracts were used, which allows a better extraction of

potential bioactive compounds. Furthermore, the differences in susceptibility of the used strains would also affect the results.

Table III.3.6. Minimum inhibitory concentration (MIC) values (mg/mL) of samples (modal values).

Samples		Strains							
		S. aureus ATCC 25923	*L. monocytogenes* LMG 16779	*E. faecalis* ATCC 29212	*B. cereus* ATCC 11778	*E. coli* ATCC 25922	*S.* Typhimurium ATCC 13311	*P. aeruginosa* ATCC 27853	*A. baumannii* LMG 1025
P. viridis		5	10	10	0.156	>10	>10	>10	5
B. caapi		1.25	2.5	10	0.313	2.5	2.5	5	0.625
P. harmala		1.25	5	5	0.625	1.25	2.5	5	0.625
M. hostilis		2.5	10	2.5	0.156	5	>10	>10	0.625
Commercial mixture		2.5	5	2.5	0.313	10	10	10	2.5
P. viridis* + *B. caapi		>10	10	>10	2.5	10	10	10	2.5
P. viridis* + *P. harmala		>10	>10	10	2.5	10	>10	10	5
M. hostilis* + *B. caapi		>10	>10	>10	2.5	5	10	5	1.25
M. hostilis* + *P. harmala		5	10	5	5	2.5	10	2.5	0.625
Controls	DMSO (%)	>20	>20	>20	>20	>20	>20	>20	>20
	Tetracycline (μg/mL)	0.06	0.06	0.06	0.06	0.06	0.24	0.06	0.24

DMSO-dimethyl sulfoxide.

Considering the antimicrobial activity demonstrated by the samples, their anti-quorum sensing properties were also evaluated (Table III.3.7). For that, the biomonitor strain *Chromobacterium violaceum* ATCC 12472 was used, which produces the pigment violacein and uses signal molecules of N-acyl homoserine lactone in order to monitor population density [59]. Analyzing the results, it was observed that, with the exception of *M. hostilis*, all samples were able to inhibit the production of violacein and, consequently, the quorum sensing. However, the samples of *B. caapi* and *P. harmala* stand out, as they produced a diameter of inhibition violacein production much higher than the other samples (13.26 mm and 13.16 mm, respectively). It should be noted that the inhibition diameters of these two samples were greater than that of resveratrol, used as a positive control.

Table III.3.7. Anti-quorum sensing activity of the samples (mean ± standard deviation).

Samples (3 mg/disc)	Diameters of inhibition of the violacein pigment production (mm)
Commercial mixture	4.31 ± 0.23
P. viridis	2.44 ± 0.05
B. caapi	13.26 ± 1.50
M. hostilis	0.00 ± 0.00
P. harmala	13.16 ± 0.04
P. viridis + *B. caapi*	3.60 ± 0.66
P. viridis + *P. harmala*	3.49 ± 0.35
M. hostilis + *B. caapi*	3.11 ± 0.28
M. hostilis + *P. harmala*	9.62 ± 1.05
DMSO (15 μL/disc)	0.00 ± 0.00
Resveratrol (5 μg/disc)	8.49 ± 0.20

DMSO-dimethyl sulfoxide.

Over the years, the antimicrobial properties of some phenolic compounds, namely protocatechuic acid [76], gentisic acid [77], catechin and epicatechin [78], and other flavonoids

[36,79,80,81,82,83] were reported. A high amount of these phenolic compounds in plants can lead to a more effective response in defense against pathogens [36,79,80,81,82,83]. Analyzing the Table III.3.1, it is possible to verify that *P. harmala* presents the highest flavonoid content. Thus, and taking into account the antimicrobial activity previously described for flavonoids, the promising results obtained with this sample may be related to this group of compounds. Similarly, the results obtained for *B. caapi* can also be related to its phytochemical composition. Observing the Table III.3.2, it is possible to verify that *B. caapi* presents considerable values of protocatechuic acid, catechin and epicatechin. As previously mentioned, the antimicrobial activity of catechin and epicatechin has been described, and their action against pathogens is known.

A. baumannii is a pathogen responsible for multidrug-resistant nosocomial infections [84]. This microorganism is often associated with bloodstream infections and pneumonia associated with ventilation, which can even be fatal [70,84]. Additionally, *A. baumannii* has a plastic genome, which allows adaptation to adverse and stressful environments [70]. The virulence factors of this pathogen are known, namely the ability to form biofilms [59]. Biofilms produced by bacteria are highly resistant, as they are protected by the extracellular matrix [85]. In addition, the lower susceptibility of Gram-negative bacteria to inhibition by plant extracts has been described [72,73]. Given the promising results of *B. caapi* and *P. harmala*, concerning the antibacterial and anti-quorum sensing activities, their anti-biofilm activity against *A. baumannii* was further evaluated. For that, biofilms formed in polystyrene coupons in the presence of the samples were observed by SEM (Figure III.3.1). Analyzing the Figure III.3.1a it is possible to verify that the biofilm of *A. baumannii* presents a three-dimensional structure with several layers of cells connected to each other. In contrast, when the biofilm was formed in the presence of *B. caapi*, the *A. baumannii* cells appear in small number, with no connection between them and without the three-dimensional structure (Figure III.3.1b). Observing Figure III.3.1c, it appears that the small number of *A. baumannii* cells are partially destroyed when the biofilm grew in the presence of *P. harmala*. These results suggest that *P. harmala* and *B. caapi* present anti-biofilm activity against *A. baumannii*. Together with the results obtained in the anti-quorum sensing test, these data allow to infer that these samples are able to inhibit biofilm formation, not only by inhibiting the bacterial growth, but also by inhibiting the cells adhesion to the surface [86].

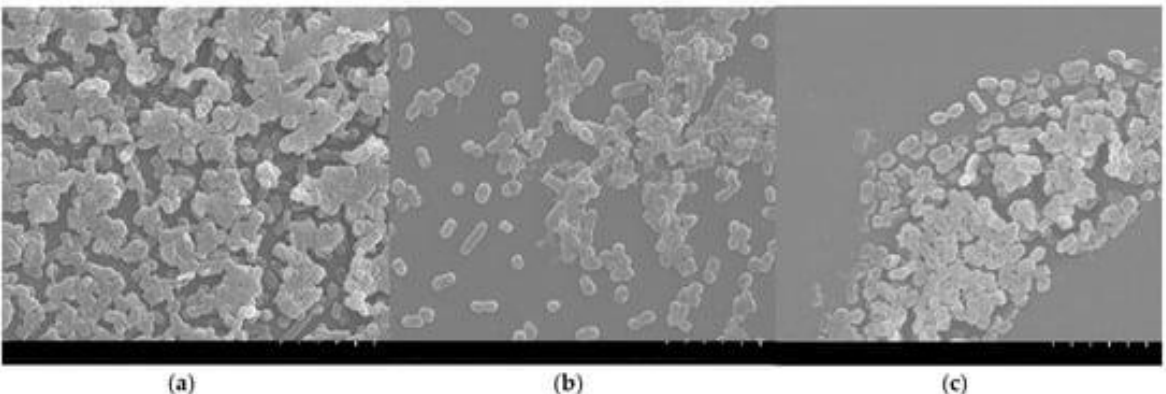


Figure III.3.1. Scanning electron microscopy (SEM) images of *Acinetobacter baumannii* biofilms formed in the presence of two samples of ayahuasca decoctions: **(a)** *A. baumannii* LMG 1025 biofilm (control); **(b)** *A. baumannii* LMG 1025 biofilm formed in the presence of *B. caapi* (0.5 × MIC); **(c)** *A. baumannii* LMG 1025 biofilm formed in the presence of *P. harmala* (0.5 × MIC). MIC—minimum inhibitory concentration; magnification 5000×.

3.3.3. Materials and Methods

3.3.3.1. Sample Preparation

The vegetal samples of *P. viridis*, *B. caapi*, *M. hostilis* and *P. harmala*, as well as the commercial mixture (without information about its composition) were acquired online from the Shayana Shop (https://www.shayanashop.com, Amsterdam, The Netherlands). The decoctions of ayahuasca were prepared according to a recipe kindly provided by Dr. Nicolás Fernández (Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina). Five decoctions of each individual plant samples were prepared. For this purpose, 0.210 g of each vegetal sample were milled in a mortar with a few drops of water. This preparation was transferred to a Schott flask and 250 mL of ultra-pure water were added. The Schott flasks were boiled at 100 °C for 4 h. Similarly, four decoctions were prepared where two of the above vegetal samples were mixed (*P. viridis* and *B. caapi*; *P. viridis* and *P. harmala*; *M. hostilis* and *B. caapi*; *M. hostilis* and *P. harmala*). Therefore, in each mixture there is a source of DMT and a source of β-carboline alkaloids, according to works previously developed by our research group [87]. Finally, the samples were cooled, filtered, frozen at −80 °C and freeze-dried.

3.3.3.2. Phytochemical Characterization and Phenolic Profile

3.3.3.2.1. Total Phenolic Compounds Determination

The total phenolic compounds were determined by the Folin–Ciocalteu colorimetric method [27]. For that, the samples were dissolved in methanol (50 mg/mL) and, subsequently, 50 µL of these solutions, or gallic acid (standard phenolic compound), were mixed with 450 µL of distilled water. Then, 2.5 mL of 0.2 N Folin–Ciocalteu reagent were added, the samples were left to stand for 5 min and, after that time, 2 mL of Na_2CO_3 (75 g/L) were added. The reaction mixtures were incubated at 30 °C for 90 min. Subsequently, the total phenolic content was determined by colorimetry (765 nm), using a standard curve prepared with methanolic solutions of gallic acid ($y = 0.001x$; $R^2 = 0.9845$). The tests were carried out in triplicate, and the results were expressed as gallic acid equivalents (mg GAE/g sample) [40].

3.3.3.2.2. Flavonoids Determination

The flavonoid content was determined by the aluminium chloride colorimetric method, following a previously described methodology, using quercetin as standard [27]. Thus, to 500 µL of each methanolic sample (50 mg/mL), 1.5 mL of methanol, 100 µL of aluminium chloride (10%, w/v), 100 µL of potassium acetate (1 M) and 2.8 mL of distilled water were added. This mixture was incubated for 30 min at room temperature. Subsequently, the flavonoid content was determined by colorimetry (415 nm), using a calibration curve prepared with methanolic solutions of quercetin (y

= 0.0146x; R^2 = 0.9964). The tests were performed in triplicate and the results were expressed as quercetin equivalents (mg QE/g of sample).

3.3.3.2.3. Determination of the Phytochemical Profile by UHPLC/ESI-QTOF-MS

The identification of secondary metabolites present in the vegetal samples was performed following a methodology previously developed by the Center of Metabolomics and Bioanalysis (CEMBIO) based on analysis by UHPLC/ESI-QTOF-MS [88,89]. For that, a methanolic extraction was performed as follows: 300 µL of methanol was added to 30 mg lyophilized powder sample. The mixture was vortexed for 2 min, sonicated for 15 min and centrifuged at 10,000× g for 5 min at 4 °C. The supernatants were then collected and transferred to a Chromacol vial (Thermo Fisher Scientific, Madrid, Spain) for LC/MS analysis. The whole procedure was performed by duplicate. Then, samples were analyzed on a 1290 Infinity series UHPLC system coupled with an electrospray ionization source (ESI) with Jet Stream technology to a 6545 iFunnel QTOF/MS system (Agilent Technologies, Waldbronn, Germany). For the separation, a volume of 2 µL was injected in a reversed-phase column (Zorbax Eclipse XDB-C18 4.6 × 50 mm, 1.8 µm, Agilent Technologies) at 40 °C. The flow rate was 0.5 mL/min with a mobile phase consisting of solvent A: 0.1% formic acid in ultrapure water, and solvent B: methanol. Gradient elution consisted of 2% B (0–6 min), 2–50% B (6–10 min), 50–95% B (11–18 min), 95% B for 2 min (18–20 min), and returned to starting conditions 2% B in one minute (20–21 min) to finally keep the re-equilibration with a total analysis time of 25 min. Detector was operated in full scan mode (m/z 50 to 1500), at a scan rate of 1 scan/s both in positive and negative ESI mode. Accurate mass measurement was assured through an automated calibrator delivery system that continuously introduced a reference solution, containing masses of m/z 121.0509 (protonated purine) and m/z 922.0098 (protonated HP-921) in positive ESI mode; whereas m/z 119.0363 (proton abstracted purine) and m/z 966.0007 (formate adduct of HP-921) in negative ESI mode. The capillary voltage was ± 4000 V for positive and negative ionization mode. The source temperature was 225 °C. The nebulizer and gas flow rate were 35 psig and 11 L/min respectively, fragmentor voltage to 75 V and a radiofrequency voltage in the octopole (OCT RF Vpp) of 750 V.

For the study, Mass Hunter Workstation Software LC/MS Data Acquisition version B.07.00 (Agilent Technologies) was used for control and acquisition of all data obtained with UHPLC/MS-QTOF.

3.3.3.3. Evaluation of Antioxidant Activity

3.3.3.3.1. DPPH Scavenging Assay

The antioxidant activity of the samples was determined by the radical scavenging activity method using the 2,2-diphenyl-1-picryhydrazyl (DPPH) free radical [52]. Briefly, to the methanolic

solutions of the samples at different concentrations (100 µL), three methanolic solutions of DPPH (3.9 mL) were added at concentrations of 0.2, 0.1242 and 0.08 mM. The control was prepared by mixing methanol (100 µL) with 3.9 mL of each DPPH solution. The reaction mixtures were incubated at room temperature for 90 min and in the absence of light. After that, the absorbances were measured at 517 nm. The radical scavenging activity was calculated using the following formula:

$$I\% = ((Abs0 - Abs1)/Abs0) \times 100 \quad (1)$$

where Abs0 corresponds to the control absorbance and Abs1 was the absorbance in the presence of the test samples at different concentrations. The IC_{50} was calculated graphically, using a linear calibration curve, plotting the sample concentrations in relation to the corresponding percentage of inhibition. The antioxidant activity was expressed as the antioxidant activity index (AAI), calculated as follows: AAI = (final concentration of DPPH in the control sample)/(IC_{50}) [52]. The AAI allowed to classify the antioxidant activity of the samples as: Poor (AAI < 0.5), moderate (0.5 < AAI ≤ 1.0), strong (1.0 < AAI < 2.0) or very strong (AAI ≥ 2.0) [52]. All tests were performed in duplicate and gallic acid was used as control.

3.3.3.3.2. β-Carotene Bleaching Test

A solution of β-carotene was prepared by dissolving 20 mg in 1 mL of chloroform. To 500 µL of this solution, 180 µL of linoleic acid, 400 mg of Tween 40 and 1 mL of chloroform were added. Then, the chloroform was evaporated on a rotary vacuum evaporator for 5 min at 45 °C. Subsequently, 100 mL of distilled water saturated with oxygen were slowly added to the mixture, which was then vigorously stirred to form an emulsion. After that, 2.5 mL of this emulsion were mixed with 300 µL of the extracts in methanol at different concentrations. A control was prepared by adding 2.5 mL of emulsion to 300 µL of methanol. The tubes were shaken and placed in a water bath at 50 °C for 1 h. After this time, the absorbances of the samples were measured at 470 nm, using an emulsion without β-carotene as blank. The measurements were performed in triplicate at 0 h (initial time) and at 1 h (final time). The antioxidant activity was measured in terms of the percentage of inhibition of β-carotene oxidation by:

$$\%\ Inhibition = (Abs^{t=1}_{sample} - Abs^{t=1}_{control})/(Abs^{t=0}_{control} - Abs^{t=1}_{control}) \quad (2)$$

where $Abs^{t=1}$ was the absorbance of the sample or control at the final incubation time and $Abs^{t=0}$ was the absorbance in the control at initial incubation time [27]. Butylated hydroxytoluene (BHT) was employed as standard antioxidant compound.

3.3.3.4. Anti-Inflammatory Activity

The determination of anti-inflammatory activity was performed by assessing the ability of the samples to inhibit protein denaturation [59]. Thus, a 1% (w/v) solution of bovine serum albumin (BSA) in phosphate buffer solution (PBS) was prepared, with the pH adjusted to 6.8 using

glacial acetic acid. Then, 900 µL of this solution was added to 100 µL of the samples previously diluted in dimethyl sulfoxide (DMSO) in tubes preheated to 37 °C. Then, the tubes were incubated at 72 °C for 10 min and after that time they were placed on ice for 10 min. A control consisting of distilled water was prepared. Finally, absorbance measurements were performed in triplicate using a microplate reader (BIO-RAD, Hercules, CA, USA) at 620 nm. The percentage of inhibition of protein denaturation was calculated using the following equation:

$$\%I = 100 - ((Abs_{sample} \times 100)/(Abs_{control})) \quad (3)$$

where $Abs_{control}$ is the absorbance of the control and Abs_{sample} is the absorbance of each sample [59]. Acetylsalicylic acid was used as positive control.

3.3.3.5. Determination of Antimicrobial Activity

The antimicrobial activity of ayahuasca decoctions was evaluated against four Gram-positive bacterial species (*Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 11778, *Listeria monocytogenes* LMG 16779 and *Enterococcus faecalis* ATCC 29212) and four Gram-negative (*Acinetobacter baumannii* LMG 1025, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922 and *Salmonella* Typhimurium ATCC 13311). Stock cultures were kept at 20% glycerol at −80 °C. All strains were subcultured 24 h before the antimicrobial tests on Brain Heart Infusion Agar (BHI-A) plates.

3.3.3.5.1. Disc Diffusion Assay

The antimicrobial activity of each extract was determined using the disc diffusion assay following the M2-A8 method, described by Clinical Laboratory and Standards Institute (CLSI). The inoculum was prepared by suspending the bacterial species in sterile saline solution to 0.5 McFarland units (about 1 to 2 × 108 colony-forming units/mL (CFU/mL)). Then, discs (6 mm in diameter) were impregnated with 15 µL of each sample (3 mg/disc). Negative controls were prepared with DMSO (15 µL/disc) and positive controls were prepared with tetracycline (30 µg/disc). The discs were placed on the inoculated agar plates, which were then incubated for 24 h at 37 °C. After this time, the inhibition zones were checked, and the diameters were measured using a digital pachymeter. All experiments were carried out in triplicate [90].

3.3.3.5.2. Resazurin Microtiter Method

The values of the minimum inhibitory concentrations (MIC) of the samples were determined using the resazurin microtiter assay [86]. Initially, the extracts were prepared in Müeller–Hinton Broth (MHB) at a concentration of 20 mg/mL, for strains *E. faecalis* and *L. monocytogenes* the culture medium used was Tryptic Soy Broth (TSB). A 96-well plate was marked and a volume of 100 µL of sample was pipetted into the first row of the plate. To all other wells, 50 µL of MHB or TSB were added, and serial twofold dilutions were performed. Then, 10 µL of

resazurin indicator solution (0.1% diluted in MHB or TSB) were added. After that, 30 µL of MHB or TSB were added and, finally, 10 µL of bacterial suspension (0.5 McFarland units) were added to each well. The tests were performed in triplicate and tetracycline was used as a positive control. The plates were incubated for 18 h at 37 °C. The color change to colorless or pink was evaluated visually and registered as positive, with MIC being considered as the lowest concentration where the color change occurred [86].

3.3.3.5.3. Anti-Quorum Sensing Properties: Solid Diffusion Assay

The anti-quorum sensing properties were evaluated using the biomonitor strain *Chromobacterium violaceum* ATCC 12472. The bacterial suspension was obtained by overnight aerobic growth in Luria-Bertani (LB) culture medium (250 rpm, 30 °C). Then, the bacterial suspension was adjusted to an OD_{620nm} of 1 and subsequently seeded on LB agar plates. Subsequently, discs were impregnated with each sample (3 mg/disc), placed on the plates and incubated for 24 h at 37 °C. After the incubation period, the anti-quorum sensing properties were evaluated by inhibiting the production of the violacein pigment around the disc, and the inhibition diameters were measured using a digital pachymeter. DMSO (15 µL/disc) was used as a negative control and resveratrol (5 µg/disc) as a positive control. The tests were performed in triplicate [59].

3.3.3.5.4. Anti-Biofilm Activity

The anti-biofilm activity of the *B. caapi* and *P. harmala* decoctions was evaluated by scanning electron microscopy (SEM) for *A. baumannii* LMG 1025. Initially, *A. baumannii* LMG 1025 was grown overnight in LB (250 rpm, 37 °C). The *A. baumannii* LMG 1025 biofilms were formed in polystyrene coupons placed in 12-well plates. Thus, 500 µL of the bacterial suspension (OD_{600nm} of 0.02) and 500 µL of the samples dissolved in LB at a concentration of 0.5× MIC were placed in each well. The growth control consisted in 500 µL of LB with 500 µL of the bacterial suspension. The plates were incubated for 24 h at 37 °C. Then, the biofilms were washed twice with sterile saline solution and fixed with 2.5% glutaraldehyde (v/v) for 4 h at 4 °C. Subsequently, the samples were washed with PBS and the dehydration was carried out in a series of ethanol for 20 min each (30, 50, 70, 80, 90% (v/v) and absolute). The samples were then left to dry overnight in a desiccator. Finally, the biofilms were coated with gold and observed by SEM of variable pressure (S-3400N; Hitachi, Tokyo, Japan), using a voltage of 20.0 kV, emission of 100.0 µA and magnification of 5000× [59,86].

3.3.4. Conclusions

This work allowed a more deepened knowledge of the phytochemical composition of samples of ayahuasca and the plant species used in the preparation of this psychoactive beverage. The ability of the samples to inhibit the lipid peroxidation and the capability to scavenge free

radicals were demonstrated, indicating the antioxidant properties of these samples. The anti-inflammatory properties by the ability to inhibit protein denaturation were also evaluated. In addition, the antimicrobial activity of the samples was tested, together with the anti-quorum sensing and anti-biofilm potential against *A. baumannii* of *B. caapi* and *P. harmala* samples. The whole set of results obtained allowed a better knowledge of ayahuasca decoctions; however, future work is necessary to fully understand the mechanisms by which the samples exert the observed effects. It should be noted that, as far as we know, this is the first work where the antimicrobial properties of *P. viridis*, *M. hostilis* and the commercial mixture were studied. It should also be noted that, in most studies where the biological properties of these samples were analyzed, extraction steps were carried out with organic solvents, whereas in the present study, water was used as solvent to mimic ayahuasca beverages.

Appendix A

Polyphenol Class	Polyphenol sub-class (Family)	NAME COMPOUND	COMMON NAME
Phenolic acids	Hydroxy benzoic acids	4-hydroxybenzoic acid	4-hydroxybenzoic acid
Phenolic acids	Hydroxy benzoic acids	3,4,5-trihydroxybenzoic acid	gallic acid
Phenolic acids	Hydroxy benzoic acids	2,5-dihydroxybenzoic acid	gentisic acid
Phenolic acids	Hydroxy benzoic acids	3,4-dihydroxybenzoic acid	protocatechuic acid
Phenolic acids	Hydroxy benzoic acids	2-Hydroxybenzoic acid	salicylic acid
Phenolic acids	Hydroxy benzoic acids	4-hydroxy-3,5-dimethoxybenzoic acid	syringic acid
Phenolic acids	Hydroxy benzoic acids	4-hydroxy-3-methoxybenzoic acid	vanillic acid
Phenolic acids	Hydroxy cinnamic acids	3,4-dihydroxycinnamic acid	caffeic acid
Phenolic acids	Hydroxy cinnamic acids	4-hydroxycinnamic acid	p-coumaric acid
Phenolic acids	Hydroxy cinnamic acids	3-(3,4-dihydroxycinnamoyl)quinate	chlorogenic acid
Phenolic acids	Hydroxy cinnamic acids	4-hydroxy-3-methoxycinnamic acid	ferulic acid
Other polyphenols	Hydroxycoumarins	6,7-dihydroxicoumarin	aesculetin
Other polyphenols	Other polyphenols	1,2-dihydroxybenzene	catechol
Monosaccharides	Monosaccharide	aldopentose	D-xylose
Flavonoids	Dihydrochalcones	dihydronaringenin	phloretin (aglycon)
Flavonoids	Dihydrochalcones	phloretin-2'-β-D-glucopyranoside	phloridzin
Flavonoids	Flavanones	naringenin (aglycone)	naringenin (aglycone)
Flavonoids	Flavanones	naringin	naringin
Flavonoids	Flavanones	hesperetin (aglycone)	hesperetin (aglycone)
Flavonoids	Flavanones	hesperidin	hesperidin
Flavonoids	Flavones	digitoflavone	luteolin (aglycone)
Flavonoids	Flavones	5,7-dihydroxyflavone	chrysin (aglycone)
Flavonoids	Flavonols	kaempferol (aglycone)	kaempferol (aglycone)
Flavonoids	Flavonols	kaempferol 3-O-glucoside	kaempferol 3-O-glucoside
Flavonoids	Flavonols	kaempferol 3-O-rutinoside	nicotiflorin
Flavonoids	Flavonols	3,5,7,2',4'-pentahydroxyflavone	morin (aglycone)
Flavonoids	Flavonols	quercetin (aglycone)	quercetin (aglycone)
Flavonoids	Flavonols	quercetin-3-O-glucoside	isoquercitrin
Flavonoids	Flavonols	quercetin-3-O-rhamnoside	quercitrin
Flavonoids	Flavonols	quercetin-3-O-rutinoside	rutin
Flavonoids	Flavonols	quercetin-3-O-galactoside	hyperosid
Flavonoids	Flavanols	(-)-epicatechin	(-)-epicatechin
Flavonoids	Flavanols	(+)-catechin	(+)-catechin
Flavonoids	Anthocyanines	cyanidin-3-O-arabinoside	cyanidin-3-O-arabinoside
Flavonoids	Anthocyanines	cyanidin-3-O-glucoside	chrysanthemin
Flavonoids	Anthocyanidin	malvidin	malvidin (aglycone)
Flavonoids	Anthocyanines	malvidin-3-galactoside	primulin
Flavonoids	Anthocyanines	pelargonidin	pelargonidin (aglycone)
Flavonoids	Anthocyanines	peonidin 3-O-glucoside	peonidin 3-O-glucoside
Flavonoids	Anthocyanines	delphinidin (aglycone)	delphinidin (aglycone)
Flavonoids	Anthocyanines	delphinidin-3-O-rutinoside	delphinidin-3-O-rutinoside
Ellagitannins	Ellagitannins	ellagic acid	ellagic acid
Benzopyrones	Benzopyrones	coumarin	coumarin
Alkaloids	Glycoalkaloid	glycoalkaloid	solanine
Alkaloids	Jasmonate	jasmonate	jasmonic acid
Alkaloids	Protoberberine	protoberberine	berberine
Acids	Acids (tricarboxilic acid)	citric acid	citric acid
Acids	Benzoic acid	cinnamic acid	cinnamic acid

Figure III.3.A1. Library of phytochemical compounds used in the analysis by UHPLC-Q/TOF-MS.

