Evaluation of *Humulus lupulus* L. Therapeutic Properties for the Treatment of Skin Diseases

Determination of Antimicrobial, Cytotoxic, Anti-inflammatory and Antioxidant Potential of *H.lupulus* Extracts

Versão final após defesa

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Resumo

A pele, é o maior órgão do corpo humano e é responsável por inúmeras funções vitais como barreira física protetora contra a invasão de microrganismos patogénicos, penetração de produtos químicos nocivos e de raios ultravioleta, mas também desempenha papéis importantes na homeostasia corporal e na elaboração de respostas imunes e sensitivas. A cooperação existente entre os vários elementos constitutivos da pele (queratinócitos, fibroblastos, células do sistema imunitário, fibras nervosas motoras e sensitivas, glândulas, vasos sanguíneos e linfáticos) e até mesmo com os microrganismos, da sua superfície é importante para manter o organismo saudável. Inúmeros distúrbios frequentes da pele como a dermatite atópica, a psoríase e a acne podem estar associadas a uma predisposição genética do indivíduo, mas também a um desequilíbrio da microflora da pele ou mesmo a uma ativação sem controlo de respostas do sistema imunitário inato e adaptativa devido muitas vezes a sobrexpressão de proteínas tais como a ciclooxigenase-2 (COX-2) responsáveis pela génese de processos inflamatórios e de stress oxidativo crónicos que levam à produção de moléculas como o óxido nítrico (NO) e espécies reativas de oxigénio (ROS) que quando em excesso se tornam tóxicos e deletérios para as células.

O *Humulus lupulus* Linnaeus, pertencente a família dos *Cannabaceae* é popularmente utilizado na confeção da cerveja e na medicina tradicional pelas suas propriedades terapêuticas relaxantes, como, por exemplo, no tratamento de casos de insónia e ansiedade. A inflorescência do *H.lupulus* é a parte da planta mais utilizada por ser onde está localizada a glândula da lupulina, órgão constituído maioritariamente por resinas 15-30% (resinas duras e macias), óleos essenciais, polifenóis, entre outros compostos minoritários responsáveis pelo carater medicinal da planta. Estes metabolitos secundários do *H.lupulus* são conhecidos por apresentarem um alto potencial anti-inflamatório, ansiolítico, antidepressivo, antioxidante e antimicrobiano.

Na presente dissertação, foi estudada a capacidade de extratos aquosos da flor, do *mix* (mistura de caules, folhas e flores), do hidrolato da flor e do hidrolato do *mix* do *H.lupulus* na diminuição da multiplicação de estirpes bacterianas Gram-positivas (*Staphylococcus aureus*, *Staphylococcus epidermidis* e *Cutibacterium acnes*) e Gram-negativas (*Escherichia coli* e *Pseudomonas aeruginosa*) pelo método de microdiluição e na alteração da atividade metabólica de células de fibroblastos 3T3 e de macrófagos RAW provenientes da pele de ratinhos através do método colorimétrico, brometo de [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] (MTT). Foi também avaliado o impacto destas substâncias na produção de NO, através do método de Griess, produção de ROS

através de moléculas fluorescentes como o H_2DCFDA, e na expressão da COX-2, agente interveniente nos processos de inflamação após ativação dos macrófagos pelo lipopolissacarideo (LPS), por *Western blot*.

Os resultados obtidos demonstram que o hidrolato da flor do *H.lupulus* apresenta maiores capacidades antibacterianas, especialmente contra as bactérias Gram-positivas, forte capacidade antioxidante, porque diminui significativamente a produção de ROS, reduz a inflamação, diminuindo a produção de NO e a expressão da COX-2 pelos macrófagos ativados pelo LPS. Os restantes extratos apresentaram também efeito anti-inflamatório e antioxidante, mas menos evidente do que o hidrolato da flor. O hidrolato do *mix* e o extrato aquoso do *mix* demonstraram um baixo efeito antibacteriano para algumas das estirpes em estudo e não alteraram significativamente a atividade metabólica dos fibroblastos 3T3 e dos macrófagos RAW.

Este estudo demonstrou que o *H.lupulus*, principalmente a inflorescência da planta (flores) possui elementos químicos com capacidades anti-inflamatórias, antioxidantes e antibacterianas, podendo ser bastante interessantes na conceção de produtos à base de lúpulo com o intuito terapêutico e de prevenção de doenças que possam surgir na pele respeitando ao mesmo tempo a sua constituição fisiológica. Contudo mais estudos são necessários de modo a se conhecer melhor o perfil dos diferentes compostos químicos do *H.lupulus* bem como de modo a se estabelecer concentrações não toxicas para as células e microrganismos que compõem a microbiota da pele.

Palavras-chave

Ciclooxigenase-2, Espécies reativas de oxigénio, Fibroblastos, *Humulus lupulus* L., Macrófagos, Metabolismo, Oxido nítrico.