3.3.5. References

1. Simão; Gonçalves; Duarte; Barroso; Cristóvão; Gallardo Toxicological Aspects and Determination of the Main Components of Ayahuasca: A Critical Review. *Medicines* **2019**, *6*, 106, doi:10.3390/medicines6040106.
2. Estrella-Parra, E.A.; Almanza-Pérez, J.C.; Alarcón-Aguilar, F.J. Ayahuasca: Uses, Phytochemical and Biological Activities. *Nat. Products Bioprospect.* 2019, *9*, 251–265.
3. Gaujac, A.; Dempster, N.; Navickiene, S.; Brandt, S.D.; De Andrade, J.B. Determination of *N,N*-dimethyltryptaminein beverages consumed in religious practices by headspace solid-phase microextraction followed by gas chromatography ion trap mass spectrometry. *Talanta* **2013**, *106*, 394–398, doi:10.1016/j.talanta.2013.01.017.
4. Domínguez-Clavé, E.; Soler, J.; Elices, M.; Pascual, J.C.; Álvarez, E.; de la Fuente Revenga, M.; Friedlander, P.; Feilding, A.; Riba, J. Ayahuasca: Pharmacology, neuroscience and therapeutic potential. *Brain Res. Bull.* 2016, *126*, 89–101.
5. dos Santos, R.G.; Bouso, J.C.; Hallak, J.E.C. Ayahuasca, dimethyltryptamine, and psychosis: a systematic review of human studies. *Ther. Adv. Psychopharmacol.* **2017**, *7*, 141–157, doi:10.1177/2045125316689030.
6. Hamill, J.; Hallak, J.; Dursun, S.M.; Baker, G. Ayahuasca: Psychological and Physiologic Effects, Pharmacology and Potential Uses in Addiction and Mental Illness. *Curr. Neuropharmacol.* **2018**, *17*, 108–128, doi:10.2174/1570159x16666180125095902.
7. McKenna, D.J. Clinical investigations of the therapeutic potential of ayahuasca: Rationale and regulatory challenges. *Pharmacol. Ther.* **2004**, *102*, 111–129, doi:10.1016/j.pharmthera.2004.03.002.
8. Callaway, J.C.; McKenna, D.J.; Grob, C.S.; Brito, G.S.; Raymon, L.P.; Poland, R.E.; Andrade, E.N.; Andrade, E.O.; Mash, D.C. Pharmacokinetics of Hoasca alkaloids in healthy humans. *J. Ethnopharmacol.* **1999**, *65*, 243–256, doi:10.1016/S0378-8741(98)00168-8.
9. Ramachandran, P.; Zhang, N.; McLaughlin, W.B.; Luo, Y.; Handy, S.; Duke, J.A.; Vasquez, R.; Ottesen, A. Sequencing the vine of the soul: Full chloroplast genome sequence of *Banisteriopsis caapi*. *Genome Announc.* **2018**, *6*, doi:10.1128/genomeA.00203-18.
10. Frison, G.; Favretto, D.; Zancanaro, F.; Fazzin, G.; Ferrara, S.D. A case of β-carboline alkaloid intoxication following ingestion of Peganum harmala seed extract. *Forensic Sci. Int.* **2008**, *179*, doi:10.1016/j.forsciint.2008.05.003.
11. Sklerov, J.; Levine, B.; Moore, K.A.; King, T.; Fowler, D. A fatal intoxication following the ingestion of 5-methoxy-*N,N*- dimethyltryptamine in an ayahuasca preparation. *J. Anal. Toxicol.* **2005**, *29*, 838–841, doi:10.1093/jat/29.8.838.
12. Dos Santos, R.G.; Balthazar, F.M.; Bouso, J.C.; Hallak, J.E.C. The current state of research on ayahuasca: A systematic review of human studies assessing psychiatric symptoms, neuropsychological functioning, and neuroimaging. *J. Psychopharmacol.* **2016**, *30*, 1230–1247, doi:10.1177/0269881116652578.
13. dos Santos, R.G.; Osório, F.L.; Crippa, J.A.S.; Hallak, J.E.C. Antidepressive and anxiolytic effects of ayahuasca: A systematic literature review of animal and human studies. *Rev. Bras. Psiquiatr.* 2016, *38*, 65–72.
14. Riba, J.; Valle, M.; Urbano, G.; Yritia, M.; Morte, A.; Barbanoj, M.J. Human pharmacology of ayahuasca: Subjective and cardiovascular effects, monoamine metabolite excretion, and pharmacokinetics. *J. Pharmacol. Exp. Ther.* **2003**, *306*, 73–83, doi:10.1124/jpet.103.049882.
15. Riba, J.; Romero, S.; Grasa, E.; Mena, E.; Carrió, I.; Barbanoj, M.J. Increased frontal and paralimbic activation following ayahuasca, the pan-amazonian inebriant. *Psychopharmacology (Berl).* **2006**, *186*, 93–98, doi:10.1007/s00213-006-0358-7.
16. De Araujo, D.B.; Ribeiro, S.; Cecchi, G.A.; Carvalho, F.M.; Sanchez, T.A.; Pinto, J.P.; de Martinis, B.S.; Crippa, J.A.; Hallak, J.E.C.; Santos, A.C. Seeing with the eyes shut: Neural basis of enhanced imagery following ayahuasca ingestion. *Hum. Brain Mapp.* **2012**, *33*, 2550–2560, doi:10.1002/hbm.21381.
17. Morales-García, J.A.; De La Fuente Revenga, M.; Alonso-Gil, S.; Rodríguez-Franco, M.I.; Feilding, A.; Perez-Castillo, A.; Riba, J. The alkaloids of *Banisteriopsis caapi*, the plant source of the Amazonian hallucinogen Ayahuasca, stimulate adult neurogenesis in vitro. *Sci. Rep.* **2017**, *7*, 1–13, doi:10.1038/s41598-017-05407-9.
18. Cameron, L.P.; Olson, D.E. Dark Classics in Chemical Neuroscience: *N, N*-Dimethyltryptamine (DMT). *ACS Chem. Neurosci.* 2018, *9*, 2344–2357.
19. Gable, R.S. Risk assessment of ritual use of oral dimethyltryptamine (DMT) and harmala alkaloids. *Addiction* 2007, *102*, 24–34.
20. Frecska, E.; Bokor, P.; Winkelman, M. The therapeutic potentials of ayahuasca: Possible effects against various diseases of civilization. *Front. Pharmacol.* 2016, *7*.
21. Cox, G.; Wright, G.D. Intrinsic antibiotic resistance: Mechanisms, origins, challenges and solutions. *Int. J. Med. Microbiol.* 2013, *303*, 287–292.
22. Rodríguez-Rojas, A.; Rodríguez-Beltrán, J.; Couce, A.; Blázquez, J. Antibiotics and antibiotic resistance: A bitter fight against evolution. *Int. J. Med. Microbiol.* 2013, *303*, 293–297.
23. Hossion, A.M.L.; Otsuka, N.; Kandahary, R.K.; Tsuchiya, T.; Ogawa, W.; Iwado, A.; Zamami, Y.; Sasaki, K. Design, synthesis, and biological evaluation of a novel series of quercetin diacylglucosides as potent anti-MRSA and anti-VRE agents. *Bioorganic Med. Chem. Lett.* **2010**, *20*, 5349–5352, doi:10.1016/j.bmcl.2010.02.060.
24. O'Donnell, F.; Smyth, T.J.P.; Ramachandran, V.N.; Smyth, W.F. A study of the antimicrobial activity of selected synthetic and naturally occurring quinolines. *Int. J. Antimicrob. Agents* **2010**, *35*, 30–38, doi:10.1016/j.ijantimicag.2009.06.031.
25. Mulyaningsih, S.; Sporer, F.; Reichling, J.; Wink, M. Antibacterial activity of essential oils from Eucalyptus and of

selected components against multidrug-resistant bacterial pathogens. *Pharm. Biol.* **2011**, *49*, 893–899, doi:10.3109/13880209.2011.553625.
26. Duarte, A.; Ferreira, S.; Silva, F.; Domingues, F.C. Synergistic activity of coriander oil and conventional antibiotics against Acinetobacter baumannii. *Phytomedicine* **2012**, *19*, 236–238, doi:10.1016/j.phymed.2011.11.010.
27. Luís, A.; Neiva, D.; Pereira, H.; Gominho, J.; Domingues, F.; Duarte, A.P. Stumps of *eucalyptus globulus* as a source of antioxidant and antimicrobial polyphenols. *Molecules* **2014**, *19*, 16428–16446, doi:10.3390/molecules191016428.
28. Dall'Agnol, R.; Ferraz, A.; Bernardi, A.P.; Albring, D.; Nör, C.; Sarmento, L.; Lamb, L.; Hass, M.; Von Poser, G.L.; Schapoval, E.E.S. Antimicrobial activity of some Hypericum species. *Phytomedicine* **2003**, *10*, 511–516, doi:10.1078/094471103322331476.
29. de Osório, F.L.; Sanches, R.F.; Macedo, L.R.; dos Santos, R.G.; Maia-De-Oliveira, J.P.; Wichert-Ana, L.; de Araujo, D.B.; Riba, J.; Crippa, J.A.; Hallak, J.E. Antidepressant effects of a single dose of ayahuasca in patients with recurrent depression: A preliminary report. *Rev. Bras. Psiquiatr.* **2015**, *37*, 13–20, doi:10.1590/1516-4446-2014-1496.
30. Sanches, R.F.; De Lima Osório, F.; Santos, R.G.D.; Macedo, L.R.H.; Maia-De-Oliveira, J.P.; Wichert-Ana, L.; De Araujo, D.B.; Riba, J.; Crippa, J.A.S.; Hallak, J.E.C. Antidepressant effects of a single dose of ayahuasca in patients with recurrent depression a SPECT study. *J. Clin. Psychopharmacol.* **2016**, *36*, 77–81, doi:10.1097/JCP.0000000000000436.
31. Grob, C.S.; McKenna, D.J.; Callaway, J.C.; Brito, G.S.; Neves, E.S.; Oberlaender, G.; Saide, O.L.; Labigalini, E.; Tacla, C.; Miranda, C.T.; et al. Human psychopharmacology of hoasca, a plant hallucinogen used in ritual context in Brazil. *J. Nerv. Ment. Dis.* **1996**, *184*, 86–94, doi:10.1097/00005053-199602000-00004.
32. Oliveira-Lima, A.J.; Santos, R.; Hollais, A.W.; Gerardi-Junior, C.A.; Baldaia, M.A.; Wuo-Silva, R.; Yokoyama, T.S.; Costa, J.L.; Malpezzi-Marinho, E.L.A.; Ribeiro-Barbosa, P.C.; et al. Effects of ayahuasca on the development of ethanol-induced behavioral sensitization and on a post-sensitization treatment in mice. *Physiol. Behav.* **2015**, *142*, 28–36, doi:10.1016/j.physbeh.2015.01.032.
33. Thomas, G.; Lucas, P.; Capler, N.R.; Tupper, K.W.; Martin, G. Ayahuasca-assisted therapy for addiction: Results from a preliminary observational study in Canada. *Curr. Drug Abuse Rev.* **2013**, *6*, 30–42, doi:10.2174/15733998113099990003.
34. Da Silveira, D.X.; Grob, C.S.; de Rios, M.D.; Lopez, E.; Alonso, L.K.; Tacla, C.; Doering-Silveira, E. Ayahuasca in adolescence: A preliminary psychiatric assessment. *J. Psychoactive Drugs* **2005**, *37*, 129–133, doi:10.1080/02791072.2005.10399792.
35. Barbosa, P.C.R.; Cazorla, I.M.; Giglio, J.S.; Strassman, R.S. A six-month prospective evaluation of personality traits, psychiatric symptoms and quality of life in Ayahuasca-Naïve Subjects. *J. Psychoactive Drugs* **2009**, *41*, 205–212, doi:10.1080/02791072.2009.10400530.
36. Rababah, T.M.; Ereifej, K.I.; Esoh, R.B.; Al-U'Datt, M.H.; Alrababah, M.A.; Yang, W. Antioxidant activities, total phenolics and HPLC analyses of the phenolic compounds of extracts from common Mediterranean plants. *Nat. Prod. Res.* **2011**, *25*, 596–605, doi:10.1080/14786419.2010.488232.
37. Ferretti, G.; Bacchetti, T.; Belleggia, A.; Neri, D. Cherry antioxidants: From farm to table. *Molecules* 2010, *15*, 6993–7005.
38. Haminiuk, C.W.I.; Maciel, G.M.; Plata-Oviedo, M.S.V.; Peralta, R.M. Phenolic compounds in fruits - an overview. *Int. J. Food Sci. Technol.* 2012, *47*, 2023–2044.
39. Stasiuk, M.; Kozubek, A. Biological activity of phenolic lipids. *Cell. Mol. Life Sci.* 2010, *67*, 841–860.
40. Santos, E.S.; Luís, Â.; Gonçalves, J.; Rosado, T.; Pereira, L.; Gallardo, E.; Duarte, A.P. *Julbernardia paniculata* and *Pterocarpus angolensis*: From ethnobotanical surveys to phytochemical characterization and bioactivities evaluation. *Molecules* **2020**, *25*, doi:10.3390/molecules25081828.
41. Hadadi, Z.; Nematzadeh, G.A.; Ghahari, S. A study on the antioxidant and antimicrobial activities in the chloroformic and methanolic extracts of 6 important medicinal plants collected from North of Iran. *BMC Chem.* **2020**, *14*, doi:10.1186/s13065-020-00683-5.
42. Cushnie, T.P.T.; Lamb, A.J. Antimicrobial activity of flavonoids. *Int. J. Antimicrob. Agents* 2005, *26*, 343–356.
43. Testai, L.; Piragine, E.; Piano, I.; Flori, L.; Da Pozzo, E.; Miragliotta, V.; Pirone, A.; Citi, V.; Di Cesare Mannelli, L.; Brogi, S.; et al. The Citrus Flavonoid Naringenin Protects the Myocardium from Ageing-Dependent Dysfunction: Potential Role of SIRT1. *Oxid. Med. Cell. Longev.* 2020, *2020*, 1–15.
44. Ma, J.; Yang, H.; Basile, M.J.; Kennelly, E.J. Analysis of polyphenolic antioxidants from the fruits of three Pouteria species by selected ion monitoring liquid chromatography-mass spectrometry. *J. Agric. Food Chem.* **2004**, *52*, 5873–5878, doi:10.1021/jf049950k.
45. Pires, A.P.S.; De Oliveira, C.D.R.; Moura, S.; Dörr, F.A.; Silva, W.A.E.; Yonamine, M. Gas chromatographic analysis of dimethyltryptamine and β-carboline alkaloids in Ayahuasca, an amazonian psychoactive plant beverage. *Phytochem. Anal.* **2009**, *20*, 149–153, doi:10.1002/pca.1110.
46. Souza, R.C.Z.; Zandonadi, F.S.; Freitas, D.P.; Tófoli, L.F.F.; Sussulini, A. Validation of an analytical method for the determination of the main ayahuasca active compounds and application to real ayahuasca samples from Brazil. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2019**, *1124*, 197–203, doi:10.1016/j.jchromb.2019.06.014.
47. Gambelunghe, C.; Aroni, K.; Rossi, R.; Moretti, L.; Bacci, M. Identification of *N,N*-dimethyltryptamine and β-carbolines in psychotropic ayahuasca beverage. *Biomed. Chromatogr.* **2008**, *22*, 1056–1059, doi:10.1002/bmc.1023.
48. McIlhenny, E.H.; Pipkin, K.E.; Standish, L.J.; Wechkin, H.A.; Strassman, R.; Barker, S.A. Direct analysis of psychoactive tryptamine and harmala alkaloids in the Amazonian botanical medicine ayahuasca by liquid chromatography-electrospray ionization-tandem mass spectrometry. *J. Chromatogr. A* **2009**, *1216*, 8960–8968,

doi:10.1016/j.chroma.2009.10.088.
49. Lesiak, A.D.; Musah, R.A. Application of ambient ionization high resolution mass spectrometry to determination of the botanical provenance of the constituents of psychoactive drug mixtures. *Forensic Sci. Int.* **2016**, *266*, 271–280, doi:10.1016/j.forsciint.2016.06.009.
50. Huhn, C.; Neusüß, C.; Pelzing, M.; Pyell, U.; Mannhardt, J.; Pütz, M. Capillary electrophoresis-laser induced fluorescence-electrospray ionization-mass spectrometry: A case study. *Electrophoresis* **2005**, *26*, 1389–1397, doi:10.1002/elps.200410163.
51. Mishra, K.; Ojha, H.; Chaudhury, N.K. Estimation of antiradical properties of antioxidants using DPPH- assay: A critical review and results. *Food Chem.* **2012**, *130*, 1036–1043, doi:10.1016/j.foodchem.2011.07.127.
52. Scherer, R.; Godoy, H.T. Antioxidant activity index (AAI) by the 2,2-diphenyl-1-picrylhydrazyl method. *Food Chem.* **2009**, *112*, 654–658, doi:10.1016/j.foodchem.2008.06.026.
53. Ali, S.K.; Hamed, A.R.; Soltan, M.M.; Hegazy, U.M.; Elgorashi, E.E.; El-Garf, I.A.; Hussein, A.A. *In vitro* evaluation of selected Egyptian traditional herbal medicines for treatment of alzheimer disease. *BMC Complement. Altern. Med.* **2013**, *13*, doi:10.1186/1472-6882-13-121.
54. Benarous, K.; Bombarda, I.; Iriepa, I.; Moraleda, I.; Gaetan, H.; Linani, A.; Tahri, D.; Sebaa, M.; Yousfi, M. Harmaline and hispidin from Peganum harmala and Inonotus hispidus with binding affinity to Candida rugosa lipase: *In silico* and *in vitro* studies. *Bioorg. Chem.* **2015**, *62*, 1–7, doi:10.1016/j.bioorg.2015.06.005.
55. Cruz, J.M.; Domínguez, J.M.; Domínguez, H.; Parajó, J.C. Antioxidant and antimicrobial effects of extracts from hydrolysates of lignocellulosic materials. *J. Agric. Food Chem.* **2001**, *49*, 2459–2464, doi:10.1021/jf001237h.
56. Dawidowicz, A.L.; Olszowy, M. Influence of some experimental variables and matrix components in the determination of antioxidant properties by β-carotene bleaching assay: Experiments with BHT used as standard antioxidant. *Eur. Food Res. Technol.* **2010**, *231*, 835–840, doi:10.1007/s00217-010-1333-4.
57. Kar, B.; Kumar, R.B.S.; Karmakar, I.; Dola, N.; Bala, A.; Mazumder, U.K.; Hadar, P.K. Antioxidant and *in vitro* anti-inflammatory activities of Mimusops elengi leaves. *Asian Pac. J. Trop. Biomed.* **2012**, *2*, S976–S980, doi:10.1016/S2221-1691(12)60346-3.
58. García-Lafuente, A.; Moro, C.; Manchón, N.; Gonzalo-Ruiz, A.; Villares, A.; Guillamón, E.; Rostagno, M.; Mateo-Vivaracho, L. *In vitro* anti-inflammatory activity of phenolic rich extracts from white and red common beans. *Food Chem.* **2014**, *161*, 216–223, doi:10.1016/j.foodchem.2014.04.004.
59. Luís, Â.; Sousa, S.; Wackerlig, J.; Dobusch, D.; Duarte, A.P.; Pereira, L.; Domingues, F. Star anise (*Illicium verum* Hook. f.) essential oil: Antioxidant properties and antibacterial activity against *Acinetobacter baumannii*. *Flavour Fragr. J.* **2019**, *34*, 260–270, doi:10.1002/ffj.3498.
60. Herraiz, T.; Guillén, H.; Arán, V.J.; Salgado, A. Identification, occurrence and activity of quinazoline alkaloids in Peganum harmala. *Food Chem. Toxicol.* **2017**, *103*, 261–269, doi:10.1016/j.fct.2017.03.010.
61. Bremner, P.; Rivera, D.; Calzado, M.A.; Obón, C.; Inocencio, C.; Beckwith, C.; Fiebich, B.L.; Muñoz, E.; Heinrich, M. Assessing medicinal plants from South-Eastern Spain for potential anti-inflammatory effects targeting nuclear factor-Kappa B and other pro-inflammatory mediators. *J. Ethnopharmacol.* **2009**, *124*, 295–305, doi:10.1016/j.jep.2009.04.035.
62. Ahmad, I.; Beg, A.Z. Antimicrobial and phytochemical studies on 45 Indian medicinal plants against multi-drug resistant human pathogens. *J. Ethnopharmacol.* **2001**, *74*, 113–123, doi:10.1016/S0378-8741(00)00335-4.
63. Cruz, C.D.; Fletcher, G.C. Assessing manufacturers' recommended concentrations of commercial sanitizers on inactivation of *Listeria monocytogenes*. *Food Control* **2012**, *26*, 194–199, doi:10.1016/j.foodcont.2012.01.041.
64. Negreiros, M. de O.; Pawlowski, Â.; Zini, C.A.; Soares, G.L.G.; Motta, A. de S.; Frazzon, A.P.G. Antimicrobial and antibiofilm activity of *Baccharis psiadioides* essential oil against antibiotic-resistant *Enterococcus faecalis* strains. *Pharm. Biol.* **2016**, *54*, 3272–3279, doi:10.1080/13880209.2016.1223700.
65. McGuinness, W.A.; Malachowa, N.; DeLeo, F.R. Vancomycin resistance in Staphylococcus aureus. *Yale J. Biol. Med.* 2017, *90*, 269–281.
66. Bottone, E.J. Bacillus cereus, a volatile human pathogen. *Clin. Microbiol. Rev.* 2010, *23*, 382–398.
67. Leimbach, A.; Hacker, J.; Dobrindt, U. *E. coli* as an all-rounder: The thin line between commensalism and pathogenicity. *Curr. Top. Microbiol. Immunol.* **2013**, *358*, 3–32, doi:10.1007/82_2012_303.
68. Wotzka, S.Y.; Nguyen, B.D.; Hardt, W.D. *Salmonella* Typhimurium Diarrhea Reveals Basic Principles of Enteropathogen Infection and Disease-Promoted DNA Exchange. *Cell Host Microbe* 2017, *21*, 443–454.
69. Pang, Z.; Raudonis, R.; Glick, B.R.; Lin, T.J.; Cheng, Z. Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and alternative therapeutic strategies. *Biotechnol. Adv.* 2019, *37*, 177–192.
70. Harding, C.M.; Hennon, S.W.; Feldman, M.F. Uncovering the mechanisms of *Acinetobacter baumannii* virulence. *Nat. Rev. Microbiol.* 2018, *16*, 91–102.
71. Gao, Y.; Jiang, X.; Lin, D.; Chen, Y.; Tong, Z. The Starvation Resistance and Biofilm Formation of *Enterococcus faecalis* in Coexistence with *Candida albicans*, *Streptococcus gordonii*, *Actinomyces viscosus*, or *Lactobacillus acidophilus*. *J. Endod.* **2016**, *42*, 1233–1238, doi:10.1016/j.joen.2016.05.002.
72. Nostro, A.; Germanò, M.P.; D'Angelo, V.; Marino, A.; Cannatelli, M.A. Extraction methods and bioautography for evaluation of medicinal plant antimicrobial activity. *Lett. Appl. Microbiol.* **2000**, *30*, 379–384, doi:10.1046/j.1472-765x.2000.00731.x.
73. Smith-Palmer, A.; Stewart, J.; Fyfe, L. Antimicrobial properties of plant essential oils and essences against five important food-borne pathogens. *Lett. Appl. Microbiol.* **1998**, *26*, 118–122, doi:10.1046/j.1472-765X.1998.00303.x.
74. Darabpour, E.; Bavi, A.; Motamedi, H.; Nejad, S. Antibacterial activity of different parts of *Peganum harmala* L. growing in Iran against multi-drug resistant bacteria - PubMed. *EXCLI J.* **2011**, *25*, 252–263.
75. Bussmann, R.W.; Malca-García, G.; Glenn, A.; Sharon, D.; Chait, G.; Díaz, D.; Pourmand, K.; Jonat, B.; Somogy, S.;

Guardado, G.; et al. Minimum inhibitory concentrations of medicinal plants used in Northern Peru as antibacterial remedies. *J. Ethnopharmacol.* **2010**, *132*, 101–108, doi:10.1016/j.jep.2010.07.048.
76. Khan, A.K.; Rashid, R.; Fatima, N.; Mahmood, S.; Mir, S.; Khan, S.; Jabeen, N.; Murtaza, G. Pharmacological activities of protocatechuic acid. *Acta Pol. Pharm. - Drug Res.* 2015, *72*, 643–650.
77. Abedi, F.; Razavi, B.M.; Hosseinzadeh, H. A review on gentisic acid as a plant derived phenolic acid and metabolite of aspirin: Comprehensive pharmacology, toxicology, and some pharmaceutical aspects. *Phyther. Res.* 2020, *34*, 729–741.
78. Reygaert, W.C. Green tea catechins: Their use in treating and preventing infectious diseases. *Biomed Res. Int.* 2018, *2018*.
79. Egea, I.; Sánchez-Bel, P.; Romojaro, F.; Pretel, M.T. Six edible wild fruits as potential antioxidant additives or nutritional supplements. *Plant Foods Hum. Nutr.* **2010**, *65*, 121–129, doi:10.1007/s11130-010-0159-3.
80. Gursoy, N.; Sarikurkcu, C.; Cengiz, M.; Solak, M.H. Antioxidant activities, metal contents, total phenolics and flavonoids of seven Morchella species. *Food Chem. Toxicol.* **2009**, *47*, 2381–2388, doi:10.1016/j.fct.2009.06.032.
81. Roy, P.; Amdekar, S.; Kumar, A.; Singh, V. Preliminary study of the antioxidant properties of flowers and roots of *Pyrostegia venusta* (Ker Gawl) Miers. *BMC Complement. Altern. Med.* **2011**, *11*, doi:10.1186/1472-6882-11-69.
82. Faustino, H.; Gil, N.; Baptista, C.; Duarte, A.P. Antioxidant activity of lignin phenolic compounds extracted from kraft and sulphite black liquors. *Molecules* **2010**, *15*, 9308–9322, doi:10.3390/molecules15129308.
83. Lee, O.H.; Lee, B.Y. Antioxidant and antimicrobial activities of individual and combined phenolics in *Olea europaea* leaf extract. *Bioresour. Technol.* **2010**, *101*, 3751–3754, doi:10.1016/j.biortech.2009.12.052.
84. Antunes, L.C.S.; Visca, P.; Towner, K.J. *Acinetobacter baumannii*: Evolution of a global pathogen. *Pathog. Dis.* 2014, *71*, 292–301.
85. Chen, H.H.; Liu, X.; Ni, C.; Lu, Y.P.; Xiong, G.Y.; Lu, Y.Y.; Wang, S.Q. Bacterial biofilms in chronic rhinosinusitis and their relationship with inflammation severity. *Auris Nasus Larynx* **2012**, *39*, 169–174, doi:10.1016/j.anl.2011.04.014.
86. Luís, Â.; Pereira, L.; Domingues, F.; Ramos, A. Development of a carboxymethyl xylan film containing licorice essential oil with antioxidant properties to inhibit the growth of foodborne pathogens. *LWT* **2019**, *111*, 218–225, doi:10.1016/j.lwt.2019.05.040.
87. Simão, A.Y.; Gonçalves, J.; Caramelo, D.; Rosado, T.; Barroso, M.; Restolho, J.; Fernández, N.; Rodilla, J.; Duarte, A.P.; Cristóvão, A.C.; et al. Determination of *N,N*-dimethyltryptamine and beta-carbolines in plants used to prepare ayahuasca beverages by means of solid-phase extraction and gas-chromatography–mass spectrometry. *SN Appl. Sci.* **2020**, *2*, 1–11, doi:10.1007/s42452-020-2296-0.
88. Gutiérrez-Albanchez, E.; Gradillas, A.; García, A.; García-Villaraco, A.; Gutierrez-Mañero, F.J.; Ramos-Solano, B. Elicitation with Bacillus QV15 reveals a pivotal role of F3H on flavonoid metabolism improving adaptation to biotic stress in blackberry. *PLoS One* **2020**, *15*, doi:10.1371/journal.pone.0232626.
89. Acero, N.; Gradillas, A.; Beltran, M.; García, A.; Muñoz Mingarro, D. Comparison of phenolic compounds profile and antioxidant properties of different sweet cherry (*Prunus avium* L.) varieties. *Food Chem.* **2019**, *279*, 260–271, doi:10.1016/j.foodchem.2018.12.008.
90. Luís, Â.; Breitenfeld, L.; Ferreira, S.; Duarte, A.P.; Domingues, F. Antimicrobial, antibiofilm and cytotoxic activities of *Hakea sericea Schrader* extracts. *Pharmacogn. Mag.* **2014**, *10*, S6–S13, doi:10.4103/0973-1296.127331.

3.4. Paper VI- Evaluation of the *In Vitro* Wound-Healing Potential of Ayahuasca

Abstract

Ayahuasca is an Amazonian drink, which contains β-carboline alkaloids and *N,N*-dimethyltryptamine. The aim of this study was to evaluate the healing potential of decoctions of a commercial mixture, four individual plants and four mixtures of two plants used in the ayahuasca preparation. Thus, the cytotoxic potential of the samples was evaluated and a wound-healing assay was performed with a NHDF cell line. Subsequently, a parallel artificial membrane permeability assay was also performed, to verify if any psychoactive compound could be absorbed by skin fibroblasts. The integrity and permeability of the cell layer were also evaluated, using the transepithelial electrical resistance assay and Lucifer yellow permeability assay, respectively. The compounds absorbed by the cell layer were quantified by high-performance liquid chromatography coupled to a diode array detector. The results showed that only one sample showed cytotoxicity and all the others promoted the migration of skin fibroblasts. Additionally, it was also verified that β-carbolynic alkaloids and *N,N*-dimethyltriptamine were not absorbed by the cell layer, and in general, did not interfere with its permeability and integrity. To the best of our knowledge, this is the first study where ayahuasca's wound-healing potential was evaluated.

Keywords: ayahuasca; wound-healing activity; PAMPA assay; HPLC-DAD

3.4.1. Introduction

Ayahuasca is a psychoactive beverage, originally prepared from the stems of *Banisteriopsis caapi* (*B. caapi*) and leaves of *Psychotria viridis* (*P. viridis*), although there are currently other variations [1,2]. Also known as *Hoasca*, *Caapi*, *Yajé/Yagé*, or *Daime*, among other names, it may have a variable composition, and more than one hundred different plants used in its preparation have been documented [1,3]. The active compounds present in this beverage are *N,N*-dimethyltryptamine (DMT) from *P. viridis* and β-carbolines from *B. caapi* [4,5]. DMT is a tryptamine with psychoactive action, which acts as an agonist of serotonergic receptors 2A (5HT2AR) and non-opioid intracellular receptor sigma 1 (SIGMAR1), exerting effects comparable to those of psilocybin, mescaline or LSD [6]. This psychoactive compound is not bioavailable when ingested orally, since it is rapidly metabolised by peripheral monoaminoxidase-A (MAO-A) [6]. However, β-carbolinic alkaloids, namely harmine, harmaline and tetrahydroharmine (THH), have inhibiting effects on MAO-A, so the co-administration of these compounds with DMT prevents their degradation [1,7]. Thus, the psychoactive compound accesses the bloodstream and later the central nervous system, exerting its psychoactive effects [8]. Additionally, THH acts as an inhibitor of serotonin reuptake, enhancing the effects of DMT [9].

Ayahuasca users describe effects as changes in hearing, visual sensations, space and time awareness or even emotional and cognitive changes [1,4,10]. Spiritual experiences, such as connections with mythical or religious entities, are also often reported [4]. From the physical point of view, it is common to experience effects such as nausea, diarrhea or vomiting [6]. Ayahuasca has been used traditionally for over a thousand years for medicinal and spiritual purposes by indigenous peoples of the Amazon [4,11]. However, in recent decades, several studies have pointed to psychological benefits, namely in the treatment of depression, anxiety, drug dependence or obsessive-compulsive disorder [8,12,13,14,15]. Other more subtle effects, such as improvement in assertiveness, trust, optimism, maturity, or decreased neuroticism, have also been associated with ayahuasca consumption [13,16,17]. Recently, the anti-inflammatory and antimicrobial activities of ayahuasca extracts have also been verified [2].

Ayahuasca consumption has been increasing in recent decades all over the world [7]. Despite all the beneficial effects reported, and there is evidence that the consumption of this substance in a controlled environment is not associated with such consequences as psychotic outbreaks, this increase in demand for the decoction has led to questions about the possible negative effects associated with its consumption; however, it has also led to interest in its potential therapeutic effects [2].

The particular case of wounds represents a major challenge for health, as there is a high resource expenditure involved in trying to improve people's well-being [18]. Thus, several studies have been carried out in an attempt to find compounds of natural origin that allow wound healing [18,19,20]. Goulart da Silva *et al.* [21] describe in their review the potential anti-inflammatory properties of ayahuasca and its implications in neurological and psychiatric diseases. The authors mention that this is probably linked to its anti-neuroinflammatory action, mainly attributed to dimethyltryptamines (*N,N*-dimethyltryptamine and 5-methoxy-*N,N*-dimethyltryptamine), which act as systemic regulators of inflammation and immune homeostasis, but also to sigma-1 receptors. The study of Dakic *et al.* [22] corroborates this potential. The authors suggest that the compound modulates anti-neuroinflammatory response through the nuclear factor of activated T cells and nuclear factor kappa B pathways, which are downregulated through the toll-like receptor and G protein-coupled receptors. These anti-inflammatory effects are consistent with reported results in which the inflammatory release of cytokines and chemokines was blocked [21].

Additionally, the antioxidant activity of ayahuasca was documented in previous studies, as well as the presence of phenolic compounds and flavonoids, which are associated with this activity, since they promote the scavenging of free radicals [2,18].

Furthermore, its anti-inflammatory and antimicrobial properties suggest the potential of ayahuasca in wound healing.

In this context, this work aimed to evaluate *in vitro* the healing activity of four individual plants, a commercial mixture and four plants generally used in the preparation of ayahuasca decoctions, using normal human dermal fibroblasts (NHDF) through a scraping test. The potential cytotoxicity of the extracts and the eventual skin absorption of the psychoactive compounds were also evaluated by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltezolium bromide (MTT) and parallel membrane permeability assay (PAMPA), respectively.

3.4.2. Results and Discussion

The demand for natural products with potential bioactive properties has increased. In previous works, ayahuasca has been shown to have some therapeutic benefits, namely antioxidant, antimicrobial and anti-inflammatory properties [2]. Thus, in this work, one decoction was prepared from a commercial mixture, four from individual plants used in the preparation of ayahuasca, and four from a mixture of plants (with two different plant materials, one a source of β-carboline alkaloids and the other a source of DMT). Wound-healing potential was assessed in all nine samples.

3.4.2.1. Evaluation of Cell Viability

The cytotoxicity of all samples was evaluated at concentrations of 250 and 500 mg/L. The MTT assay was used for this purpose. This is a method employed used in the detection of cellular alterations at the metabolic level [23]. This assay consists of the conversion, by action of a mitochondrial reductase, of the yellow dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] into the purple dye formazan [9,23]. After analysing the results, it was found that the *P. harmala* sample, at both concentrations, showed a high decrease in cell viability, so this sample was eliminated in the subsequent phase of this study (Table III.4.1). All other samples showed no decrease in cell viability. To our knowledge, no studies have evaluated the cytotoxicity of ayahuasca samples on human skin fibroblasts, so it is not possible to establish a comparison. However, in a study that evaluated the cytotoxicity of *B. caapi* extracts in six cell lines, no cytotoxicity was observed [24]. In another study by Katchborian-Neto *et al.* [25], where the cytotoxicity of ayahuasca samples in SH-SY5Y cells was evaluated, it was found that there was no decrease in cell viability. On the other hand, studies where cancer cells were exposed to ayahuasca samples showed a decrease in cell viability [26,27].

Table III.4.1. Cell viability after exposure to extracts. The values are expressed as mean ± SD.

Samples	Cell viability (%)	
	250 mg/L	**500 mg/L**
P. viridis	150.24 ± 0.18	130.22 ± 0.08
B. caapi	91.72 ± 0.17	134.80 ± 0.09
P. harmala	38.07 ± 0.03	24.91 ± 0.001
M. hostilis	95.38 ± 0.11	121.79 ± 0.00
Commercial mixture	77.86 ± 0.11	98.35 ± 0.01
P. viridis* + *B. caapi	110.25 ± 0.08	229.85 ± 0.07
P. viridis* + *P. harmala	74.11 ± 0.06	174.54 ± 0.02
M. hostilis* + *B. caapi	111.89 ± 0.12	160.62 ± 0.03
M. hostilis* + *P. harmala	136.19 ± 0.03	132.42 ± 0.09

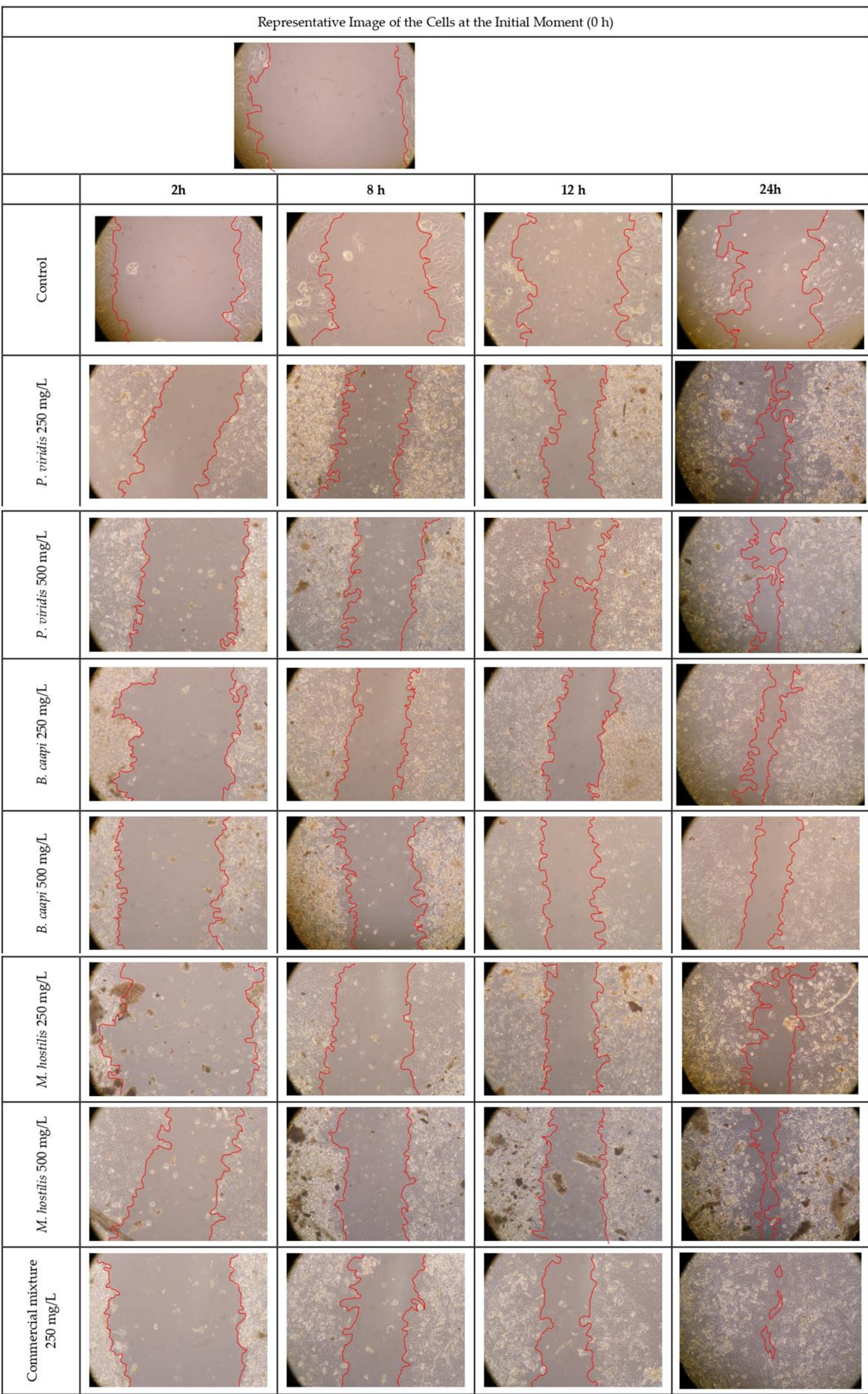
3.4.2.2. Evaluation of the *In Vitro* Wound-Healing Activity

Natural products have an important capacity in the reconstruction of skin lesions, as they lead to the proliferation of fibroblasts. Plant extracts have been reported to contain cell adhesion molecules, growth factors, and signalling molecules, which aid in the regeneration process and consequent wound healing [28].

In the present study, the healing potential of 8 decoctions used in the preparation of ayahuasca was evaluated, using the wound scratch test. The evolution of the scratch created was monitored using microscopic images (Table III.4.2) and the distance between the margins of the lesion was calculated (Table III.4.3). Analysing the images that showed the evolution of the distance between the margins of the lesion and comparing them with the control samples, it was possible to verify that, in general, all the samples showed a great decrease in the lesion, and after 24 h of incubation, the samples of *M. hostilis* + *P. harmala* at 500 mg/L and *P. viridis* + *B. caapi* at 250 mg/L showed the best results. However, analysing the evolution of the distance calculated, only the sample of the commercial mixture at 250 mg/L after 2 h of incubation did not show a significant decrease. All other samples at different evaluation times, as well as the commercial mixture at 250 mg/L at the other times (8 h, 12 h and 24 h) showed a significant decrease in lesion margins compared to the control.

These results are indicative of the healing activity of the samples tested. It is possible to observe in the images of Table III.4.2 the migration of the fibroblasts incubated with the samples at different concentrations. In these images, it was possible to verify that the lesions closed over time, which was in accordance with the distance calculated between the margins of the lesion. As far as we know, to date there are no studies where the healing potential of ayahuasca has been evaluated, and it is not possible to compare the results now obtained. However, these results can be explained by the antioxidant and anti-inflammatory activity previously studied in these samples [2], since it is reported that antioxidant activity and healing properties coexist in plant extracts [28]. Wound healing consists of the reconstruction of the lesion, involving several interactions between epithelial cells, growth factors, cytokines and chemokines. It has been reported that natural products, namely plant extracts, are involved in the proliferation of fibroblasts and keratinocytes, and may contain cell adhesion molecules, growth factors and cell signalling molecules, which can promote lesion reconstruction [28]. This *in vitro* assay, which, unlike conventional assays used to determine the healing properties of plant material, was non-invasive, allowed the screening of several samples with antibacterial, anti-inflammatory and antioxidant properties, which are important in wound healing [2,20].

Table III.4.2. Microscopic images obtained from the scratch wound-healing assay with the samples of ayahuasca (magnification: 100×). The margins of the scratch appear in red.



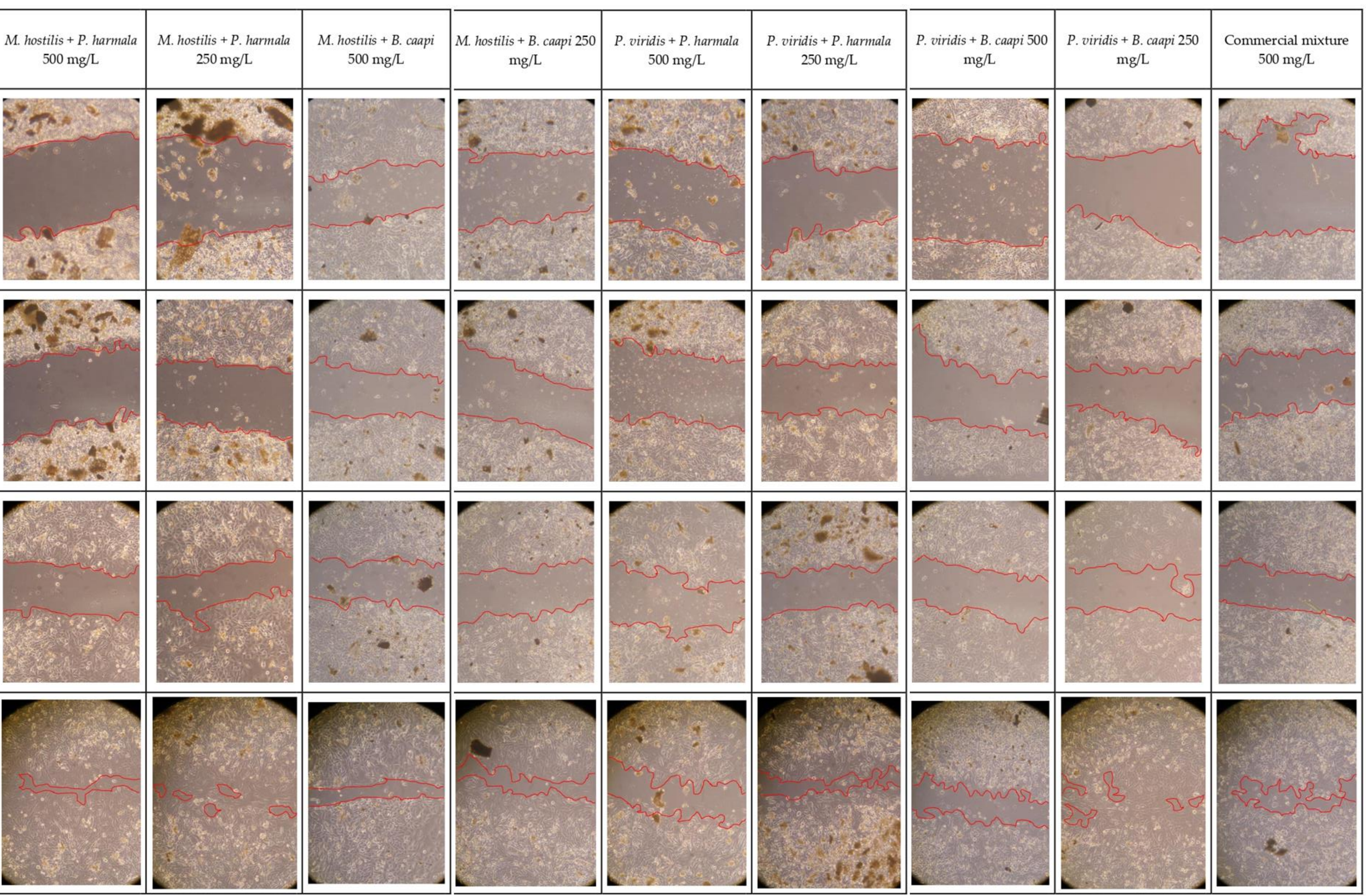

M. hostilis + P. harmala 500 mg/L
M. hostilis + P. harmala 250 mg/L
M. hostilis + B. caapi 500 mg/L
M. hostilis + B. caapi 250 mg/L
P. viridis + P. harmala 500 mg/L
P. viridis + P. harmala 250 mg/L
P. viridis + B. caapi 500 mg/L
P. viridis + B. caapi 250 mg/L
Commercial mixture 500 mg/L

Table III.4.3. Calculated mean difference between the distance of the injury of the negative control and the samples.

Samples	0h	2h	*p*-Value	8h	*p*-Value	12h	*p*-Value	24h	*p*-Value
Control	3.8	3.79	-	3.13	-	2.93	-	2.65	-
***P. viridis* 250 mg/L**		2.36	<0.001	2.06	<0.001	1.5	<0.001	0.53	<0.001
***P. viridis* 500 mg/L**		2.97	<0.001	2	<0.001	1.64	<0.001	0.54	<0.001
***B. caapi* 250 mg/L**		3.23	<0.001	1.71	<0.001	1.42	<0.001	0.75	<0.001
***B. caapi* 500 mg/L**		3.08	<0.001	2.24	<0.001	1.42	<0.001	1	<0.001
***M. hostilis* 250 mg/L**		4.14	<0.001	2.43	<0.001	1.49	<0.001	0.81	<0.001
***M. hostilis* 500 mg/L**		2.43	<0.001	2.07	<0.001	1.76	<0.001	0.4	<0.001
Commercial mixture 250 mg/L		3.79	1	2.05	<0.001	1.39	<0.001	0.49	<0.001
Commercial mixture 500 mg/L		3.14	<0.001	1.85	<0.001	1.11	<0.001	0.69	<0.001
***P. viridis* + *B. caapi* 250 mg/L**		2.1	0.001	1.38	<0.001	1.23	<0.001	0.25	<0.001
***P. viridis* + *B. caapi* 500 mg/L**		2.73	<0.001	1.5	<0.001	1.29	<0.001	0.63	<0.001
***P. viridis* + *P. harmala* 250 mg/mL**		1.95	<0.001	1.44	<0.001	1.26	<0.001	0.37	<0.001
***P. viridis* + *P. harmala* 500 mg/L**		2.15	<0.001	1.98	<0.001	1.34	<0.001	0.8	<0.001
***M. hostilis* + *B. caapi* 250 mg/L**		2.08	<0.001	1.65	<0.001	1.38	<0.001	0.47	<0.001
***M. hostilis* + *B. caapi* 500 mg/L**		1.72	<0.001	1.38	<0.001	1.2	<0.001	0.33	<0.001
***M. hostilis* + *P. harmala* 250 mg/L**		2.83	<0.001	1.71	<0.001	1.07	<0.001	0.3	<0.001
***M. hostilis* + *P. harmala* 500 mg/L**		2.73	<0.001	2	<0.001	1.47	<0.001	0.2	<0.001

Ayahuasca has psychoactive compounds in its constitution, and since the samples showed healing potential, we evaluated whether compounds such as DMT, harmine, harmaline, harmol, harmalol and THH can cross the NHDF cell layer, being absorbed and becoming accessible to the bloodstream. For this, a PAMPA assay was performed, and parameters such as permeability and cell layer integrity were also evaluated.

3.4.2.3. Evaluation of the Electrical Resistance of the Cell Transendothelial Membrane

The integrity of the cell layer was assessed using the TEER assay, the results being shown in Table III.4.4. This assay, which is able to assess changes in intercellular junctions and monitor the integrity of the cell monolayer, was performed before and after incubation with the extracts [29]. After incubation with the extracts, a new TEER measurement was performed, where it was possible to verify that during cell incubation with the sample *P. viridis* + *P. harmala* at 500 mg/L there were significant changes in the integrity of the cell monolayer. In all other samples there was no significant change in cell layer integrity. To date, there are no studies with ayahuasca samples where the TEER assay was performed on NHDF cells. However, in another study carried out by our research group, where the TEER assay was performed in intestinal adenocarcinoma cells, after incubation with ayahuasca samples, no significant changes were observed in the integrity of the cells, however the concentrations of the samples used were much lower [9].

Table III.4.4. TEER values obtained before and after incubation with the extracts. The values are expressed as mean ± SD. Statistically significant values were considered if $p < 0.05$.

Samples	TEER (Ω cm²)		
	Before	**After**	***p*-Value**
Control	924 ± 124.45	1166 ± 217.79	0.306
***P. viridis* 250 mg/L**	1166 ± 31.11	1056 ± 62.23	0.155
***P. viridis* 500 mg/L**	1122 ± 155.56	1188 ± 248.90	0.781
***B. caapi* 250 mg/L**	1309 ± 140.01	1100 ± 124.45	0.255
***B. caapi* 500 mg/L**	1023 ± 202.23	1430 ± 93.34	0.123
***M. hostilis* 250 mg/L**	1342 ± 93.34	1320 ± 124.45	0.860
***M. hostilis* 500 mg/L**	869 ± 171.12	968 ± 186.68	0.636
Commercial mixture 250 mg/L	1265 ± 233.35	1056 ± 0.00	0.333
Commercial mixture 500 mg/L	836 ± 62.23	1144 ± 124.45	0.089
***P. viridis* + *B. caapi* 250 mg/L**	858 ± 31.11	1254 ± 217.79	0.126
***P. viridis* + *B. caapi* 500 mg/L**	1078 ± 217.79	1386 ± 31.11	0.186
***P. viridis* + *P. harmala* 250 mg/L**	1254 ± 31.11	1298 ± 31.11	0.293
***P. viridis* + *P. harmala* 500 mg/L**	1056 ± 62.23	1298 ± 31.11	0.039
***M. hostilis* + *B. caapi* 250 mg/L**	979 ± 202.23	1342 ± 155.56	0.182
***M. hostilis* + *B. caapi* 500 mg/L**	1331 ± 202.23	1276 ± 124.45	0.774
***M. hostilis* + *P. harmala* 250 mg/L**	924 ± 186.68	1056 ± 0.00	0.403
***M. hostilis* + *P. harmala* 500 mg/L**	968 ± 124.45	1210 ± 155.56	0.228

3.4.2.4. Evaluation of Cell Monolayer Permeability

Cell layer permeability was evaluated by the Lucifer yellow permeability assay, the results being shown in Table III.4.5. This assay was used to assess the permeability of a cell monolayer by

measuring the passive diffusion of specific molecules, as is the case with Lucifer yellow [30]. Analysing the results, it was possible to verify that, compared to the control, significant changes only occurred when the cells were incubated with the *P. viridis* + *P. harmala* sample at 500 mg/L. Studies suggest that permeability and TEER measurement are inversely related; that is, a decrease in TEER values is accompanied by an increase in permeability [31,32]. Comparing the results obtained in this assay with the results obtained in the TEER assay, we verified that they were in agreement. These results suggest that, with the exception of the sample of *P. viridis* + *P. harmala* at 500 mg/L, there were no changes in cell barrier function or intracellular spaces, and consequently, in membrane permeability [31,32]. So far, there are no studies with samples of ayahuasca where this assay has been carried out in NHDF cells, so it is not possible to establish a comparison. However, as previously mentioned, our research group carried out a study in which the Lucifer yellow permeability assay was performed on intestinal adenocarcinoma cells, and after incubation with ayahuasca samples, no significant changes were observed in the permeability of the cells [9]. However, it should be noted that the concentrations of the samples used were much lower [9].

Table III.4.5. Percentage of permeability of NHDF cells after incubation with the extracts. The values are expressed as mean ± SD. Statistically significant values were considered if $p < 0.05$.

Samples	Permeability (%)	*p*-Value
Control	13.55 ± 0.51	-
***P. viridis* 250 mg/L**	14.69 ± 3.04	0.652
***P. viridis* 500 mg/L**	13.75 ± 2.49	0.922
***B. caapi* 250 mg/L**	13.40 ± 2.65	0.947
***B. caapi* 500 mg/L**	13.41 ± 2.76	0.951
***M. hostilis* 250 mg/L**	15.46 ± 2.98	0.465
***M. hostilis* 500 mg/L**	14.40 ± 2.74	0.709
Commercial mixture 250 mg/L	13.80 ± 2.74	0.910
Commercial mixture 500 mg/L	12.33 ± 0.73	0.193
***P. viridis* + *B. caapi* 250 mg/L**	12.69 ± 0.29	0.174
***P. viridis* + *B. caapi* 500 mg/L**	12.51 ± 2.46	0.617
***P. viridis* + *P. harmala* 250 mg/L**	11.33 ± 1.29	0.151
***P. viridis* + *P. harmala* 500 mg/L**	11.29 ± 0.38	0.037
***M. hostilis* + *B. caapi* 250 mg/L**	12.51 ± 2.55	0.628
***M. hostilis* + *B. caapi* 500 mg/L**	13.66 ± 0.05	0.789
***M. hostilis* + *P. harmala* 250 mg/L**	14.52 ± 1.45	0.467
***M. hostilis* + *P. harmala* 500 mg/L**	13.85 ± 0.12	0.501

3.4.2.5. Characterisation of the Main Compounds after Cell Incubation

After performing the PAMPA assay, aliquots were collected from the basolateral part of each well and the amounts of the compounds present in the aliquots were quantified by HPLC-DAD. This analytical method, previously developed and validated, proved to be linear between 0.16 and 10.00 µg/mL for harmol, THH, harmaline and harmine; between 0.31 and 10.00 µg/mL for harmalol and between 0.031 and 1.00 µg/mL for DMT, with coefficients of determination higher than 0.997. The intra- and interday precision revealed coefficients of variation below 15%, and the accuracy was within the range of ±15%. The limits of quantification and detection obtained were 0.31 µg/mL for all compounds, except for DMT, where these values were 0.031 µg/mL [9]. When the analytical method was developed, the concentrations of the aforementioned compounds present in the ayahuasca decoctions were also determined. The concentrations of DMT ranged from 4.50 to 10.50 µg/mg extract (6.50 µg/mg in *P. viridis*, 10.50 µg/mg in *M. hostilis*, 10.40 µg/mg in the commercial mixture, 4.50 µg/mg in the mixture of *P. viridis* + *B. caapi*, 6.50 µg/mg in the mixture of *P. viridis* + *P. harmala*, 8.00 µg/mg in the mixture of *M. hostilis* + *B. caapi* and 8.50 µg/mg in the mixture of *M. hostilis* + *P. harmala*.

Concerning β-carbolines, harmine concentrations ranged between 0.02 and 12.00 µg/mg extract (10.00 µg/mg for *B. caapi*, 12.00 µg/mg for *P.harmala*, 0.02 µg/mg for the commercial mixture, 0.48 µg/mg for the mixture of *P. viridis* + *B. caapi*, 0.30 µg/mg for the mixture of *P. viridis* + *P. harmala*, 0.82 µg/mg for the mixture of *M. hostilis* + *B. caapi* and 9.00 µg/mg for the mixture of *M. hostilis* + *P. harmala*); harmaline concentrations ranged from 0.07 to 17.00 µg/mg extract (4.68 µg/mg for *B. caapi*, 17.00 µg/mg for *P. harmala*, 0.37 µg/mg for the commercial mixture, 0.07 µg/mg for the mixture of *P. viridis* + *B. caapi*, 0.48 µg/mg for the mixture of *P. viridis* + *P. harmala*, 0.12 µg/mg for the mixture of *M. hostilis* + *B. caapi* and 13.5 µg/ mg for the *M. hostilis* + *P. harmala* mixture).

For THH, the concentrations ranged between 0.63 and 5.00 µg/mg extract (5.00 µg/mg for *B. caapi*, 3.05 µg/mg for *P. harmala*, 2.09 µg/mg for the commercial mixture, 2.50 µg/mg for the mixture of *P. viridis* + *B. caapi*, 0.63 µg/mg for the mixture of *P. viridis* + *P. harmala*, 1.90 µg/mg for the mixture of *M. hostilis* + *B. caapi* and 3.44 µg/mg for the mixture of *M. hostilis* + *P. harmala*).

harmol was also detected in extracts of samples of *B. caapi* (0.14 µg/mg), *P. harmala* (0.02 µg/mg), commercial mixture (0.01 µg/mg), *P. viridis* + *B. caapi* (0.01 µg/mg), *P. viridis* + *P. harmala* (0.02 µg/mg), *M. hostilis* + *B. caapi* (0.03 µg/mg), *M. hostilis* + *P. harmala* (0.06 µg/mg) and hamalol in the samples of *B. caapi* (0.05 µg/mg), *P. harmala* (0.66 µg/mg), *P. viridis* + *P. harmala* (0.08 µg/mg), *M. hostilis* + *B. caapi* (0.04 µg/ mg) and *M. hostilis* + *P. harmala* (0.36 µg/mg). These results are also presented in the study y Gonçalves *et al.* [9]. Regarding the aliquots collected in the present study when performing the PAMPA test, it was possible to verify that DMT, harmine, harmaline, THH, harmol and harmalol were not detected. To the best of our knowledge, PAMPA assays with ayahuasca have not been previously performed in NHDF cells. However, in a study carried out by our research team, where this same assay was performed in Caco2 cells, the

presence of these compounds was verified [9]. It should be noted that in that work, an *in vitro* digestion was carried out, which may have facilitated the passage of the compounds [9]. Additionally, during that process, the DMT and β-carbolines present in the initial extracts were metabolised in compounds of smaller size, which more easily crossed the cell layer [9].

3.4.3. Materials and Methods

3.4.3.1. Chemicals and Materials

The analytical standards of DMT, harmine, THH, harmaline, harmalol and harmol were kindly provided by Nal von Minden, GmbH (Regensburg, Germany). Roswell Park Memorial Institute (RPMI) medium, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and Lucifer yellow were obtained from Sigma-Aldrich (Sintra, Portugal). Formic acid and dimethyl sulfoxide (99.9% of purity) were purchased from Sigma-Aldrich (Sintra, Portugal). Methanol (HPLC grade) was obtained from Fischer Chemical (Loughborough, UK). Deionised water was obtained from a Milli-Q System (Millipore, Billerica, MA, USA).

3.4.3.2. Sample and Work Solutions Preparation

Individual stock solutions of harmaline, harmol, harmalol, harmine and DMT were prepared at 1 mg/mL in methanol, and the working solutions were prepared from these by serial dilutions in methanol.

The vegetal samples were acquired online from Shayana Shop (https://www.shayanashop.com, Amsterdam, The Netherlands) (accessed on 25 May 2019). Ayahuasca decoctions were prepared according to a traditional recipe, provided by a consumer who was admitted to the emergency department with symptoms of intoxication. Thus, 0.210 g of each of the four plant samples—leaves of *P. viridis*, stem remains of *B. caapi*, root bark of *M. hostilis* and seeds of *P. harmala*—and the commercial mixture were weighed and then milled in a mortar with some water drops. After that, 250 mL of ultrapure water were added and the mixture was transferred to a flask and boiled for 4 h [2,9,27]. The four decoctions of the plant mixtures were prepared in the same way, with two of the above plant samples being mixed for each (*P. viridis* and *P. harmala*; *P. viridis* and *B. caapi*; *M. hostilis* and *P. harmala*; *M. hostilis* and *B. caapi*). After 4 h, the samples were cooled, filtered, frozen at −80 °C and lyophilised.

For application in cell culture, all lyophilised samples were prepared in RPMI medium at two concentrations (250 and 500 mg/L). These concentrations were used in other studies by our research group, in which antioxidant and anti-inflammatory properties were evaluated in other samples [2,18,20].

3.4.3.3. Cell Culture

The normal human dermal fibroblast (NHDF) cell line was cultured in RPMI medium supplemented with 0.01 M HEPES, 0.02 M L-glutamine, 0.001 M sodium pyruvate, 1% antibiotic mixture and 10% of foetal bovine serum, and then incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were used between passages 23 and 28.

For the wound-healing and PAMPA assay, the cells were seeded in culture inserts placed in 12 multi-well plates (at a cell density of 6×10^4). On the other hand, for the MTT assay, the cells were seeded in 96 multi-well plates at a cell density of 0.5×10^4.

3.4.3.3.1. Cytotoxicity

The cytotoxicity of the samples was evaluated by the cell viability assay using the MTT method. Thus, after the cells reached confluence in 96-well plates, they were exposed to the samples for 24 h. The negative control consisted of RPMI medium. After the incubation time, the medium from all the wells was removed and replaced with an MTT solution (0.5 mg/mL). Then, the cells were incubated at 37 °C for 3 h. After that, the MTT solution was removed and the formazan crystals were dissolved in dimethylsulfoxide (DMSO), the absorbance being measured at 570 nm in a microplate reader. All the assays were performed in triplicate.

3.4.3.3.2. Wound-Healing Assay

The assay was carried out in accordance with previously developed studies [18,19,20]. For this purpose, the selected samples were submitted to the wound scratch assay to assess their healing activity. Thus, after the NHDF cells reached confluence, the RPMI medium was removed, and a scratch was created by scraping in a straight line with a p200 micropipette tip. Then, reference points were marked on the microplate, so that the same point was always visualised. The wells were washed with PBS and after that, samples were added to the cells, using RPMI medium as a negative control. After that, the plates were placed under a phase contrast microscope and images were acquired at the initial time (t = 0 h). Then the cells were incubated at 37 °C with the samples and new images were taken after 2, 8, 12 and 24 h. The distances between the lesion margins were evaluated using a digital image analysis tool (IC Measure software version 2.0.0.161, The Imaging Source, Bremen, Germany). All assays were performed in triplicate.

3.4.3.3.3. Parallel Artificial Membrane Permeability Assay

The potential crossing of psychoactive compounds by NHDF cells was evaluated using the PAMPA assay. Thus, after a confluent monolayer was formed, the samples were placed in contact with it by adding 500 μL of each sample to the apical chamber. The cells were incubated at 37 °C and after 2, 8, 12 and 24 h aliquots of 250 μL were collected from the basolateral chamber.

Subsequently, the collected aliquots were analysed by high performance liquid chromatography coupled to diode array detector (HPLC-DAD). All assays were performed in triplicate.

3.4.3.3.4. Transepithelial Electrical Resistance Assay

Cell monolayer integrity, as well as tight junction changes, were assessed by measuring transepithelial electrical resistance (TEER). Thus, before and after the PAMPA assay, TEER was measured using a transepithelial resistance meter electrode (EVOM2, World Precision Instrument, Sarasora, FL, USA). Thus, the electrode was equilibrated with culture medium and then placed in each well, making a 90° angle. The longest part remained in the basolateral chamber, while the shortest part remained in the apical chamber. The assay was performed in triplicate and the TEER value was determined according to the following equation:

TEER value = (mean of the resistances of each well-mean of the resistance of blank) × insert area (1)

3.4.3.3.5. Lucifer Yellow Permeability Assay

Cell monolayer permeability changes were evaluated by the Lucifer yellow permeability assay. Thus, after performing the PAMPA assay, the medium of the apical and basolateral compartments was removed and replaced with 500 µL of Lucifer yellow solution (0.1 mg/mL) in the apical chamber and 1.5 mL of Hank's balanced salt solution (HBSS) in the basolateral chamber. Cells were incubated at 37 °C for 1 h. After this, 200 µL were collected from each basolateral chamber and transferred to a 96-well plate. A Lucifer yellow (0.1 mg/mL) solution was used as a positive control and HBSS buffer was used as a blank. Then fluorescence was measured at 485 nm (excitation) and 535 nm (emission) using a spectrofluorometer. The permeability percentage was calculated as follows:

% permeability = (mean of fluorescence of each well-fluorescence of blank)/(fluorescence of positive control-fluorescence of blank) × 100 (2)

3.4.3.4. Instrumental and Chromatographic Conditions

The quantification of DMT, harmine, harmaline, THH, harmol and harmalol was performed in an HPLC-DAD (Agilent Technologies, Soquímica, Lisbon, Portugal). The injection volume was 50 µL and the flow rate was 1.5 mL/min. The stationary phase consisted of a YMC-Triart PFP analytical column (5 m, 4.6 i.d. × 150 mm) coupled to a Guard-c support (4 × 10 mm) and a Triart PFP pre-column (5 m, 3 × 10 mm), all from YMC Europe GMBH (Solítica, Lisbon, Portugal). The oven temperature was maintained at 25 °C. The mobile phase consisted of 0.1% formic acid in methanol in line A, and 0.1% formic acid in water in line B. Elution was performed in gradient mode and included 5% A (0–2 min), 50% A (2–32 min) and again, 5% A (32–40 min).

Finally, harmaline and harmalol were detected at 360 nm, DMT and THH at 278 nm and harmine and harmol at 246 nm. The sampler temperature was set at 4 °C.

3.4.3.5. Statistical Analysis

The results were expressed as mean values with standard deviations (SD). The Student's *t*-test was employed, and statistically significant values were considered when $p < 0.05$ (*).

3.4.4. Conclusions

Ayahuasca samples showed a great healing potential, which can be seen in the microscopic images collected. After performing the PAMPA assay with the extracts, it was also possible to verify that they did not show cytotoxicity, and the integrity of the cell monolayer remained unchanged, except for one sample, as well as its permeability. This suggests that most samples did not interfere with intercellular junctions. It should be noted that the present study is the first work where these tests were carried out simultaneously, and additionally, it was an *in vitro* test; therefore, conclusions must be drawn with caution.

3.4.5. References

1. Houle, S.K.D.; Evans, D.; Carter, C.A.; Schlagenhauf, P. Ayahuasca and the traveller: A scoping review of risks and possible benefits. *Travel Med. Infect. Dis.* **2021**, *44*, doi:10.1016/J.TMAID.2021.102206.
2. Gonçalves, J.; Luís, Â.; Gradillas, A.; García, A.; Restolho, J.; Fernández, N.; Domingues, F.; Gallardo, E.; Duarte, A.P. Ayahuasca Beverages: Phytochemical Analysis and Biological Properties. *Antibiot. (Basel, Switzerland)* **2020**, *9*, 1–21, doi:10.3390/ANTIBIOTICS9110731.
3. Gonçalves, J.; Luís, Â.; Gallardo, E.; Duarte, A.P. Psychoactive Substances of Natural Origin: Toxicological Aspects, Therapeutic Properties and Analysis in Biological Samples. *Molecules* **2021**, *26*, doi:10.3390/MOLECULES26051397.
4. Ruffell, S.G.D.; Netzband, N.; Tsang, W.; Davies, M.; Butler, M.; Rucker, J.J.H.; Tófoli, L.F.; Dempster, E.L.; Young, A.H.; Morgan, C.J.A. Ceremonial Ayahuasca in Amazonian Retreats-Mental Health and Epigenetic Outcomes From a Six-Month Naturalistic Study. *Front. psychiatry* **2021**, *12*, doi:10.3389/FPSYT.2021.687615.
5. Simão, A.Y.; Antunes, M.; Marques, H.; Rosado, T.; Soares, S.; Gonçalves, J.; Barroso, M.; Andraus, M.; Gallardo, E. Recent bionalytical methods for the determination of new psychoactive substances in biological specimens. *Bioanalysis* **2020**, *12*, 1557–1595, doi:10.4155/BIO-2020-0148.
6. Durante, Ĺ.; Dos Santos, R.G.; Bouso, J.C.; Hallak, J.E. Risk assessment of ayahuasca use in a religious context: self-reported risk factors and adverse effects. *Rev. Bras. Psiquiatr.* **2021**, *43*, 362–369, doi:10.1590/1516-4446-2020-0913.
7. Malcolm, B.J.; Lee, K.C. Ayahuasca: An ancient sacrament for treatment of contemporary psychiatric illness? *Ment. Heal. Clin.* **2017**, *7*, 39–45, doi:10.9740/MHC.2017.01.039.
8. Brabec de Mori, B. The Power of Social Attribution: Perspectives on the Healing Efficacy of Ayahuasca. *Front. Psychol.* **2021**, *12*, doi:10.3389/FPSYG.2021.748131.
9. Gonçalves, J.; Castilho, M.; Rosado, T.; Luís, Â.; Restolho, J.; Fernández, N.; Gallardo, E.; Duarte, A.P. *In Vitro* Study of the Bioavailability and Bioaccessibility of the Main Compounds Present in Ayahuasca Beverages. *Molecules* **2021**, *26*, doi:10.3390/MOLECULES26185555.
10. McKenna, D.J. Clinical investigations of the therapeutic potential of ayahuasca: rationale and regulatory challenges. *Pharmacol. Ther.* **2004**, *102*, 111–129, doi:10.1016/J.PHARMTHERA.2004.03.002.
11. Miller, M.J.; Albarracin-Jordan, J.; Moore, C.; Capriles, J.M. Chemical evidence for the use of multiple psychotropic plants in a 1,000-year-old ritual bundle from South America. *Proc. Natl. Acad. Sci. U. S. A.* **2019**, *116*, 11207–11212, doi:10.1073/PNAS.1902174116.
12. Da Silva, F.S.; Silva, E.A.S.; De Sousa, G.M.; Maia-De-oliveira, J.P.; De Soares-Rachetti, V.P.; De Araujo, D.B.; Sousa, M.B.C.; Lobão-Soares, B.; Hallak, J.; Galvão-Coelho, N.L. Acute effects of ayahuasca in a juvenile non-human primate model of depression. *Brazilian J. Psychiatry* **2019**, *41*, 280, doi:10.1590/1516-4446-2018-0140.
13. Barbosa, P.C.R.; Cazorla, I.M.; Giglio, J.S.; Strassman, R.S. A six-month prospective evaluation of personality traits, psychiatric symptoms and quality of life in ayahuasca-naïve subjects. *J. Psychoactive Drugs* **2009**, *41*, 205–

212, doi:10.1080/02791072.2009.10400530.
14. Palhano-Fontes, F.; Barreto, D.; Onias, H.; Andrade, K.C.; Novaes, M.M.; Pessoa, J.A.; Mota-Rolim, S.A.; Osório, F.L.; Sanches, R.; Dos Santos, R.G.; et al. Rapid antidepressant effects of the psychedelic ayahuasca in treatment-resistant depression: a randomized placebo-controlled trial. *Psychol. Med.* **2019**, *49*, 655–663, doi:10.1017/S0033291718001356.
15. de Osório, F.L.; Sanches, R.F.; Macedo, L.R.; dos Santos, R.G.; Maia-De-Oliveira, J.P.; Wichert-Ana, L.; de Araujo, D.B.; Riba, J.; Crippa, J.A.; Hallak, J.E. Antidepressant effects of a single dose of ayahuasca in patients with recurrent depression: a preliminary report. *Rev. Bras. Psiquiatr.* **2015**, *37*, 13–20, doi:10.1590/1516-4446-2014-1496.
16. Barbosa, P.C.R.; Giglio, J.S.; Dalgalarrondo, P. Altered States of Consciousness and Short-Term Psychological After-Effects Induced by the First Time Ritual Use of Ayahuasca in an Urban Context in Brazil. *https://doi.org/10.1080/02791072.2005.10399801* **2011**, *37*, 193–201, doi:10.1080/02791072.2005.10399801.
17. Da Silveira, D.X.; Grob, C.S.; de Rios, M.D.; Lopez, E.; Alonso, L.K.; Tacla, C.; Doering-Silveira, E. Ayahuasca in Adolescence: A Preliminary Psychiatric Assessment. *https://doi.org/10.1080/02791072.2005.10399792* **2011**, *37*, 129–133, doi:10.1080/02791072.2005.10399792.
18. Afonso, A.M.; Gonçalves, J.; Luís, Â.; Gallardo, E.; Duarte, A.P. Evaluation of the *In Vitro* Wound-Healing Activity and Phytochemical Characterization of Propolis and Honey. *Appl. Sci. 2020, Vol. 10, Page 1845* **2020**, *10*, 1845, doi:10.3390/APP10051845.
19. Felician, F.F.; Yu, R.H.; Li, M.Z.; Li, C.J.; Chen, H.Q.; Jiang, Y.; Tang, T.; Qi, W.Y.; Xu, H.M. The wound healing potential of collagen peptides derived from the jellyfish Rhopilema esculentum. *Chinese J. Traumatol. = Zhonghua chuang shang za zhi* **2019**, *22*, 12–20, doi:10.1016/J.CJTEE.2018.10.004.
20. Santos, E.S.; Luís, Â.; Gonçalves, J.; Rosado, T.; Pereira, L.; Gallardo, E.; Duarte, A.P. *Julbernardia paniculata* and *Pterocarpus angolensis*: From Ethnobotanical Surveys to Phytochemical Characterization and Bioactivities Evaluation. *Molecules* **2020**, *25*, doi:10.3390/MOLECULES25081828.
21. da Silva, M.G.; Daros, G.C.; de Bitencourt, R.M. Anti-inflammatory activity of ayahuasca: therapeutical implications in neurological and psychiatric diseases. *Behav. Brain Res.* **2021**, *400*, doi:10.1016/J.BBR.2020.113003.
22. Dakic, V.; Minardi Nascimento, J.; Costa Sartore, R.; MacIel, R.D.M.; De Araujo, D.B.; Ribeiro, S.; Martins-De-Souza, D.; Rehen, S.K. Short term changes in the proteome of human cerebral organoids induced by 5-MeO-DMT. *Sci. Reports 2017 71* **2017**, *7*, 1–13, doi:10.1038/s41598-017-12779-5.
23. Kumar, P.; Nagarajan, A.; Uchil, P.D. Analysis of Cell Viability by the MTT Assay. *Cold Spring Harb. Protoc.* **2018**, *2018*, 469–471, doi:10.1101/PDB.PROT095505.
24. Samoylenko, V.; Rahman, M.M.; Tekwani, B.L.; Tripathi, L.M.; Wang, Y.H.; Khan, S.I.; Khan, I.A.; Miller, L.S.; Joshi, V.C.; Muhammad, I. *Banisteriopsis caapi*, a unique combination of MAO inhibitory and antioxidative constituents for the activities relevant to neurodegenerative disorders and Parkinson's disease. *J. Ethnopharmacol.* **2010**, *127*, 357–367, doi:10.1016/J.JEP.2009.10.030.
25. Katchborian-Neto, A.; Santos, W.T.; Nicácio, K.J.; Corrêa, J.O.A.; Murgu, M.; Martins, T.M.M.; Gomes, D.A.; Goes, A.M.; Soares, M.G.; Dias, D.F.; et al. Neuroprotective potential of Ayahuasca and untargeted metabolomics analyses: applicability to Parkinson's disease. *J. Ethnopharmacol.* **2020**, *255*, doi:10.1016/J.JEP.2020.112743.
26. Santos, B.W.L.; Moreira, D.C.; Borges, T.K.D.S.; Caldas, E.D. Components of Banisteriopsis caapi, a Plant Used in the Preparation of the Psychoactive Ayahuasca, Induce Anti-Inflammatory Effects in Microglial Cells. *Molecules* **2022**, *27*, doi:10.3390/MOLECULES27082500.
27. Simão, A.Y.; Gonçalves, J.; Gradillas, A.; García, A.; Restolho, J.; Fernández, N.; Rodilla, J.M.; Barroso, M.; Duarte, A.P.; Cristóvão, A.C.; et al. Evaluation of the Cytotoxicity of Ayahuasca Beverages. *Molecules* **2020**, *25*, doi:10.3390/MOLECULES25235594.
28. Talekar, Y.P.; Apte, K.G.; Paygude, S. V.; Tondare, P.R.; Parab, P.B. Studies on wound healing potential of polyherbal formulation using *in vitro* and *in vivo* assays. *J. Ayurveda Integr. Med.* **2017**, *8*, 73–81, doi:10.1016/J.JAIM.2016.11.007.
29. Elbrecht, D.H.; Long, C.J.; Hickman, J.J. Transepithelial/endothelial Electrical Resistance (TEER) theory and applications for microfluidic body-on-a-chip devices Keywords TEER Body-on-a-chip Barrier tissue Blood-brain barrier Organ Endothelial cells Epithelial cells Human-on-a-chip. *J Rare Dis Res Treat* **2016**, *1*, 46–52.
30. Colombini, A.; Perego, S.; Ardoino, I.; Marasco, E.; Lombardi, G.; Fiorilli, A.; Biganzoli, E.; Tettamanti, G.; Ferraretto, A. Evaluation of a possible direct effect by casein phosphopeptides on paracellular and vitamin D controlled transcellular calcium transport mechanisms in intestinal human HT-29 and Caco2 cell lines. *Food Funct.* **2013**, *4*, 1195–1203, doi:10.1039/C3FO60099H.
31. Satsu, H.; Yokoyama, T.; Ogawa, N.; Fujiwara-Hatano, Y.; Shimizu, M. The changes in the neuronal PC12 and the intestinal epithelial Caco-2 cells during the coculture. The functional analysis using an *in vitro* coculture system. *Cytotechnology* **2001**, *35*, 73–79, doi:10.1023/A:1008136103927.
32. Piccolino, M.; Neyton, J.; Gerschenfeld, H.M. Decrease of gap junction permeability induced by dopamine and cyclic adenosine 3':5'-monophosphate in horizontal cells of turtle retina. *J. Neurosci.* **1984**, *4*, 2477–2488, doi:10.1523/JNEUROSCI.04-10-02477.1984.

3.5. Paper VII- The role of Ayahuasca in colorectal adenocarcinoma cell survival, proliferation and oxidative stress

Abstract

The psychedelic beverage ayahuasca is originally obtained by *Banisteriopsis caapi* (*B. caapi*) (BC) and *Psychotria viridis* (*P. viridis*) (PV). However, sometimes these plant species are replaced by others that mimic the original effects, such as *Mimosa hostilis* (*M. hostillis*) (MH) and *Peganum harmala* (*P. harmala*) (PH). Its worldwide consumption and the number of studies on its potential therapeutic effects has increased. This study aimed to evaluate the anticancer properties of ayahuasca in human colorectal adenocarcinoma cells. Thus, the maximum inhibitory concentration (IC_{50}) of decoctions of MH, PH and a mixture of these (MHPH) was determined. The activities of caspases 3 and 9 were evaluated, and the cell proliferation index was determined through immunocytochemical analysis (Ki-67). Two fluorescent probes were used to evaluate the production of oxidative stress and the activity of the antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx) was also evaluated. It was demonstrated that exposure to the extracts significantly induced apoptosis in Caco-2 cells, while decreasing cell proliferation. MH and MHPH samples significantly reduced oxidative stress and significantly increased glutathione peroxidase activity. No significant differences were found in SOD activity. Overall, it was demonstrated that the decoctions have a potential anticancer activity in Caco-2 cells.

Keywords: Ayahuasca; Caco2; Viability; Apoptosis; Oxidative stress; Cellular Proliferation

3.5.1. Introduction

Ayahuasca is a hallucinogenic beverage from South America [1]. Traditionally, it was used by indigenous tribes in the Amazon for medicinal purposes and divine rituals [2]. The term ayahuasca is made up of the terms "*aya*" and "*wasca*" and means "vine of the dead" or "vine of the soul" [1,3]. This psychoactive beverage is obtained by boiling scrapings stem of *Banisteriopsis caapi* (Spruce ex Griseb.) C. V. Morton (*B. caapi*) (BC) and leaves of *Psychotria viridis* Ruiz & Pav. (*P. viridis*) (PV) resulting in a brownish, thick and oily drink [3]. Many interations of this preparation have developed over time, and at this point, certain equivalents that can take the place of PV are known (*Malouetia tamaquarina* A.DC., *Brugmansia suaveolens* (Willd.) Sweet, *Psychotria carthagenensis* Jacq., *Nicotiana tabacum* L., among others) and BC (*Peganum harmala* L. (PH), tetrahydroharmine and harmine [1,4].

The hallucinogenic character of ayahuasca is due to the presence of *N,N*-Dimethyltryptamine (DMT) from PV [5]. This compound is a serotonin receptor (5-HT1A/2A/2C) agonist, which when ingested alone is harmless, as it is metabolized by peripheral MAO-A [5].

However, this beverage also contains β-carboline alkaloids (harmine, tetrahydroharmine (THH) and harmaline) that come from BC [1,5]. This class of compounds is able to temporarily inhibit MAO-A, allowing DMT to access the bloodstream and subsequently the central nervous system [1]. Additionally, THH also inhibits serotonin reuptake enhancing the effects of DMT [6].

The rise in popularity of this psychoactive drink is also due to the fact that it is often seen as a natural remedy used for millennia to cure various ailments [7]. In spite of being used in traditional medicine, it is also consumed recreationally all over the world, representing a concern, since the laws that regulate this consumption are ambiguous and very variable [5,8].

Ayahuasca consumption is characterized by a set of physical symptoms, such as vomiting, nausea and diarrhoea, but also psychological effects such as changes in the perception of time and space, visual, auditory and cognitive effects [2,5]. However, several studies described the use of ayahuasca in the treatment of psychological problems such as depression, anxiety, addiction and psychological disorders [5]. Additionally, other studies have reported the ayahuasca antimicrobial, anti-inflammatory, and healing properties [9,10]. Furthermore, there is a great demand for ayahuasca as an alternative medicine for several diseases, including cancer, and its potential as a possible treatment for some types of cancer has been described [11].

Medical reports or clinical data about the potential of ayahuasca in treating cancer are almost non-existent [11]. However, isolated pieces of research work have suggested it could be interesting to be exploited in cancer therapy. A study available in the literature revealed that a liver cancer patient undergoing surgery to remove part of the organ achieved regression of carcinoembryonic antigens and remained in remission for five years after replacing the recommended chemotherapy with religious ayahuasca sessions [11]. Two other cases of prostate and ovarian cancer patients revealed significant improvements in the levels of prostate-specific antigen and CEA-125, respectively, after treatment with ayahuasca [12]. Favourable results from taking ayahuasca were also described in patients diagnosed with uterine [13] and breast [14] cancers. The present evidence, though scarce, strongly incites curiosity about investigating the anticancer role of ayahuasca and how it is propitiated from a mechanistic perspective.

Colorectal cancer is one of the main causes of death in the world, despite being studied for years [12]. In the previous decade, colon cancer accounted for 8.5% of all deaths worldwide and 9.7% of all cancer cases [13]. Sometimes, the cancer recurs demonstrating that the available treatments are incomplete and may not be durable in the long term [14]. Therefore, finding new therapies or treatment adjuvants remains a scientific and clinical challenge. This study aimed to assess the impact of Ayahuasca decoctions on the modulation of several characteristics of colorectal cancer cells, namely, cell viability, apoptosis, cell proliferation and oxidative stress.

3.5.2. Results and discussion

In previous studies carried out by our research group, the main compounds present in the commercial mixture also used here in the preparation of ayahuasca extracts were determine [6,9]. After preparing the extracts, as described below, they were subjected to analysis by ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC/ESI-QTOF-MS) [9]. Briefly, after comparison with a phytochemical library of 48 standards, it was

possible to quantify protocatechuic acid, 4-hydroxybenzoic acid, salicylic acid, (+)-catechin, (-)-epicatechin, quercetin-3-*O*-galactoside, quercetin-3- *O*-glucoside and quercetin-3-*O*-rutinoside [9]. In other studies, the main constituents of ayahuasca were characterized by high-performance liquid chromatography with diode-array detection (HPLC-DAD) [6] and gas chromatography coupled to mass spectrometry [15]. After preparing the commercial mixture, it was possible to quantify DMT, THH, harmine, harmaline and harmol [6]. However, it is important to highlight that in other extracts the concentrations of each compound in each ayahuasca sample can vary greatly, depending on the proportion used by each consumer, the preparation methods, the different concentrations of the compound between the plants, as well as its purity [6]. The toxicity of the extracts from the ayahuasca decoctions used herein was also evaluated in Caco-2 cell line, in another study carried out by our research group [6]. The results showed that the extracts did not present cytotoxicity.

3.5.2.1. Determination of The Half-maximal Inhibitory Concentration (IC_{50})

The human colorectal adenocarcinoma Caco-2 cell line was chosen as our study model. Besides the cancer-like phenotype, this cell line is widely used to mimic the intestinal mucosa barrier in absorption studies [16], gaining great pertinence in the present work as Ayahuasca is consumed as a beverage. The IC50 values for all Ayahuasca extracts in Caco-2 cells were determined by using the MTT assay and are shown in Table III.5.1.

Table III.5.1. IC_{50} values of ayahuasca extracts in Caco-2 cells after treatment for 24 h.

Sample	IC_{50} (µg/mL) ± SD
PV	715.62 ± 0.05
BC	558.42 ± 0.08
PH	338.99 ± 0.05
MH	553.58 ± 0.02
Commercial Mixture	365.42 ± 0.02
PVBC	623.54 ± 0.07
PVPH	375.97 ± 0.02
MHBC	366.17 ± 0.05
MHPH	276.97 ± 0.05

Legend: PVBC (*P. viridis* and *B. caapi*); PVPH (*P. viridis* and *P. harmala*); MHBC (*M. hostilis* and *B. caapi*); MHPH (*M. hostilis* and *P. harmala*).

By evaluating the data, it was able to verify that the extract that presented a lower IC_{50} value, and therefore, requires a lower concentration to decrease the viability of Caco-2 cells, was the MHPH (IC_{50}=276.97 µg/mL). The PH, commercial mixture, MHBC and PVPH extracts followed, with IC_{50} values in the range of 300 µg / mL, and the MH and BC extracts with IC_{50} values in the order of 500 µg / mL. The extracts with higher IC_{50} values were PVBC and PV with IC_{50} values of

623.54 µg / mL and 715.62 µg / mL, respectively. As far as we know, there are no studies determining the IC_{50} values of Ayahuasca extracts in Caco-2 cells. Katchborian-Neto *et al.* [17] evaluated the citotoxicity in cardioyocytes (CC_{50}), these authors verifying values greater than 200 µg/mL for DMT and Ayahuasca (*P. viridis* and *B. Caapi*).

Considering the obtained results, the sample that presented the lowest IC_{50} value was MHPH, therefore it was selected to pursue the work. In the same way, the two samples that constitute the previous mixture (MH and PH) were also chosen to carry out the following assays, in order to understand the influence of each of these extracts on the results obtained for the MHPH sample.

3.5.2.2. Ayahuasca Extracts Affected Apoptosis in Caco-2 Cells

Apoptotic programmed cell death is a critical biological process in maintaining tissue homeostasis, and serving as a natural barrier to cancer development. Thus, impaired apoptotic responses significantly contribute to tumour progression and treatment resistance [18]. Herein, apoptosis was determined by measuring the activity of the executioner caspase-3, a target player in apoptotic cell death at the crossroad of the intrinsic and extrinsic apoptotic pathways [19] (Figure III.5.1.). Observing the results presented in Figure III.5.1., it was possible to see that, in general, Ayahuasca extracts increased caspase-3 activity in Caco-2 cell line. After exposing cells to PH and MHPH extracts, caspase-3 activity increased significantly, in comparison to the control group (2.87 ± 0.35 and 2.73 ± 0.12-fold increase, respectively, $p < 0.05$, Figure III.5.1.A). Regarding the MH extract, the results showed a more pronounced effect increasing caspase-3 activity (3.46 ± 0.73-fold increase, $p < 0.01$, Figure III.5.1.A).

In many cell line models, measuring caspase-3 activity has been utilized as a means of determining the rate of apoptosis. [19–21]. Therefore, the results obtained clearly demonstrate the ability of Ayahuasca extracts to induce apoptosis in Caco-2 cells, which is in accordance with the existing literature. In a study developed by Shabani *et al.* [22], where the triggering of apoptosis in the MDA-MB-231 breast cancer cell line was evaluated by PH extract, an increase in the induction of apoptosis through the intrinsic pathway was observed. Another study in breast cancer cell lines (MDA-MB-231 and MCF-7) also demonstrated an increase in apoptosis induced by harmine present in PH [23]. Li *et al.* [24] evaluated the induction of apoptosis in human gastric cancer cells, caused by harmine present in PH, and found an increase in the induction of apoptosis. Similar results were verified by Zhang *et al.* [25] and by Wang *et al.* [26]. Similarly, it was found that the same compound induced apoptosis in B16F-10 melanoma cells [27], in human colorectal carcinoma SW620 cells [28] and in non-small cell lung cancer (NSCLC) cells [29]. Otherwise, harmaline, present in PH, was responsible for arresting the cell cycle and inducing apoptosis in the glioblastoma cell line [30]. Other studies were carried out, where only the anticancer effects of synthetic or isolated β-carbolines were evaluated. It was found that harmine, harmaline, harmol or harmalol were able to increase the rate of apoptosis in human liver carcinoma cells [31,32], two lung tumour cell lines [33] and kidney adenocarcinoma cells [34].

As far as our knowledge, no research was done to assess how MH extracts affect cancer cell apoptosis. On the other hand, it is possible to verify that this extract causes a significant increase in caspase-3 activity based on the results shown in Figure III.5.1.A. Thus, we can infer that the MH extract will be the most promising extract in inducing apoptosis in Caco-2 cells.

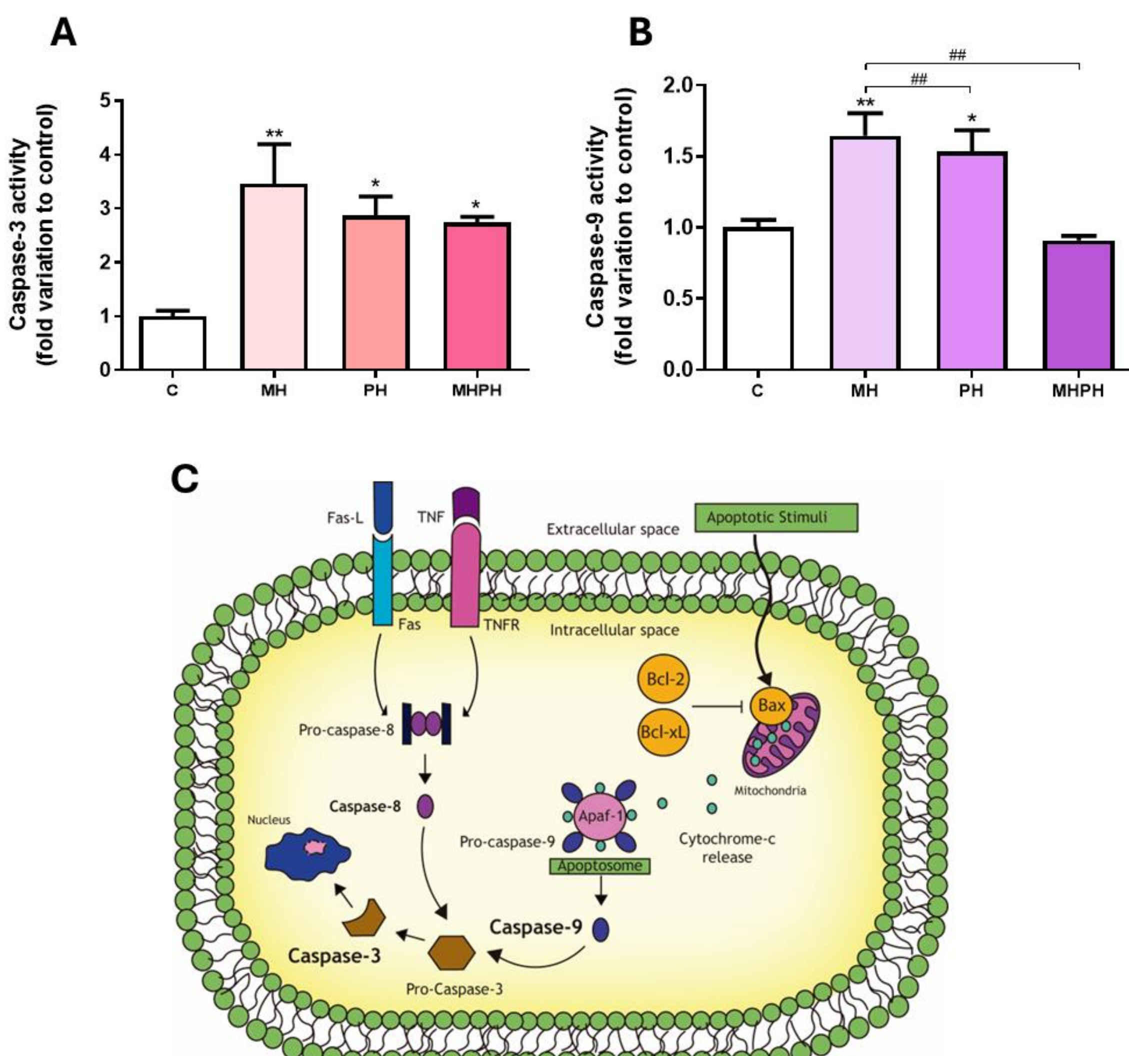


Figure III.5.1. Caspase-3 (A) and caspase-9 (B) activity in Caco-2 cells behind the treatment with 276.97 µg / mL MH, PH and MHPH extracts for 24 h (n = 3). When compared to the control group, the error bars show the mean ± S.E.M. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.01$ (##). (ANOVA p values: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), or $p < 0.0001$ (****)) (C) Intrinsic and extrinsic pathways of apoptosis. Two dif-ferent mechanisms can cause apoptosis.: the receptor-mediated (extrinsic) and the mitochondrial (intrinsic). The death receptors triggering the extrinsic pathway (e.g. Fas and tumour necrosis factor receptor, TNFR) are located at the plasma membrane and activated by their ligands (Fas-L and TNF, respectively), prompting the activation of the initiator caspase-8. The intrinsic route is triggered by several apoptotic stimuli that increase the ratio of proapoptotic (e.g. Bax)/anti-apoptotic (e.g. Bcl-2, Bcl-xL) mitochondrial proteins, causing the mitochondria to release cytochrome c. In the cytoplasm, cytochrome-c, pro-caspase-9 and the protease activating factor (Apaf-1) form the apoptosome, activating the initiator caspase-9. Pro-caspase-3 is where both routes converge that, after cleavage, becomes the active effector caspase-3, determining an end and irreversible point of apoptosis.

In order to understand which of the apoptotic pathways are being activated (intrinsic or extrinsic) in response to Ayahuasca extracts treatment, the activity of caspase-9 was determined. This is the initiator caspase associated with the activation of the intrinsic (mitochondrial) pathway of apoptosis (Figure III.5.1.C) [35]. Currently, the intrinsic apoptotic pathway is widely implicated as a barrier to the carcinogenic process [18]. Observing the results presented in Figure III.5.1.B, it was possible to verify that there was a significant increase in Caco-2 cells' caspase-9 activity, following exposure to PH and MH extracts ($p < 0.05$ and $p < 0.01$, respectively, Figure III.5.1.B). Thus, the results obtained suggest that apoptosis in cells exposed to PH and MH extracts occurred due to activation of the intrinsic pathway. These results are in accordance with the available scientific literature, showing that PH extracts are capable of inducing apoptosis in cancer cells, by activating the intrinsic pathway [22,28,36]. In a study developed by Elansary *et al.* [36], the activation capacity of caspase-9 was evaluated in cancer cells treated with PH extract compared to control. An increase in caspase-9 activity was found in T lymphocyte lineage (Jurkat), bladder cancer (T24), colorectal adenocarcinoma (HT-29), breast cancer (MCF-7) and HeLa cells [36]. Other study, developed by Liu *et al.* [28], found that harmine, present in PH extracts, was also capable of activating caspase-9 in SW620 cells. Otherwise, no studies were found evaluating the effect of MH on caspase-9 activity.

Exposing Caco-2 cells to the MHPH extract, despite increasing the activity of caspase-3, did not alter caspase-9 activity, indicating that the apoptotic process may also be driven by the activation of the extrinsic pathway (Figure III.5.1.C). To our knowledge, no studies were performed evaluating the effect of the MHPH mixture on caspase-9 activation in Caco-2 cells, and further research is needed to disclose the involvement of membrane cell death receptors (extrinsic pathway) triggering apoptosis.

3.5.2.3. Ayahuasca Extracts Affected Cellular Proliferation in Caco-2 Cells

Uncontrolled cell proliferation is one of the most recognized hallmarks of cancer [18]. Through Ki-67 immunofluorescence investigations, the proliferation index of Caco-2 cells treated with Ayahuasca extracts was determined. It was established the number of Ki-67 positive cells there were in relation to all cells (Figure III.5.2). Ki-67 is widely used as a cell proliferation marker, as it is found in the nuclei of cells in a proliferative process at any stage of the cell division cycle [37]. Contrariwise, it is absent in cells that are not proliferating [37].

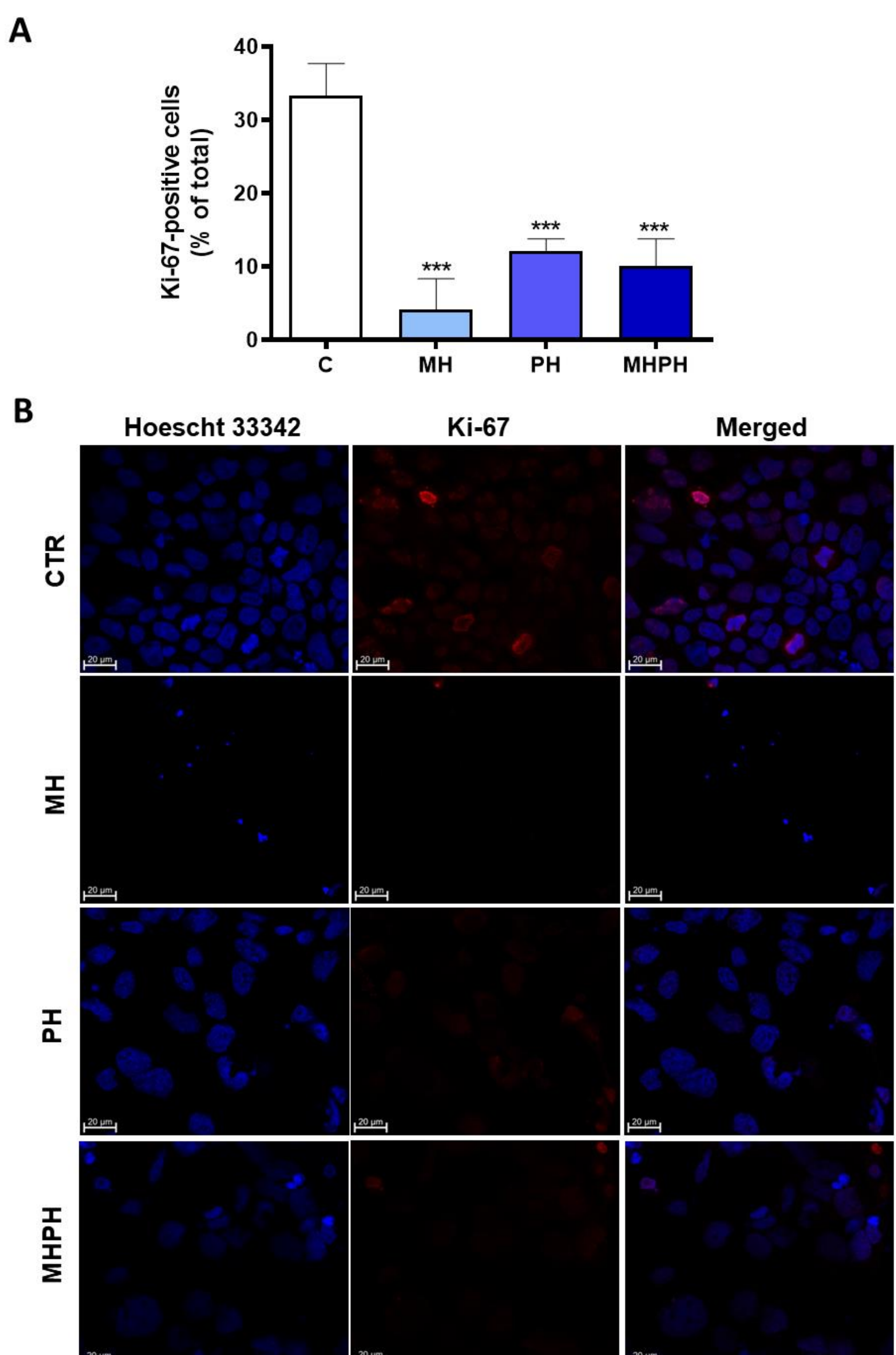


Figure III.5.2. Proliferation index of Caco-2 cells after treatment with 276.97 µg / mL MH, PH and MHPH extracts for 24 h determined by Ki-67 immunofluorescence analysis (n = 3). (A) Proportion of cells that are positive for Ki-67 compared to all cells. The fold variation between the treated and untreated control groups is used to express the results. Mean ± S.E.M. is shown by error bars; *** $p < 0.001$. (ANOVA p values: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), or $p < 0.0001$(****)) (B) Confocal microscopy photos of the control and treated groups that are representative of the Ki-67 labelling (red). Images were captured at a magnification of 630x using the Zeiss LSM 710 laser scanning confocal microscope. Hoechst 33342 is used to stain the nuclei (blue).

Ki-67 proliferation index of Caco-2 cells was significantly reduced in all treated groups compared to the control (Figure III.5.2.A). It was possible to verify that cells treated with the MH extract showed a greater reduction in the number of Ki-67 labelled cells (Figure III.5.2.A and 2B) and, therefore, a greater reduction in cell proliferation index ($p < 0.001$). The cell proliferation index of treated cells with MHPH and PH extracts was significantly lower than that of untreated cells ($p < 0.001$ for both groups). So far, there are no studies evaluating the influence of MH extracts on proliferation of cancer cells; however, some studies with PH extracts were developed. Wang et al. [38] verified that PH demonstrated antiproliferative effects in human lung cancer cells (A549). Other studies have shown similar results in gastric cancer cells [25,26], breast cancer cell lines (MDA-MB-231 and MCF-7) [23], oesophageal squamous cell carcinoma [39], NSCLC [29,40], glioblastoma [30], carcinoma (Med-mek and UCP-Med) and sarcoma (UCP-Med and Sp2/O-Ag14) [41,42] and human colorectal carcinoma (SW620) [28].

3.5.2.4. Ayahuasca Extracts Affected Oxidative Damage and Activity of Antioxidant Enzymes in Caco-2 Cells

Lipids are the main macromolecules in the constitution of cell membranes and are highly sensitive to oxidative stress [35]. Damage to the structure and function of the lipid bilayer can compromise cell integrity [43], and this is one of the reasons why reactive oxygen species (ROS) have been considered in cancer therapy [44]. On the contrary, chronic increased levels of ROS are associated with the onset and development of cancer [45]. Therefore, as we have previously characterized the phytochemical profile of Ayahuasca extracts [9] confirming its composition enriched in several compounds with antioxidant properties (Figure III.5.3.), we decided to investigated their effect in modulating oxidative stress in Caco-2 cells.

Figure III.5.3. Chemical structure of the active compounds present in ayahuasca extracts. Protocatechuic acid (A), 4-hydroxybenzoic acid (B), salicylic acid (C), (+)-catechin (D), (-)-epicatechin (E), gentilic acid (F).

In this study, ROS levels were determined by two different methods, with concordant results obtained. Results presented in Figure III.5.4. show that the MH and MHPH extracts significantly reduced oxidative stress, as indicated by the diminished ROS levels (DCFDA, both $p < 0.0001$; DHE, $p < 0.0001$ and $p < 0.001$, respectively). On the other hand, no significant changes were observed with the PH extract. So far, there were no studies evaluating ROS levels in cancer cells after treatment with Ayahuasca. However, there are several studies where the phytochemical profile of these extracts was evaluated, being found that compounds with antioxidant properties are part of their composition, which help to combat oxidative damage [9,36]. At first sight, these results appear controversial as a reduction of ROS levels may be considered a cell survival stimulus rather than an anti-tumoral one. However, it is important to enforce that chronic excessive cellular oxidative stress is widely perceived as a key factor in cancer development [46,47]. Thus, the ability of Ayahuasca extracts to reduce ROS levels may represent another possible anti-cancer mechanism tha tdeserves further investigation.

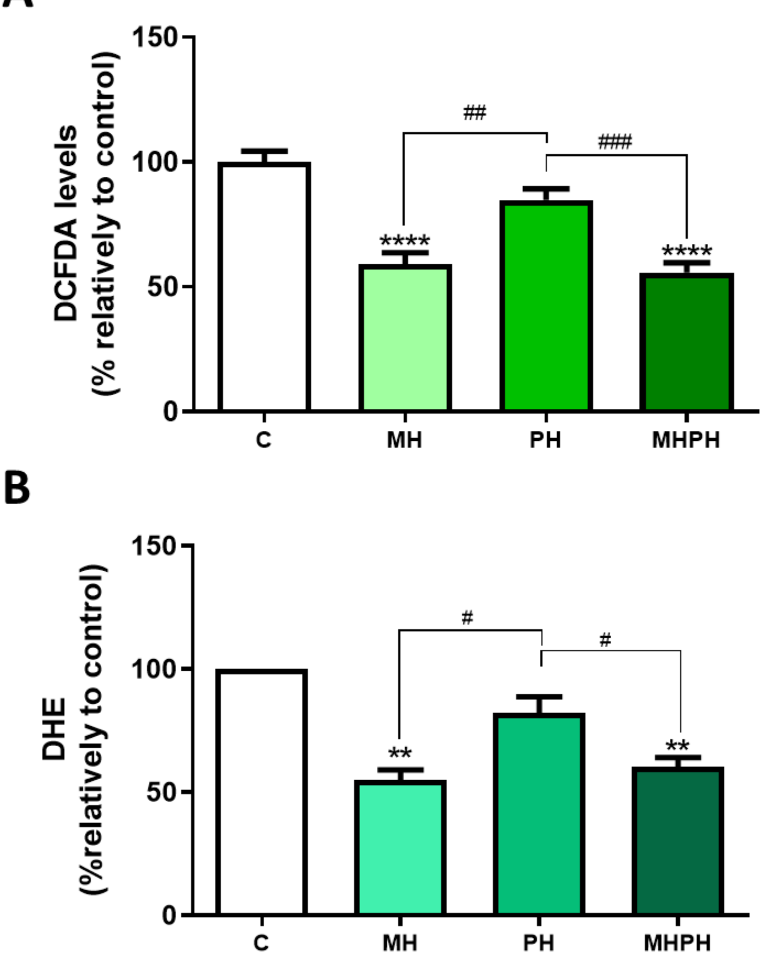


Figure III.5.4. Cellular ROS production in Caco-2 cells after treatment with 276.97 µg/mL MH, PH and MHPH extracts for 24 h measured using dichlorofluorescein diacetate (DCFDA, A) or dihydroethidium (DHE, B) (n = 3). The fold variation between the treated and untreated control groups is used to express the results. The mean ± S.E.M., $p < 0.05$ (#), $p < 0.01$ (##), $p < 0.001$ (###), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****) are indicated by the error bars (ANOVA p values: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), or $p < 0.0001$ (****)).

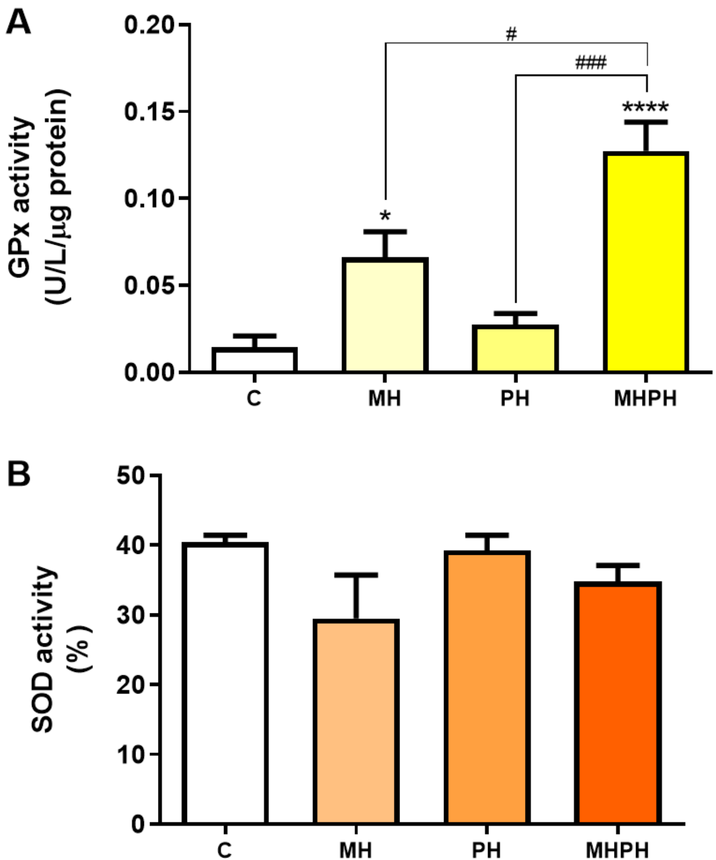


Figure III.5.5. Activity of glutathione peroxidase (GPx, A) and superoxide dismutase (SOD, B) in Caco-2 cells behind the treatment with 276.97 µg / mL MH, PH and MHPH extracts for 24 h (n = 3). Enzyme activity is normalized to protein content. When compared to the control group, the error bars show the mean ± S.E.M., $p < 0.05$ (#), $p < 0.001$ (###), $p < 0.05$ (*), , and $p < 0.0001$ (****) (ANOVA p values: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), or $p < 0.0001$ (****)).

Another feature of cancer cells is the upregulated expression of antioxidant defence enzymes to maintain ROS levels within ranges that allow for avoiding cell death [48]. However, low levels of antioxidant enzymes have also been reported in certain types of cancer (e.g. bladder, cervical, breast), and mostly depending on the stage of disease [49–51]. GPx and SOD enzymes play an important role in the defence against oxidative stress [35]. When evaluating GPx activity (Figure III.5.5.A) there was a significant increase after exposure to the MH and MHPH extracts ($p < 0.05$ and $p < 0.0001$, respectively) and a non-significant increase in the PH sample. However, no significant differences were found in the evaluation of SOD activity (Figure III.5.5.B). These results indicated that the tested Ayahuasca extracts have a good potential for minimizing oxidative damage in Caco-2 cells by increasing the activity of the GPx enzyme, though not affecting the SOD activity. In fact, Bourogaa *et al.* [52] evaluated the protective effects of the PH extract in the chronic treatment with ethanol. After treatment with PH extract, GPx activity increased [52]. The same results were verified for SOD activity [52]. These results can be justified by the antioxidant activity of the PH extract, which may be involved in an inhibition effect on damage caused by ROS, leading

to an increase in endogenous antioxidant activity [52]. In addition to the antioxidant activity attributed to the PH extract, it may also be involved in the scavenging of free radicals and inhibition of lipid peroxidation [52]. However, so far, no research has been done with MH samples, evaluating the activity of antioxidant enzymes.

Ayahuasca decoctions (MH, PH and MHPH) were able to induce apoptosis and reduce the viability and proliferative activity of Caco-2 cells. Additionally, it was demonstrated that oxidative stress levels decreased in the presence of extracts, and despite no significant differences were detected in SOD activity, the results of GPx activity suggest that the extracts can trigger a defence response against oxidative stress. A study previously carried out by our research group evaluated the phytochemical profile of these Ayahuasca extracts [9]. It was found that several compounds with antioxidant properties, such as protocatechuic acid, 4-hydroxybenzoic acid, salicylic acid, (+)-catechin[53], (-)-epicatechin [54], gentisic acid acid [55] are part of its composition. Therefore, it is likely to assume that these compounds may be involved in combating oxidative damage [9,36] justifying the results obtained herein .

3.5.3. Material and Methods

3.5.5.1. Plant Material and Preparation of Extracts

On May 25, 2019, we obtained vegetable samples via the internet from Shayana Shop (https://www.shayanashop.com, Amsterdam, The Netherlands). "World Flora Online" (www.worldfloraonline.org) was used to verify the full names of botanical plants. The five vegetal samples (commercial mixture, PV leaves, BC stem scraps, PH seeds, and MH root bark) were weighed in order to prepare ayahuasca decoctions. Following that, the plant material was milled in a mortar with a few drops of water before being moved and mixed with 250 mL of ultrapure water in a Schott flask. The mixture was then brought to a boil for 4 h at 100 °C. Similarly, four decoctions were prepared by mixing two of the selected plants (PV and PH (PVPH); PV and BC (PVBC); MH and PH (MHPH) MH and BC (MHBC)). Following filtration, the samples were frozen at 80 °C and then placed in the freeze dryer until they were completely freeze-dried.

3.5.3.2. Cell Culture and Treatment

Caco-2 cells were purchased from the American Type Culture Collection (ATCC) (Accession number: HTB-37) and maintained in Roswell Park Memorial Institute (RPMI) 1640 culture medium (Sigma-Aldrich, Sintra, Portugal), supplemented with 10% foetal bovine serum (FBS) and 1% antibiotic mixture (Sigma-Aldrich), at 37°C in an air incubator with a humidified atmosphere of 5% CO_2. For analysis of the effects of ayahuasca extracts on cell viability, Caco-2 cells were cultured in 96-well plates (cat. number 734-2802 Avantor, VWR, Amadora Portugal) with 1, 50, 250, 500, 750 and 1000 μg / mL of extract, prepared in culture medium, for 24 h. Apoptosis, cell proliferation (96-well plates) and oxidative stress (96-well plates) were assessed using extracts at 276.97 μg / mL

(concentration corresponding to the IC_{50} of the MHPH sample) for a treatment time of 24 h. For all assays RPMI medium was used as a negative control.

3.5.3.3. Cell Viability Assay

The MTT assay was used to analyse cell viability. This method consists in the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide into its insoluble formazan (Sigma-Aldrich). Cells were exposed to MTT until formazan crystals were obtained (3 to 4 hours), which were thereafter dissolved with 200 µL of dimethyl sulfoxide (DMSO). The absorbance at 570 nm was measured using the xMark™ microplate absorption spectrophotometer (Bio-Rad Laboratories, Hercules, CA). All experiments were performed in three independent assays [6].

3.5.3.4. Protein Extraction

By homogenizing the Caco-2 cells in the appropriate volume of radioimmunoprecipitation (RIPA) buffer—150 mM NaCl, 1% Nonidet-P40 substitute, 0.5% Nadeoxycholate, 0.1% SDS, 50 mM Tris pH 8.0, and 1 mM EDTA—supplemented with 10% PMSF and 1% protease inhibitor cocktail (Sigma-Aldrich), the total protein was extracted from the cells. The homogenates of cells were centrifuged at 14000 rpm for 20 min at 4°C after being on ice for 20 min and shaken periodically. Using the bicinchoninic acid (BCA) test (Thermo Fisher Scientific, Rockford, USA), the total protein content of the supernatant was determined [35].

3.5.3.5. Caspase-3 and Caspase-9 Activity Assays

The activity of Caspase-3 and Caspase-9 was determined using the Caspase-3 Assay Kit (Sigma-Aldrich) and Caspase-9 Colorimetric Assay Kit (Sigma-Aldrich), respectively. By quantifying the release of the p-nitroaniline chromophore group (pNA) through cleavage of their respective substrates (Ac-DEVD-pNA and LEHD-pNA, respectively), the activities were ascertained spectrophotometrically. Consequently, an appropriate volume of reaction buffer (25 mM HEPES, pH 7.5, 0.1% 3-[(3-cholamidopropyl) dimethylammonium]-1-propanesulfonate, 10% sucrose, and 10 mM dithiothreitol (DTT) containing 200 mM of substrate) was incubated with 3 µL of total protein extracts at 37°C overnight. An xMark™ microplate absorption spectrophotometer (Bio-Rad Laboratories, Hercules, CA) was used to measure the release of pNA at 405 nm. Caspases 3 and 9 activity was estimated by extrapolating the amount of released pNA using a standard free pNA curve [35].

3.5.3.6. Ki-67 Fluorescent Immunocytochemistry

After 10 min of paraformaldehyde (4%) fixation, Caco-2 cells were permeabilized for 5 min using Triton (1%) solution. Following this, cells were incubated for 1 h at room temperature in

phosphate buffer saline (PBS) with 0.1% Tween 20® (PBST) and 20% FBS as a blocking phase. Following a washing step, the cells were treated for 1 h at room temperature with the primary rabbit anti-Ki-67 antibody (1:50, no. 16667, Abcam, Cambridge, UK). Following that, cells were treated for another hour at room temperature with Alexa fluor 546 goat anti-rabbit IgG secondary antibody (1:500, Invitrogen). The specificity of the immunostaining was evaluated by excluding the primary antibody and staining the cell nuclei for 10 min with Hoechst 33342 (5 µg / mL, Invitrogen). Following a wash, Dako fluorescent mounting media (Dako, Glostrup, Denmark) was used to fix the coverslips on the slide. Images were acquired using a Zeiss LSM 710 confocal laser scanning microscope (Carl Zeiss, Göttingen, Germany), and the proliferation index was estimated by counting the number of Ki-67-positive cells and Hoechst-stained nuclei in 10 randomly selected fields for each section at 63x magnification. The ratio between the number of Ki-67-stained cells and the total number of nuclei was calculated [35].

3.5.3.7. Cellular Reactive Oxygen Species (ROS) Level Measurements

The ROS level was determined using two fluorescent probes: DCFDA (Sigma-Aldrich) and DHE, which measures cytosolic superoxide (Sigma-Aldrich). DCFDA is oxidized by ROS and transformed into fluorescent 2',7'-dichlorofluorescein, which is the basis for the DCFDA assay. Following 24 h exposure to Ayahuasca extracts, culture medium was removed, and cells were treated for 1 h at 37 °C with 50 µM DCFDA prepared in the culture medium. The emitted fluorescence was read in a spectrofluorometer (SpetroMax GeminiEM; Molecular Devices) at 485 (excitation) and 535 nm (emission) [56]. On the other hand, red fluorescent ethidium bromide is created when superoxide (O_2^-) dehydrogenates blue fluorescent DHE. Likewise, cells were treated with 100 µM DHE in culture media at 37°C for 20 min after being exposed to the stimuli for 24 h. A spectrofluorometer (SpetroMax GeminiEM) was used to measure the produced fluorescence (excitation 515 nm; emission 605 nm) [57].

3.5.3.8. Glutathione Peroxidase Assay

Using a commercial kit (Calbiochem, Darmstadt, Germany), GPx activity was assessed in accordance with the manufacturer's instructions. The method focuses on the oxidation of glutathione (GSH) to oxidized glutathione (GSSG), which occurs at a temperature of 25°C and is catalyzed by GPx. The actions of glutathione reductase (GR) and NADPH convert GSSG back into GSH. The absorbance at 340 nm (xMarkTM Microplate Absorbance Spectrophotometer (Bio-Rad)), which is proportional to GPx activity, decreases when NADPH is oxidized to NADP+ [35].

3.5.3.9. Superoxide Dismutase Assay

The commercial SOD Assay Kit-WST (Sigma-Aldrich) was used to measure SOD activity in accordance with the manufacturer's instructions. Briefly, the reduction of the WST-1 substrate (tetrazolium salt) with a superoxide anion results in the production of a water-soluble formazan colour. The activity of xanthine oxidase (inhibited by SOD) is linearly related to the rate of formazan production. The xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad) was used to measure the formazan production at 450 nm in relation to the amount of superoxide anion and the reduction reaction at 37°C. The percent inhibition rate of the reaction corresponds to the SOD activity [35].

3.5.3.10. Statistical Analysis

The statistical analyses were all carried out with GraphPad Prism8. Tukey's test was conducted after the results of the Student's *t* test or ANOVA to determine the statistical significance of the various groups. If $p < 0.05$(*), $p < 0.01$(**), $p < 0.001$(***), or $p < 0.0001$(****), there were significant differences. Every experimental result is displayed as mean ± S.E.M.

3.5.4. Conclusion

Ayahuasca decoctions were able to induce apoptosis and reduce the viability and proliferative activity of Caco-2 cells. Oxidative stress levels decreased in the presence of Ayahuasca extracts and, although no significant differences were observed in SOD activity, the results of GPx activity suggest that extracts can trigger a defence response against oxidative damage. The current findings have added to our understanding of the biological effects of Ayahuasca extracts, demonstrating the anticancer properties of this natural product. Moreover, this study opens new research lines to further explore the potential of Ayahuasca extracts as anticancer agents by in-depth studies, namely *in vivo* assays and clinical trials. Future studies should fully address the molecular mechanisms through which ayahuasca extracts exert their tumour suppressor effects by evaluating tumour-specific markers precursors of disease. Moreover, the anticancer potential of ayahuasca over other cell lines should be envisaged.

3.5.5. References

1. Houle, S.K.D.; Evans, D.; Carter, C.A.; Schlagenhauf, P. Ayahuasca and the traveller: A scoping review of risks and possible benefits. Travel Med. Infect. Dis. 2021, 44, doi:10.1016/J.TMAID.2021.102206.

2. Gonçalves, J.; Luís, Â.; Gallardo, E.; Duarte, A.P. A Systematic Review on the Therapeutic Effects of Ayahuasca. Plants 2023, 12, 2573, doi:10.3390/PLANTS12132573/S1.

3. Palhano-Fontes, F.; Soares, B.L.; Galvão-Coelho, N.L.; Arcoverde, E.; Araujo, D.B. Ayahuasca for the Treatment of Depression. Curr. Top. Behav. Neurosci. 2022, 56, 113–124, doi:10.1007/7854_2021_277/COVER.

4. Simão; Gonçalves; Duarte; Barroso; Cristóvão; Gallardo Toxicological Aspects and Determination of the Main Components of Ayahuasca: A Critical Review. Medicines 2019, 6, 106, doi:10.3390/medicines6040106.

5. Maia, L.O.; Daldegan-Bueno, D.; Wießner, I.; Araujo, D.B.; Tófoli, L.F. Ayahuasca's therapeutic potential: What we know - and what not. Eur. Neuropsychopharmacol. 2023, 66, 45–61, doi:10.1016/J.EURONEURO.2022.10.008.

6. Gonçalves, J.; Castilho, M.; Rosado, T.; Luís, Â.; Restolho, J.; Fernández, N.; Gallardo, E.; Duarte, A.P. In Vitro Study of the Bioavailability and Bioaccessibility of the Main Compounds Present in Ayahuasca Beverages. Molecules 2021, 26, doi:10.3390/MOLECULES26185555.

7. Barabasz-Gembczyk, A.; Kucia, K. Ayahuasca - potential therapeutic properties in psychiatry. Research review. Psychiatr. Pol. 2020, 54, 381–389, doi:10.12740/PP/103364.

8. Gonçalves, J.; Luís, Â.; Gallardo, E.; Duarte, A.P. Psychoactive Substances of Natural Origin: Toxicological Aspects, Therapeutic Properties and Analysis in Biological Samples. Molecules 2021, 26, 1397.

9. Gonçalves, J.; Luís, Â.; Gradillas, A.; García, A.; Restolho, J.; Fernández, N.; Domingues, F.; Gallardo, E.; Duarte, A.P. Ayahuasca Beverages: Phytochemical Analysis and Biological Properties. Antibiot. 2020, Vol. 9, Page 731 2020, 9, 731, doi:10.3390/ANTIBIOTICS9110731.

10. Gonçalves, J.; Luís, Â.; Gallardo, E.; Duarte, A.P. Evaluation of the In Vitro Wound-Healing Potential of Ayahuasca. Mol. 2022, Vol. 27, Page 5760 2022, 27, 5760, doi:10.3390/MOLECULES27185760.

11. Schenberg, E.E. Ayahuasca and cancer treatment. SAGE open Med. 2013, 1, 205031211350838, doi:10.1177/2050312113508389.

12. Gupta, R.; Bhatt, L.K.; Johnston, T.P.; Prabhavalkar, K.S. Colon cancer stem cells: Potential target for the treatment of colorectal cancer. Cancer Biol. Ther. 2019, 20, 1068, doi:10.1080/15384047.2019.1599660.

13. Arnold, M.; Sierra, M.S.; Laversanne, M.; Soerjomataram, I.; Jemal, A.; Bray, F. Global patterns and trends in colorectal cancer incidence and mortality. Gut 2017, 66, 683–691, doi:10.1136/GUTJNL-2015-310912/-/DC1.

14. Han, L.; Shi, S.; Gong, T.; Zhang, Z.; Sun, X. Cancer stem cells: therapeutic implications and perspectives in cancer therapy. Acta Pharm. Sin. B 2013, 3, 65–75, doi:10.1016/J.APSB.2013.02.006.

15. Simão, A.Y.; Gonçalves, J.; Caramelo, D.; Rosado, T.; Barroso, M.; Restolho, J.; Fernández, N.; Rodilla, J.; Duarte, A.P.; Cristóvão, A.C.; et al. Determination of N,N-dimethyltryptamine and beta-carbolines in plants used to prepare ayahuasca beverages by means of solid-phase extraction and gas-chromatography–mass spectrometry. SN Appl. Sci. 2020, 2, 1–11, doi:10.1007/S42452-020-2296-0/TABLES/6.

16. Van Breemen, R.B.; Li, Y. Caco-2 cell permeability assays to measure drug absorption. Expert Opin. Drug Metab. Toxicol. 2005, 1, 175–185, doi:10.1517/17425255.1.2.175.

17. Katchborian-Neto, A.; Santos, M.F.C.; Vilas-Boas, D.F.; dos Santos, E.G.; Veloso, M.P.; Bueno, P.C.P.; Caldas, I.S.; Soares, M.G.; Dias, D.F.; Chagas-Paula, D.A. Immunological Modulation and Control of Parasitaemia by Ayahuasca Compounds: Therapeutic Potential for Chagas's Disease. Chem. Biodivers. 2022, 19, e202200409, doi:10.1002/CBDV.202200409.

18. Hanahan, D.; Weinberg, R.A. Hallmarks of cancer: The next generation. Cell 2011, 144, 646–674, doi:10.1016/J.CELL.2011.02.013/ATTACHMENT/68024D79-3A9C-46C4-930B-640934F11E2E/MMC1.PDF.

19. Porter, A.G.; Jänicke, R.U. Emerging roles of caspase-3 in apoptosis. Cell Death Differ. 1999 62 1999, 6, 99–104, doi:10.1038/sj.cdd.4400476.

20. Cardoso, H.J.; Figueira, M.I.; Vaz, C. V.; Carvalho, T.M.A.; Brás, L.A.; Madureira, P.A.; Oliveira, P.J.; Sardão, V.A.; Socorro, S. Glutaminolysis is a metabolic route essential for survival and growth of prostate cancer cells and a target of 5α-dihydrotestosterone regulation. Cell. Oncol. (Dordr). 2021, 44, 385–403, doi:10.1007/S13402-020-00575-9.

21. Cardoso, H.J.; Vaz, C. V.; Carvalho, T.M.A.; Figueira, M.I.; Socorro, S. Tyrosine kinase inhibitor imatinib modulates the viability and apoptosis of castrate-resistant prostate cancer cells dependently on the glycolytic environment. Life Sci. 2019, 218, 274–283, doi:10.1016/J.LFS.2018.12.055.

22. Hashemi Sheikh Shabani, S.; Seyed Hasan Tehrani, S.; Rabiei, Z.; Tahmasebi Enferadi, S.; Vannozzi, G.P. Peganum harmala L.'s anti-growth effect on a breast cancer cell line. Biotechnol. reports (Amsterdam, Netherlands) 2015, 8, 138–143, doi:10.1016/J.BTRE.2015.08.007.

23. Yao, P.; Yao, P.; Ku, X.; Yang, J. Harmine suppresses the malignant phenotypes and PI3K activity in breast cancer. Anticancer. Drugs 2023, 34, 373–383, doi:10.1097/CAD.0000000000001462.

24. Li, C.; Wang, Y.; Wang, C.; Yi, X.; Li, M.; He, X. Anticancer activities of harmine by inducing a pro-death autophagy and apoptosis in human gastric cancer cells. Phytomedicine 2017, 28, 10–18, doi:10.1016/J.PHYMED.2017.02.008.

25. Zhang, H.; Sun, K.; Ding, J.; Xu, H.; Zhu, L.; Zhang, K.; Li, X.; Sun, W. Harmine induces apoptosis and inhibits tumor cell proliferation, migration and invasion through down-regulation of cyclooxygenase-2 expression in gastric cancer. Phytomedicine 2014, 21, 348–355, doi:10.1016/J.PHYMED.2013.09.007.

26. Wang, Y.; Wang, C.; Jiang, C.; Zeng, H.; He, X. Novel mechanism of harmaline on inducing G2/M cell cycle arrest and apoptosis by up-regulating Fas/FasL in SGC-7901 cells. Sci. Rep. 2015, 5, doi:10.1038/SREP18613.

27. Hamsa, T.P.; Kuttan, G. Harmine activates intrinsic and extrinsic pathways of apoptosis in B16F-10 melanoma. Chin. Med. 2011, 6, doi:10.1186/1749-8546-6-11.

28. Liu, J.; Li, Q.; Liu, Z.; Lin, L.; Zhang, X.; Cao, M.; Jiang, J. Harmine induces cell cycle arrest and mitochondrial pathway-mediated cellular apoptosis in SW620 cells via inhibition of the Akt and ERK signaling pathways. Oncol. Rep. 2016, 35, 3363–3370, doi:10.3892/OR.2016.4695.

29. Shen, J.; Wang, B.; Zhang, T.; Zhu, N.; Wang, Z.; Jin, J.; He, Y.; Hu, M. Suppression of Non-Small Cell Lung Cancer Growth and Metastasis by a Novel Small Molecular Activator of RECK. Cell. Physiol. Biochem. 2018, 45, 1807–1817, doi:10.1159/000487872.

30. Vahedi, M.M.; Shahini, A.; Mottahedi, M.; Garousi, S.; Shariat Razavi, S.A.; pouyamanesh, G.; Afshari, A.R.; Ferns, G.A.; Bahrami, A. Harmaline exerts potentially anti-cancer effects on U-87 human malignant glioblastoma cells in vitro. Mol. Biol. Rep. 2023, 50, 4357–4366, doi:10.1007/S11033-023-08354-Z.

31. Xu, B.; Li, M.; Yu, Y.; He, J.; Hu, S.; Pan, M.; Lu, S.; Liao, K.; Pan, Z.; Zhou, Y.; et al. Effects of harmaline on cell growth of human liver cancer through the p53/p21 and Fas/FasL signaling pathways. Oncol. Lett. 2018, 15, 1931, doi:10.3892/OL.2017.7495.

32. Sarkar, S.; Bhattacharjee, P.; Bhadra, K. DNA binding and apoptotic induction ability of harmalol in HepG2: Biophysical and biochemical approaches. Chem. Biol. Interact. 2016, 258, 142–152, doi:10.1016/J.CBI.2016.08.024.

33. Roy, S.; Mohammad, T.; Gupta, P.; Dahiya, R.; Parveen, S.; Luqman, S.; Hasan, G.M.; Hassan, M.I. Discovery of Harmaline as a Potent Inhibitor of Sphingosine Kinase-1: A Chemopreventive Role in Lung Cancer. ACS Omega 2020, 5, 21550–21560, doi:10.1021/ACSOMEGA.0C02165/ASSET/IMAGES/MEDIUM/AO0C02165_M007.GIF.

34. Ghosh, T.; Sarkar, S.; Bhattacharjee, P.; Jana, G.C.; Hossain, M.; Pandya, P.; Bhadra, K. In vitro relationship between serum protein binding to beta-carboline alkaloids: a comparative cytotoxic, spectroscopic and calorimetric assays. J. Biomol. Struct. Dyn. 2020, 38, 1103–1118, doi:10.1080/07391102.2019.1595727.

35. Silva, G.R.; Vaz, C. V.; Catalão, B.; Ferreira, S.; Cardoso, H.J.; Duarte, A.P.; Socorro, S. Sweet Cherry Extract Targets the Hallmarks of Cancer in Prostate Cells: Diminished Viability, Increased Apoptosis and Suppressed Glycolytic Metabolism. Nutr. Cancer 2020, 72, 917–931, doi:10.1080/01635581.2019.1661502.

36. Elansary, H.O.; Szopa, A.; Kubica, P.; Ekiert, H.; Al-Mana, F.A.; El-Shafei, A.A. Polyphenols of Frangula alnus and Peganum harmala Leaves and Associated Biological Activities. Plants (Basel, Switzerland) 2020, 9, 1–15, doi:10.3390/PLANTS9091086.

37. Gerdes, J.; Lemke, H.; Baisch, H.; Wacker, H.H.; Schwab, U.; Stein, H. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J. Immunol. 1984, 133, 1710–1715, doi:10.4049/JIMMUNOL.133.4.1710.

38. Wang, C.; Zhang, Z.; Wang, Y.; He, X. Cytotoxic Constituents and Mechanism from Peganum harmala. Chem. Biodivers. 2016, 13, 961–968, doi:10.1002/CBDV.201500384.

39. Zhang, Y.; Shi, X.; Xie, X.; Laster, K.V.; Pang, M.; Liu, K.; Hwang, J.; Kim, D.J. Harmaline isolated from Peganum harmala suppresses growth of esophageal squamous cell carcinoma through targeting mTOR. Phyther. Res. 2021, 35, 6377–6388, doi:10.1002/PTR.7289.

40. Hamsa, T.P.; Kuttan, G. Studies on Anti-metastatic and Anti-invasive Effects of Harmine Using Highly Metastatic Murine B16F-10 Melanoma Cells. J. Environ. Pathol. Toxicol. Oncol. 2011, 30, 123–137, doi:10.1615/JENVIRONPATHOLTOXICOLONCOL.V30.I2.40.

41. Lamchouri, F.; Settaf, A.; Cherrah, Y.; Zemzami, M.; Lyoussi, B.; Zaid, A.; Atif, N.; Hassar, M. Antitumour principles from Peganum harmala seeds. Therapie 1999, 54, 753–758.

42. Lamchouri, F.; Settaf, A.; Cherrah, Y.; Hassar, M.; Zemzami, M.; Atif, N.; Nadori, E.B.; Zaid, A.; Lyoussi, B. In vitro cell-toxicity of Peganum harmala alkaloids on cancerous cell-lines. Fitoterapia 2000, 71, 50–54, doi:10.1016/S0367-326X(99)00117-3.

43. Halliwell, B.; Chirico, S.; Crawford, M.A.; Bjerve, K.S.; Gey, K.F. Lipid peroxidation: its mechanism, measurement, and significance. Am. J. Clin. Nutr. 1993, 57, doi:10.1093/AJCN/57.5.715S.

44. Perillo, B.; Di Donato, M.; Pezone, A.; Di Zazzo, E.; Giovannelli, P.; Galasso, G.; Castoria, G.; Migliaccio, A. ROS in cancer therapy: the bright side of the moon. Exp. Mol. Med. 2020, 52, 192, doi:10.1038/S12276-020-0384-2.

45. Klaunig, J.E.; Kamendulis, L.M.; Hocevar, B.A. Oxidative Stress and Oxidative Damage in Carcinogenesis. http://dx.doi.org/10.1177/0192623309356453 2009, 38, 96–109, doi:10.1177/0192623309356453.

46. Azmanova, M.; Pitto-Barry, A. Oxidative Stress in Cancer Therapy: Friend or Enemy? ChemBioChem 2022, 23, e202100641, doi:10.1002/CBIC.202100641.

47. Hayes, J.D.; Dinkova-Kostova, A.T.; Tew, K.D. Oxidative Stress in Cancer. Cancer Cell 2020, 38, 167, doi:10.1016/J.CCELL.2020.06.001.

48. Xing, F.; Hu, Q.; Qin, Y.; Xu, J.; Zhang, B.; Yu, X.; Wang, W. The Relationship of Redox With Hallmarks of Cancer: The Importance of Homeostasis and Context. Front. Oncol. 2022, 12, doi:10.3389/FONC.2022.862743.

49. Gupta, R.K.; Patel, A.K.; Kumari, R.; Chugh, S.; Shrivastav, C.; Mehra, S.; Sharma, A.N. Interactions between oxidative stress, lipid profile and antioxidants in breast cancer: a case control study. Asian Pac. J. Cancer Prev. 2012, 13, 6295–6298, doi:10.7314/APJCP.2012.13.12.6295.

50. Badjatia, N.; Satyam, A.; Singh, P.; Seth, A.; Sharma, A. Altered antioxidant status and lipid peroxidation in Indian patients with urothelial bladder carcinoma. Urol. Oncol. 2010, 28, 360–367, doi:10.1016/J.UROLONC.2008.12.010.

51. Balasubramaniyan, N.; Subramanian, S.; Govindasamy, S. Status of antioxidant systems in human carcinoma of uterine cervix. Cancer Lett. 1994, 87, 187–192, doi:10.1016/0304-3835(94)90221-6.

52. Bourogaa, E.; Jarraya, R.M.; Damak, M.; Elfeki, A. Hepatoprotective activity of Peganum harmala against ethanol-induced liver damages in rats. Arch. Physiol. Biochem. 2015, 121, 62–67, doi:10.3109/13813455.2015.1016974.

53. Van Hung, P. Phenolic Compounds of Cereals and Their Antioxidant Capacity. Crit. Rev. Food Sci. Nutr. 2016, 56, 25–35, doi:10.1080/10408398.2012.708909.

54. Ruiz-Riaguas, A.; Fernández-de Córdova, M.L.; Llorent-Martínez, E.J. Phenolic profile and antioxidant activity of Euonymus japonicus Thunb. Nat. Prod. Res. 2022, 36, 3445–3449, doi:10.1080/14786419.2020.1855641.

55. Khatib, M. Al; Costa, J.; Spinelli, D.; Capecchi, E.; Saladino, R.; Baratto, M.C.; Pogni, R. Homogentisic Acid and Gentisic Acid Biosynthesized Pyomelanin Mimics: Structural Characterization and Antioxidant Activity. Int. J. Mol. Sci. 2021, 22, 1–13, doi:10.3390/IJMS22041739.

56. Wojtala, A.; Bonora, M.; Malinska, D.; Pinton, P.; Duszynski, J.; Wieckowski, M.R. Methods to monitor ROS production by fluorescence microscopy and fluorometry. Methods Enzymol. 2014, 542, 243–262, doi:10.1016/B978-0-12-416618-9.00013-3.

57. Rocha, S.M.; Saraiva, T.; Cristóvão, A.C.; Ferreira, R.; Santos, T.; Esteves, M.; Saraiva, C.; Je, G.; Cortes, L.; Valero, J.; et al. Histamine induces microglia activation and dopaminergic neuronal toxicity via H1 receptor activation. J. Neuroinflammation 2016, 13, doi:10.1186/S12974-016-0600-0.

3.6. Paper VIII- The role of ayahuasca in cell viability and oxidative stress in gastric adenocarcinoma cell line

Abstract

Ayahuasca, a psychoactive beverage native to the Amazon, originally derived from *Banisteriopsis caapi* stem scrapings and *Psychotria viridis* leaves, exhibits hallucinogenic properties due to *N,N*-dimethyltryptamine. When combined with β-carbolines, it enters the bloodstream and central nervous system, inhibiting monoamine oxidase-A. Over time, therapeutic effects have been associated to ayahuasca consumption. This study assessed the impact of extracts from three plant decoctions used in ayahuasca preparation on the gastric adenocarcinoma cell line (AGS). MTT reduction assays selected *B. caapi*, *Mimosa hostilis*, and *Peganum harmala* samples as most effective. Lactate dehydrogenase activity evaluated membrane integrity loss, while oxidative stress induction was measured using dihydroethidium and 2',7'-dichlorodihydrofluorescein diacetate probes. Results revealed apoptosis induction in AGS cells, with all three samples significantly reducing oxidative stress.

Keywords: ayahuasca, AGS, apoptosis, oxidative stress

3.6.1. Introduction

Ayahuasca, a psychoactive beverage native to the Amazon, originally derived from *Banisteriopsis caapi* stem scrapings and *Psychotria viridis* leaves, exhibits hallucinogenic properties due to *N,N*-dimethyltryptamine. When combined with β-carbolines, it enters the bloodstream and central nervous system, inhibiting monoamine oxidase-A. Over time, therapeutic effects have been associated to ayahuasca consumption. This study assessed the impact of extracts from three plant decoctions used in ayahuasca preparation on the gastric adenocarcinoma cell line (AGS). MTT reduction assays selected *B. caapi*, *Mimosa hostilis*, and *Peganum harmala* samples as most effective. Lactate dehydrogenase activity evaluated membrane integrity loss, while oxidative stress induction was measured using dihydroethidium and 2',7'-dichlorodihydrofluorescein diacetate probes. Results revealed apoptosis induction in AGS cells, with all three samples significantly reducing oxidative stress.

3.6.2. Results and Discussion

The materials and methods are described in the supplementary material (Supplementary information). Table III.6.1. summarizes the results of the 3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide (MTT) reduction assay. *P. harmala* had the lowest IC_{50}, followed by *B. caapi* and *M. hostilis*, indicating their potency in reducing AGS cell viability at lower concentrations. Thus, these samples were chosen to pursue the work.

Table III.6.1. IC_{50} values of ayahuasca extracts after 24h of incubation with AGS cells.

Sample	IC_{50} (μg/mL) ± SD
P. viridis	419.35 ± 0.04
B. caapi	245.02 ± 0.02
P. harmala	122.76 ± 0.02
M. hostilis	275.55 ± 0.06
P. viridis* + *B. caapi	283.65 ± 0.05
P. viridis* + *P. harmala	379.12 ± 0.01
M. hostilis* + *B. caapi	385.68 ± 0.02
M. hostilis* + *P. harmala	477.27 ± 0.01

Membrane integrity was assessed by measuring LDH activity released into the medium, as depicted in Figure III.6.1. Overall, there was a noticeable rise in released LDH activity, suggesting an apoptosis/necrosis increase in cells after exposure to the three extracts, however only in the *P. harmala* sample was this increase statistically significant. To our knowledge, there are no studies where LDH activity has been evaluated after exposure to these extracts. Several studies have assessed the induction of apoptosis, compromising the integrity of the cell membrane, after exposure to *P. harmala* extracts e.g. [9–13], *P. harmala* also triggered necrosis in gastric cancer cells and in tumour-bearing mice [14]. However, the effects of *B. caapi* and *M. hostilis* extracts on inducing necrosis and apoptosis has not been explored before.

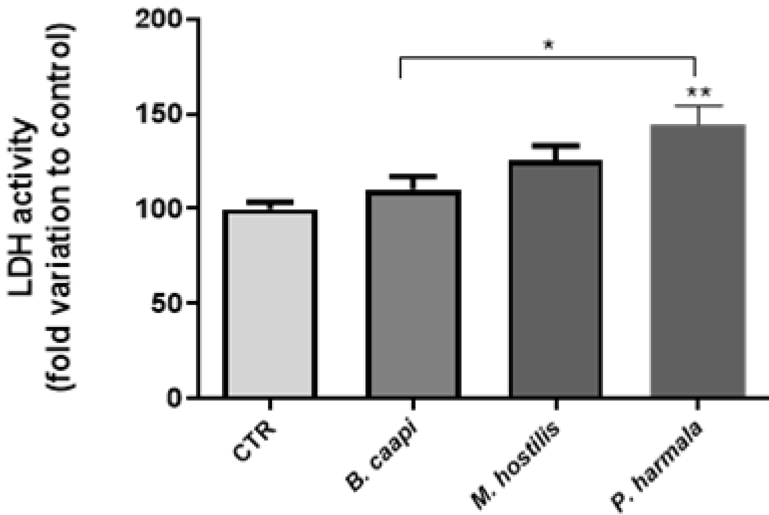


Figure III.6.1. LDH released by AGS cells after treatment with extracts at concentrations corresponding to IC_{50} values obtained in the MTT assay (n=4). Error bars indicate mean ± SD. $p < 0.05$(*), $p < 0.01$(**) when compared with control.

Reactive oxygen species (ROS) levels were assessed using two methods, as illustrated in Figures III.6.2. and III.6.3.

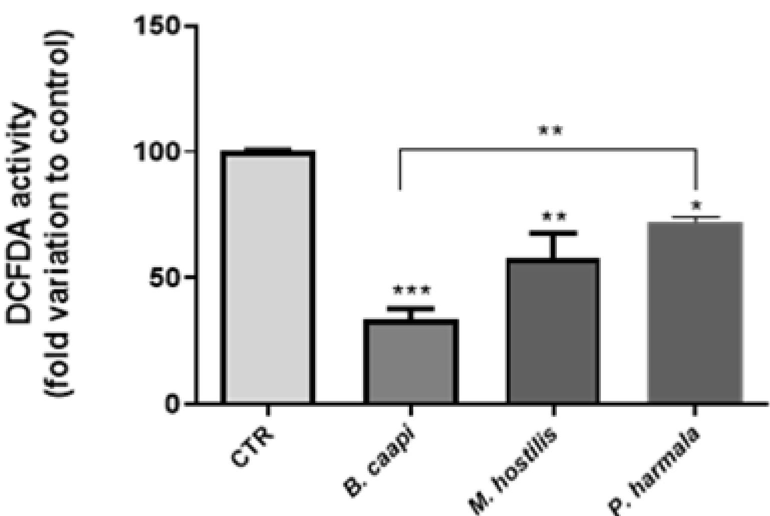


Figure III.6.2. Cellular ROS production induced after treatment with IC_{50} values extracts for 24 h, measured using DCFDA. Results are expressed as fold variation relatively to the control untreated group. Error bars indicate mean ± SD. $p < 0.05$(*), $p < 0.01$(**) and $p < 0.001$(***).

All extracts demonstrated the ability to significantly decrease oxidative stress in both assays. Increased ROS levels are often associated with the development of cancer [15,16].

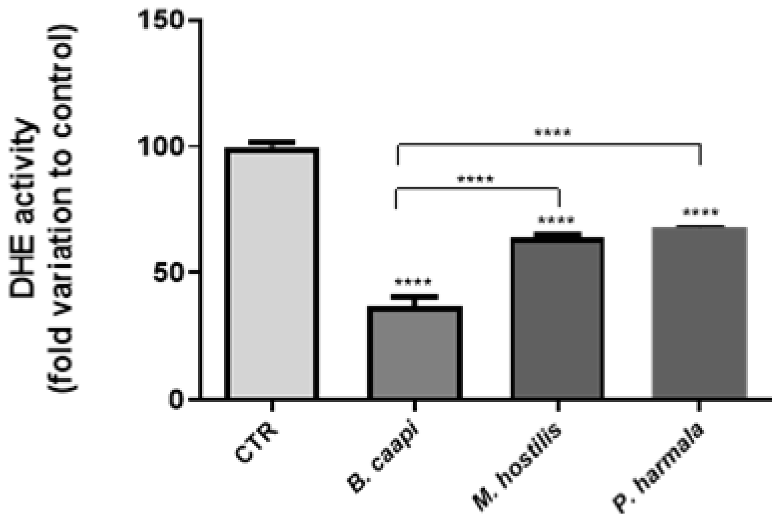


Figure III.6.3. Cellular ROS production induced after treatment with IC_{50} values extracts for 24 h, measured using DHE. Results are expressed as fold variation relatively to the control untreated group. Error bars indicate mean ± SD. $p < 0.0001$(****).

However, the assessment of ROS levels in cancer cell lines post-exposure to *P. harmala*, *M. hostilis* and *B. caapi* extracts has not been investigated. In gastric cancer, ROS generated by NOX4 stimulate cell proliferation by inducing GLI1 expression [17]. Additionally, we must consider that ROS and oxidative stress compromise lipids, which are the main macromolecules present in the constitution of cell membranes [16]. These macromolecules are highly sensitive to states of

oxidative stress and the damage that may be caused to them can compromise the integrity of cells [18](Figure III.6.4). Thus, ROS have been considered in cancer therapy [19]. However, increased levels of ROS are also associated with cancer development [15]. Thus, the observed reduction in basal ROS may contribute to the anticancer effects of the extracts. Nevertheless, alternative mechanisms cannot be discarded. Previous studies identified antioxidant compounds in the extracts used in the present study, suggesting their role in combating oxidative damage [5,20]. *P. harmala* extract contained protocatechuic acid, 4-hydroxybenzoic acid, gentilic acid, salicylic acid, (+)-catechin, (-)-epicatechin, and quercetin-3-*O*-retinoside. *B. caapi* extract featured protocatechuic acid, salicylic acid, (+)-catechin, (-)-epicatechin, quercetin-3-*O*-glucoside, quercetin-3-*O*-rutinoside, and phlorizin. *M. hostilis* extract included protocatechuic, 4-hydroxybenzoic, and salicylic acids, along with two flavonoids ((+)-catechin and (-)-epicatechin) [5]. Studies suggest these components play crucial roles in reducing ROS, which might justify the results observed in the present study. [3,4,21,22].

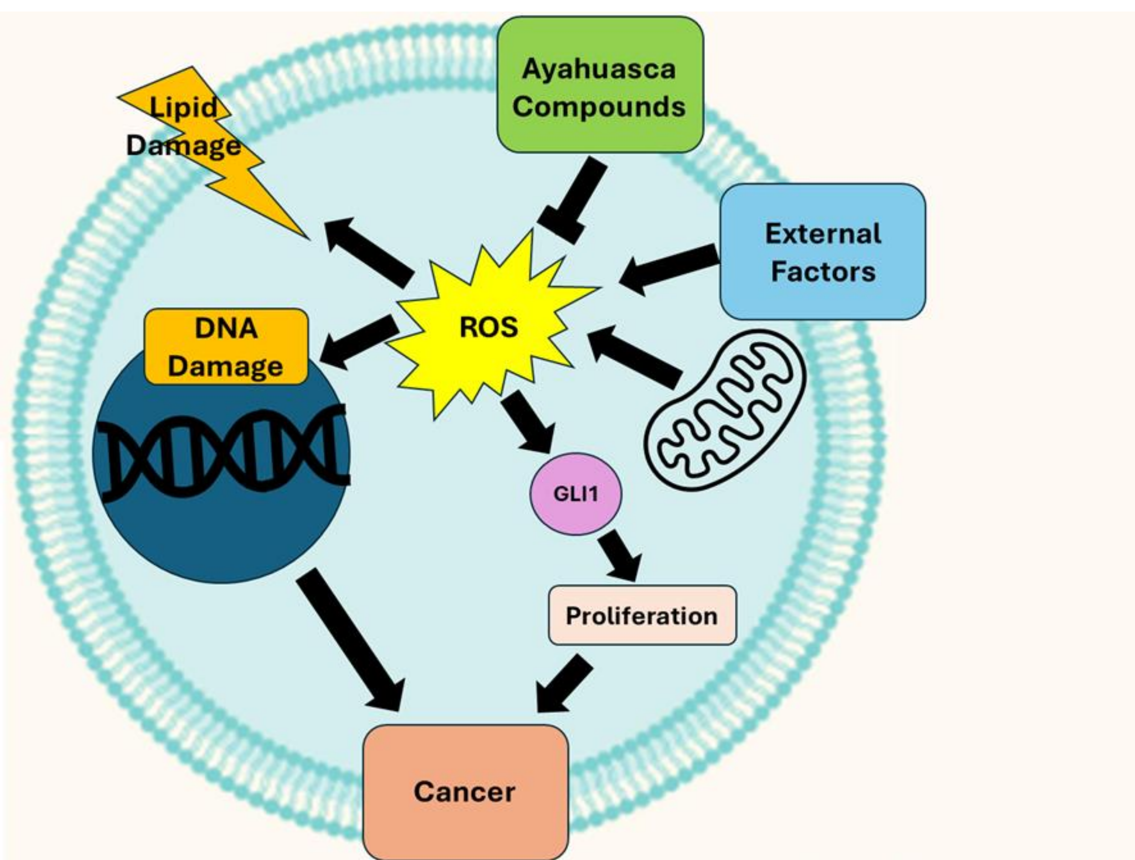


Figure III.6.4. Mechanism of action of ayahuasca in reducing oxidative damage. ROS produced by external factors and mitochondria are inhibited by the compounds present in ayahuasca. Consequently, lipid damage and other damages that can cause cancer development are inhibited.

3.6.3 Materials and Methods

3.6.3.1. Plant material preparation

Vegetal samples were acquired online from Shayana Shop (https://www.shayanashop.com, Amsterdam, The Netherlands) (accessed on 25th May 2019). Ayahuasca decoctions were prepared individually by weighing and milleding 0.210 g of *P. viridis*, *B. caapi*, *P. harmala*, or *M. hostilis* with water. After that, the mixture was transferred to a Schott flask, 250 mL of ultrapure water were added, and the preparation was boiled at 100°C for 4 h. Similarly, four decoctions were prepared where two of the above plant samples were mixed (*P. viridis* and *B. caapi*; *P. viridis* and *P. harmala*; *M. hostilis* and *B. caapi*; *M. hostilis* and *P. harmala*). Afterwards, the resulting extracts were filtered, frozen at 80°C and lyophilized.

3.6.3.2. Cell culture and treatment

Gastric adenocarcinoma (AGS) cells were purchased from the American Type Culture Collection (ATCC) (LGC Standards S.L.U., Spain) and maintained in Dulbecco's Modified Eagle Medium (DMEM) with GlutaMAX (Gibco Laboratories; Lenexa, KS), supplemented with 10% foetal bovine serum and 1% antibiotic mixture (Penicillin-Streptomycin and Antibiotic Antimycotic) (Sigma-Aldrich, Sintra, Portugal), at 37 °C in an incubator with a humidified atmosphere containing 5% CO_2. For cell viability assays, the AGS cells were cultured in 96-well plates (cat. number 734-2802 Avantor, VWR, Amadora Portugal) with 1, 50, 250, 500, 750 and 1000 µg/mL of extract solution for 24 h and the IC_{50} for each extract calculated. To evaluate the effects of ayahuasca extract on oxidative stress, the cells were cultured in 96-well plates and the extracts were incubated for 24 h at the concentration corresponding to the IC_{50}.

3.6.3.3. MTT Reduction Assay

Formed formazan crystals, resulting from MTT reduction (Sigma-Aldrich, Sintra, Portugal), were dissolved and homogenized in 200 µL of dimethyl sulfoxide (DMSO). Absorbance was measured at 570 nm using an xMarkTM microplate absorption spectrophotometer (Bio-Rad Laboratories, Hercules, CA). All experiments were conducted in three independent assays.

3.6.3.4. Lactate desidrogenase (LDH) assay

After incubating samples with the plant extracts, culture medium aliquots were collected to assess LDH activity. Measurement occurred at room temperature using a commercial kit (CyQUANT™ LDH Cytotoxicity Assay Kit, Thermo Fisher Scientific, Alfagene, Portugal) as per the manufacturer's instructions. The assay was conducted in four independent trials, and LDH activity was calculated using a follow equation:

$$\% \text{ Cytotoxicity} = ((\text{Abs}_{\text{Sample}} - \text{Blank})/(\text{Abs}_{\text{Control}} - \text{Blank})) \times 100$$

The blank consisted in phosphate buffered saline (PBS).

3.6.3.5. Cellular Reactive Oxygen Species (ROS) levels

ROS levels were assessed using fluorescent probes, DHE and DCFDA. Cells were stimulated for 24 h with plant extracts, then incubated for 20 min with 100 μM DHE at 37 °C and red fluorescence was measured (excitation 515 nm; emission 605 nm). The DCFDA assay involved exposing cells to ayahuasca extracts for 24 h, followed by a 1-hour incubation with 50 μM DCFDA at 37 °C, measuring fluorescence at 485 (excitation) and 535 nm (emission). Assays were conducted in four independent trials.

3.6.3.6. Statistical Analysis

The statistical significance of the different groups was assessed using the Student's *t* test or ANOVA, followed by the Tukey test. Significant differences were considered when $p < 0.05$(*), $p < 0.01$(**), $p < 0.001$(***) or $p < 0.0001$(****). All experimental data are shown as mean ± SD.

3.6.4. Conclusion

Ayahuasca extracts from *P. harmala*, *B. caapi*, and *M. hostilis* induced cytotoxicity in AGS cells, evidenced by membrane damage and reduced viability. Notably, the extracts significantly reduced oxidative stress, possibly due to their phytochemical composition. These results provide an initial insight into ayahuasca's biological effects on AGS cells.

3.6.5. References

1. Gonçalves, J.; Luís, Â.; Gallardo, E.; Duarte, A.P. A Systematic Review on the Therapeutic Effects of Ayahuasca. *Plants* **2023**, *12*, 2573, doi:10.3390/PLANTS12132573/S1.
2. Van Hung, P. Phenolic Compounds of Cereals and Their Antioxidant Capacity. *Crit. Rev. Food Sci. Nutr.* **2016**, *56*, 25–35, doi:10.1080/10408398.2012.708909.
3. Ruiz-Riaguas, A.; Fernández-de Córdova, M.L.; Llorent-Martínez, E.J. Phenolic profile and antioxidant activity of Euonymus japonicus Thunb. *Nat. Prod. Res.* **2022**, *36*, 3445–3449, doi:10.1080/14786419.2020.1855641.
4. Khatib, M. Al; Costa, J.; Spinelli, D.; Capecchi, E.; Saladino, R.; Baratto, M.C.; Pogni, R. Homogentisic Acid and Gentisic Acid Biosynthesized Pyomelanin Mimics: Structural Characterization and Antioxidant Activity. *Int. J. Mol. Sci.* **2021**, *22*, 1–13, doi:10.3390/IJMS22041739.
5. Gonçalves, J.; Luís, Â.; Gradillas, A.; García, A.; Restolho, J.; Fernández, N.; Domingues, F.; Gallardo, E.; Duarte, A.P. Ayahuasca Beverages: Phytochemical Analysis and Biological Properties. *Antibiot. 2020, Vol. 9, Page 731* **2020**, *9*, 731, doi:10.3390/ANTIBIOTICS9110731.
6. Yin, W.; Wang, J.; Jiang, L.; James Kang, Y. Cancer and stem cells. *Exp. Biol. Med.* **2021**, *246*, 1791, doi:10.1177/15353702211005390.
7. Thrift, A.P.; El-Serag, H.B. Burden of Gastric Cancer. *Clin. Gastroenterol. Hepatol.* **2020**, *18*, 534, doi:10.1016/J.CGH.2019.07.045.

8. Schenberg, E.E. Ayahuasca and cancer treatment. *SAGE open Med.* **2013**, *1*, 205031211350838, doi:10.1177/2050312113508389.

9. Zhang, H.; Sun, K.; Ding, J.; Xu, H.; Zhu, L.; Zhang, K.; Li, X.; Sun, W. Harmine induces apoptosis and inhibits tumor cell proliferation, migration and invasion through down-regulation of cyclooxygenase-2 expression in gastric cancer. *Phytomedicine* **2014**, *21*, 348–355, doi:10.1016/J.PHYMED.2013.09.007.

10. Wang, Y.; Wang, C.; Jiang, C.; Zeng, H.; He, X. Novel mechanism of harmaline on inducing G2/M cell cycle arrest and apoptosis by up-regulating Fas/FasL in SGC-7901 cells. *Sci. Rep.* **2015**, *5*, doi:10.1038/SREP18613.

11. Li, C.; Wang, Y.; Wang, C.; Yi, X.; Li, M.; He, X. Anticancer activities of harmine by inducing a pro-death autophagy and apoptosis in human gastric cancer cells. *Phytomedicine* **2017**, *28*, 10–18, doi:10.1016/J.PHYMED.2017.02.008.

12. Hashemi Sheikh Shabani, S.; Seyed Hasan Tehrani, S.; Rabiei, Z.; Tahmasebi Enferadi, S.; Vannozzi, G.P. *Peganum harmala* L.'s anti-growth effect on a breast cancer cell line. *Biotechnol. reports (Amsterdam, Netherlands)* **2015**, *8*, 138–143, doi:10.1016/J.BTRE.2015.08.007.

13. Vahedi, M.M.; Shahini, A.; Mottahedi, M.; Garousi, S.; Shariat Razavi, S.A.; pouyamanesh, G.; Afshari, A.R.; Ferns, G.A.; Bahrami, A. Harmaline exerts potentially anti-cancer effects on U-87 human malignant glioblastoma cells in vitro. *Mol. Biol. Rep.* **2023**, *50*, 4357–4366, doi:10.1007/S11033-023-08354-Z.

14. Fan, Y.; Zeng, F.; Ma, L.; Zhang, H. Effects of β-carboline alkaloids from *Peganum harmala* on the FAK/PI3K/AKT/Mtor pathway in human gastric cancer cell line SGC-7901 and tumor-bearing mice. *Pak. J. Pharm. Sci.* **2021**, *34*, 891–898, doi:10.36721/PJPS.2021.34.3.REG.891-898.1.

15. Klaunig, J.E.; Kamendulis, L.M.; Hocevar, B.A. Oxidative Stress and Oxidative Damage in Carcinogenesis. *http://dx.doi.org/10.1177/0192623309356453* **2009**, *38*, 96–109, doi:10.1177/0192623309356453.

16. Silva, G.R.; Vaz, C. V.; Catalão, B.; Ferreira, S.; Cardoso, H.J.; Duarte, A.P.; Socorro, S. Sweet Cherry Extract Targets the Hallmarks of Cancer in Prostate Cells: Diminished Viability, Increased Apoptosis and Suppressed Glycolytic Metabolism. *Nutr. Cancer* **2020**, *72*, 917–931, doi:10.1080/01635581.2019.1661502.

17. Tang, C.T.; Lin, X.L.; Wu, S.; Liang, Q.; Yang, L.; Gao, Y.J.; Ge, Z.Z. NOX4-driven ROS formation regulates proliferation and apoptosis of gastric cancer cells through the GLI1 pathway. *Cell. Signal.* **2018**, *46*, 52–63, doi:10.1016/J.CELLSIG.2018.02.007.

18. Halliwell, B.; Chirico, S.; Crawford, M.A.; Bjerve, K.S.; Gey, K.F. Lipid peroxidation: its mechanism, measurement, and significance. *Am. J. Clin. Nutr.* **1993**, *57*, doi:10.1093/AJCN/57.5.715S.

19. Perillo, B.; Di Donato, M.; Pezone, A.; Di Zazzo, E.; Giovannelli, P.; Galasso, G.; Castoria, G.; Migliaccio, A. ROS in cancer therapy: the bright side of the moon. *Exp. Mol. Med.* **2020**, *52*, 192, doi:10.1038/S12276-020-0384-2.

20. Elansary, H.O.; Szopa, A.; Kubica, P.; Ekiert, H.; Al-Mana, F.A.; El-Shafei, A.A. Polyphenols of *Frangula alnus* and *Peganum harmala* Leaves and Associated Biological Activities. *Plants (Basel, Switzerland)* **2020**, *9*, 1–15, doi:10.3390/PLANTS9091086.

21. C. Maiyo, F.; Moodley, R.; Singh, M. Cytotoxicity, Antioxidant and Apoptosis Studies of Quercetin-3-*O* Glucoside and 4-(β-D-Glucopyranosyl-1→4-α-L-Rhamnopyranosyloxy)-Benzyl Isothiocyanate from Moringa oleifera. *Anticancer. Agents Med. Chem.* **2016**, *16*, 648–656, doi:10.2174/1871520615666151002110424.

22. Tan, S.; Ke, Z.; Zhou, C.; Luo, Y.; Ding, X.; Luo, G.; Li, W.; Shi, S. Polyphenol Profile, Antioxidant Activity, and Hypolipidemic Effect of Longan Byproducts. *Molecules* **2023**, *28*, doi:10.3390/MOLECULES28052083.

Chapter 4

SUMMARIZING DISCUSSION

4.1. Summarizing discussion

The consumption of substances of natural origin has been increasing over the years, both for recreational and medicinal use. Regarding recreational use, the search for new sensations and experiences is what motivates most consumers. There are several reasons that triggers the search for new alternative natural therapies. The lack of access to hospital treatments is one of the main motivations; however, the search for more natural treatments or the lack of effectiveness in the conventional ones is also one of the main reasons for the search. Ayahuasca is one of the natural products used for both purposes. Additionally, there are religious and spiritual beliefs that motivate its use.

The bioactive properties of ayahuasca have been reported since ancient times. However, there is a great lack of studies that prove the benefits associated with its use. The lack of understanding of the mechanisms of action and the lack of studies that allow an evaluation and of the therapeutic and bioactive properties associated with the consumption of ayahuasca, therefore becomes crucial. It is also extremely important to understand the phytochemical composition and respective properties of ayahuasca extracts, to which consumers have access, as well as the development of analytical methods that allow this characterization.

Therefore, initially a review of plant species, which are used recreationally, medicinally, or religiously, given their properties was conducted. It wasnoticed that one of the main concerns about these products is the weak legislation that regulates their consumption or their constituents. The analytical methods available for the detection and quantification of the constituents of that plant species were also reviewed. It was verified that there are developed methodologies; however, they mostly use classic extraction techniques, where higher volumes of organic solvents are used. The use of miniaturized techniques has not proven to be very common for detecting this type of compounds. Subsequently, a systematic review was carried out on the therapeutic properties associated with the consumption of ayahuasca. It was evident that most studies reviewed, focused on the use of ayahuasca as a treatment for psychological/psychiatric disorders. Most of the available studies are clinical trials or studies that use individuals with beliefs in religions based on ayahuasca or who consume this drink recurrently and, therefore, may present biased results. Other studies consist of placebo-controlled clinical trials and therefore present more reliable results. However, it is curious that the number of existing *in vitro* and *in vivo* tests is quite small compared to those using volunteers.

One of the objectives of this thesis was the development of an analytical method that would allow identifying and quantifying the main constituents of ayahuasca decoctions. Three miniaturized sample cleaning techniques were tested to eliminate impurities and pre-concentrate the analytes, so that their detection is better. This step is extremely important, as it allows to reduce simultaneously the amount of sample and organic solvent used. For the first time, a comparative study was carried out between three miniaturized techniques, choosing QuEChERS as the most promising technique for extracting the compounds present in ayahuasca decoctions. Using the response surface approach helped to perform this optimization more quickly and with reliable results. Although the equipment used was not coupled to a more sensitive detector, as is the case of

the mass spectrometry detector, the method proved to be linear in the range of concentrations tested, selective, precise, and accurate. Additionally, the extraction method showed very acceptable recoveries.

Despite the worldwide use of ayahuasca, the behavior of ayahuasca constituents throughout the digestive process is not fully understood. Therefore, one of the objectives of this thesis was also related to understanding the behavior of the main compounds of this decoction throughout a simulated digestion process divided into three stages: salivary, gastric, and duodenal. This process made it possible to verify the variation in concentrations of ayahuasca compounds between stages of digestion. Through *in vitro* simulation of the intestinal wall, using the Caco-2 cell line, it was also found that the compounds crossed the cell monolayer, becoming bioavailable. The study also allowed us to verify that the constituents of ayahuasca do not interfere with either the integrity or the permeability of the cell monolayer.

One of the objectives of this thesis was to characterize at a phytochemical level the ayahuasca decoctions, determining some classes of compounds that are commonly associated with the biological properties of plants, namely phenolics and flavonoids. Thus, the determination of phenolic compounds in the extracts, carried out using the Folin-Ciocalteu reagent method, revealed that all samples presented high concentrations of total phenols. Similarly, they were shown to have flavonoids in their constitution, when determined using the aluminium chloride colorimetric method. The analysis by UHPLC-Q/TOF-MS allowed to realize that the phenolic compounds in the samples are made up of two groups of compounds: hydroxybenzoic acids and flavonoids. Although these compounds are associated with certain beneficial effects, the biological activity of compounds of natural origin is normally due to the joint action of their different constituents, and there may be synergistic reactions, additive or potentiating effects.

The determination of bioactive and therapeutic properties was also a objective of this thesis. Determining the antioxidant activity can sometimes be difficult, as this process is complex and depends on several factors. Thus, the antioxidant activity of the extracts under study was evaluated using the DPPH and the β-carotene/linoleic acid system methods. The first method allowed to verify that most samples presented very strong or strong antioxidant activity and only the *P. harmala* sample presented poor antioxidant activity. These results are in agreement with the content of phenolic compounds in each plant, given that the samples have the highest antioxidant activity. Likewise, the β-carotene/linoleic acid system also allowed to verify that this same sample presented the highest IC_{50} value and, consequently, the lowest antioxidant activity. In contrast, the commercial mixture, and the *P. viridis* sample showed the highest antioxidant activity.

The determination of anti-inflammatory activity was carried out by determining the ability to inhibit protein denaturation, which is an important indicator of an inflammation process, as this effect can occur when tissue is damaged. The results revealed that the samples with the best anti-inflammatory activity are *P. harmala*, *M. hostilis*, the mixture of *M. hostilis* + *P. harmala* and the commercial mixture. The anti-inflammatory potential of the *P. harmala* sample had already been described before in the literature.

The search for therapies of natural origin has been increasing and with this interest comes the need to evaluate possible therapeutic potentials, particularly at the microbiological level. Thus,

from the analysis of the results of the antimicrobial activity obtained by the disk diffusion method, it was found that six of the samples inhibited bacterial growth in all strains (*P. viridis*, *B. caapi*, *P. harmala*, *M. hostilis*, *M. hostilis* + *B. caapi* and *M. hostilis* + *P. harmala*) and the two strains most susceptible to inhibition were *S. aureus* and *A. baumannii*. The MIC values were also determined using the agar dilution method and it was concluded that the strains for which the lowest MIC values were observed and, therefore, more susceptible to the action of the samples, were *B. cereus* and *A. baumannii*. On the other hand, the samples that showed the best MIC values and the highest antimicrobial activity were the *B. caapi* and *P. harmala* samples. The anti-quorum sensing properties of the samples were also evaluated, using the *Chromobacterium violaceum* strain. The *B. caapi* and *P. harmala* samples were the samples with the most promising results. The anti-biofilm activity of the *B. caapi* and *P. harmala* samples was evaluated in the *A. baumannii* strain, and it was found that the samples presented anti-biofilm activity against this strain.

Given the promising properties obtained in the evaluation of antioxidant, anti-inflammatory and antimicrobial activities, the healing potential of ayahuasca decoctions was also evaluated *in vitro*. Through the MTT cytotoxicity test, it was possible to rule out the use of *P. harmala* extract, since at the tested concentrations it showed cytotoxicity. The evaluated extracts showed good wound healing potential, with emphasis on the mixtures of *P. viridis* + *B. caapi* and *M. hostilis* + *P. harmala*, where a greater evolution of the simulated *in vitro* injury was verified. Additionally, the psychoactive compound DMT and β-carbolines were not absorbed through the simulated skin barrier in a PAMPA trial. These results may be related to the phytochemical composition of the extracts, namely phenolic compounds, particularly flavonoids.

Finally, the anticancer properties of the extracts were also evaluated *in vitro*. After determining the IC_{50} in Caco-2 cells, it was found that the samples of *M. hostilis*, *P. harmala* and the mixture of *M. hostilis* + *P. harmala* were those that presented a lower value, therefore being the most promising. Caspase-3 activity was evaluated, and the cell proliferation index was determined through immunocytochemical analysis of the cell proliferation marker Ki-67, revealing a reduction in cell proliferation and a pro-apoptotic effect. The extracts also demonstrated the ability to reduce oxidative stress, assessed using two fluorescent probes: 2',7'-dichlorodihydrofluorescein diacetate and dihydroethidium. The activity of antioxidant enzymes was also evaluated. The activity of Superoxide Dismutase did not change significantly; however, the obtained results by evaluating the activity of Glutathione Peroxidase suggest that the extracts lead to the triggering of a defense response against oxidative stress.

The anticancer properties of the extracts were also evaluated in AGS cells. After determining the IC_{50}, it was found that the samples of *P. harmala*, *B. caapi* and *M. hostilis* were those that presented the most promising values. Thus, the ability to induce apoptosis was evaluated by indirect measurement of Lactate dehydrogenase, and a pro-apoptotic effect was verified in the three extracts. Finally, the induction of oxidative stress was evaluated using two fluorescent probes: 2',7'-dichlorodihydrofluorescein diacetate and dihydroethidium. A reduction in oxidative stress was observed after incubation with the three extracts tested.

Overall, this work contributed to a better understanding of the bioactive and therapeutic properties of ayahuasca decoctions. Extracts of *B. caapi* and *P. harmala* demonstrated the most

promising antimicrobial activity, while mixtures of *P. viridis* + *B. caapi* and *M. hostilis* + *P. harmala* showed the best healing potential. Regarding the anticancer activity, it was found that extracts of *M. hostilis*, *P. harmala* and the mixture of *M. hostilis* + *P. harmala* presented the best results in Caco-2 cells. Otherwise, in AGS cells, extracts from *P. harmala*, *B. caapi* and *M. hostilis* showed the most promising results in terms of anticancer activity. The bioactive properties of some of these extracts were studied for the first time in this work. Determining the phytochemical profile of ayahuasca extracts was also crucial to understanding some properties presented by the extracts. The work carried out in this thesis allowed to verify that ayahuasca extracts are an important source of bioactive compounds, which could be used as alternatives to the existing therapies. Carrying out this study also allowed to understand the antioxidant and anti-inflammatory potential of the compounds present in the studied extracts, namely polyphenols.

Chapter 5

CONCLUSION AND FUTURE PERSPECTIVES

5.1. Conclusions and future perspectives

The interest in ayahuasca has been increasing over the years, both for recreational and religious use, and for therapeutic use. Over the years, several health benefits associated with ayahuasca consumption have been highlighted and the number of studies has also been increasing. However, contrary to what would be expected, most of these studies consist of clinical trials or observational studies, which use volunteers or regular consumers of ayahuasca. Most trials focus on the treatment of illnesses or psychological imbalances; however, other promising results in different areas point to ayahuasca as a potential alternative therapy.

The development of analytical methods that allow the detection and quantification of the main ayahuasca compounds is increasingly important. The use of miniaturized extraction techniques is not yet widely used in ayahuasca samples. In the present work was possible to compare for the first time three miniaturized extraction techniques on ayahuasca decoctions. The QuEChERS technique proved to be the most efficient in extracting the main ayahuasca compounds. The analytical method developed in HPLC-DAD proved to be precise and accurate for a linearity range between 0.16 and 10 µg/mL (0.016 and 1 µg/mL for DMT), with LOD and LLOQ of 0.16 µg/mL (0.016 µg/ mL for DMT).

The bioavailability and bioaccessibility of ayahuasca decoctions were evaluated *in vitro*. β-Carbolines and DMT were released from the matrix, becoming bioaccessible. After digestion, ayahuasca compounds became bioavailable, as they were able to cross the simulated intestinal barrier *in vitro*. However, compounds that were not subjected to the digestive process were able to become bioavailable as well.

The ayahuasca samples were characterized regarding some classes of secondary metabolites, namely phenolics and flavonoids. The *M. hostilis* sample presented the highest content of total phenols, while the *P. harmala* sample presented more flavonoids. The ability of the samples to scavenge free radicals and inhibit lipid peroxidation were demonstrated, indicating the antioxidant properties of these samples. The samples of *M. hostilis*, the mixtures of *M. hostilis* and *B. caapi* and *M. hostilis* and *P. harmala* were the ones that showed the best antioxidant activity by the DPPH method, while the commercial mixture and *P. viridis* samples showed the best ability to inhibit lipid peroxidation. Additionally, the samples showed anti-inflammatory capacity through the ability to inhibit protein denaturation. The same samples also demonstrated antimicrobial properties, anti-*quorum sensing* and anti-biofilm potential, highlighting the role of the *B. caapi* and *P. harmala* samples against *A. baumannii*.

Furthermore, ayahuasca decoctions shown to have great potential for wound healing. Performing the scratch assay allowed verifying *in vitro* the migration of fibroblasts after incubation with the extracts. It should be noted that samples of *P. viridis* and *B. caapi* and *M. hostilis* and *P. harmala* showed the best healing potential. It is also worth noting that the skin absorption of the hallucinogenic compound DMT and β-carbolines was also evaluated, and it was noted that these compounds did not cross the skin barrier.

When evaluating the anticancer activity of the samples, it was found that decoctions of *P. harmala*, *M. hostilis* and the mixture of *M. hostilis* and *P. harmala* could reduce the viability and

cell proliferation of Caco-2 cells, also having a pro-apoptotic effect. The extracts also demonstrated the ability to decrease the oxidative stress, and although no significant differences were found in SOD activity, the results of GPx activity suggest that the extracts lead to the triggering of a defence response against oxidative stress.

P. harmala, *B. caapi* and *M. hostilis* samples also demonstrated anticancer properties in AGS cells. The extracts showed a pro-apoptotic effect, as well as a reduction in oxidative stress.

In light of the aforementioned findings, this research has significantly advanced our understanding of the biological properties associated with different ayahuasca decoctions, thereby emphasizing the potential for its application as an alternative therapeutic approach. Otherwise, the development of new analytical methodologies associated with promising miniaturized extraction techniques in the purification of the main constituents of ayahuasca was also achieved.

However, it is crucial to acknowledge that despite the promising results, the studies conducted are currently in the preliminary stage and predominantly encompass *in vitro* tests. Consequently, we must interpret these findings with caution. Therefore, it would be prudent to continue with more comprehensive investigations.

Expanding upon the insights gained in this research, it becomes imperative to investigate its applicability in the advancement of alternative therapies or even new topical drugs with healing, anti-inflammatory and antimicrobial properties. Given the knowledge acquired with this work, it would also be interesting to develop alternative therapies or even new topical drugs with healing, anti-inflammatory and antimicrobial properties.