Resumo Alargado

A pele, maior órgão do corpo humano, desempenha inúmeras funções vitais. É responsável pela defesa do organismo contra a penetração de corpos estranhos (microrganismos patogénicos ou compostos químicos nocivos) provenientes do meio ambiente. Participa na termorregulação corporal, na regulação da eliminação de fluidos corporais, na homeostase, mas também na elaboração de respostas imunes inatas ou adaptativas em caso de invasão de patógenos. A pele é dividida essencialmente em três camadas designadas respetivamente de epiderme, derme e hipoderme ou subcutânea. Estas camadas são constitucionalmente diferentes e permanecem conectadas entre si graças a junções existentes entre as células subjacentes (junções aderentes, desmossomas, *gap and tight junctions*). A cooperação que existe entre os vários elementos constitutivos da pele (queratinócitos, fibroblastos, células do sistema imunitário, fibras nervosas motoras e sensitivas, glândulas, vasos sanguíneos e linfáticos) e o relacionamento com a microbiota da sua superfície é importante para manter o organismo protegido contra invasores patogénicos. O microbioma da pele é constituído por uma grande diversidade de bactérias, vírus e fungos. Estes microrganismos estabelecem uma relação intrínseca com o sistema imunitário mediando muitas das respostas imunes. Inúmeros distúrbios comuns da pele como a dermatite atópica, a psoríase e a acne podem estar associadas a uma predisposição genética do individuo, mas também a um desequilíbrio do microbioma da pele ou mesmo a uma ativação sem controlo de respostas do sistema imunitário inato e adaptativo devido muitas vezes a sobrexpressão de proteínas como a COX-2, mediadores de processos inflamatórios e de stress oxidativo que levam a produção de moléculas como o NO e ROS que, por sua vez, quando em excesso, se tornam tóxicas e deletérias para as células. Muitos dos microrganismos associados ao desenvolvimento de doenças desenvolvem variedades resistentes a antibióticos o que torna estas infeções mais difíceis de serem tratadas como é o exemplo das provocadas por *Staphylococcus aureus* resistente a meticilina (MRSA).

O processo inflamatório é uma resposta que o organismo elabora devido a invasão de corpos estranhos no organismo, como agentes patogénicos. Os macrófagos, um dos tipos de células responsáveis pela imunidade inata e adaptativa, quando ativados por LPS, por interferon-ϒ (INF-ϒ) ou outros mediadores como citoquinas, produzem NO e ROS de modo a expulsarem o corpo invasor. LPSs são macromoléculas constitutivas da parede celular de bactérias Gram-negativas e são constituídas essencialmente de lípidos e de polissacarídeos. O NO é produto da ativação dos macrófagos por um agente patogénico e é

sintetizado pela NOS2 (enzima homodimérica), num processo complexo de oxido-redução que se realiza nas mitocôndrias, a partir da L-arginina e de oxigénio, independente do fluxo de cálcio (Ca^{2+}). Durante a inflamação a expressão de COX-2, que é uma isoenzima associada a dor na inflamação, é ativada. A sua ativação permite a síntese de prostaglandinas E2 (PGE2) e tromboxanos a partir de ácido araquidónico. Ao longo do processo de inflamação também são sintetizados pelos macrófagos, no nível das mitocôndrias, ROS. Os ROS são moléculas altamente reativas e que são originadas por reações de oxido-redução a partir do oxigénio (O_2). Estas moléculas em excesso ou quando o sistema protetor antioxidante está deficiente origina situações de stress oxidativo, que por sua vez está implicado no aparecimento de muitos distúrbios da pele como o envelhecimento precoce ou mesmo cancro.

O *Humulus lupulus* Linnaeus*,* é uma planta dioica, perene, trepadeira, oriunda de regiões de clima temperado. As espécies do género *Humulus* apresentam diferenças entre si de acordo com a sua distribuição geográfica e com as suas características morfológicas. A glândula de lupulina presente na inflorescência da planta é popularmente utilizada na confeção da cerveja por causa do seu aroma e sabor característicos. Esta particularidade deve-se à sua constituição, essencialmente resinas (α-ácidos e β-ácidos) e óleo essencial que ali são sintetizados, e que se encontram acumulados. Na medicina tradicional a planta *H.lupulus* é conhecida por ser rica em terpenos, xanthohumol, lumulene, lupulona, mirceno e β-cariofileno responsáveis pelas suas propriedades anti-inflamatórias, ansiolítica, antidepressiva, antioxidante, antimicrobiana e fungicida, entre outras. A inflorescência da planta fêmea do *H.lupulus* é a parte da planta com maior valor comercial sendo que a masculina é apenas utilizada para fins de aperfeiçoamento da espécie. A planta é maioritariamente comercializada sob a forma de grânulos (60%) e na forma de extratos (25%). Existem diversos métodos para a obtenção de extratos distinguindo-se os metanolico, hidro/alcoólico, de CO_2 supercrítico, aquoso obtido por extração líquido/líquido, os óleos essenciais e os hidrolatos que são obtidos por hidrodestilação. Diferentes partes da planta (flores, folhas, caules, rizomas e sementes) podem ser utilizadas para a obtenção dos extratos.

Na presente dissertação, foi avaliada a composição química dos hidrolatos da flor e do *mix* (mistura de caules, folhas e flores) através do método de cromatografia gasosa acoplado a espectrometria de massa (GC-MS). A atividade antimicrobiana foi determinada pelo método de microdiluiçao, em placas de 96 poços, tendo por finalidade observar a capacidade dos extratos aquosos da flor, do *mix* (mistura de caules, folhas e flores), do hidrolato da flor e do hidrolato do *mix* de *H.lupulus* na diminuição da multiplicação de estirpes bacterianas Gram-positivas (*Staphylococcus aureus*, *Staphylococcus epidermidis* e *Cutibacterium acnes*) e Gram-negativas (*Escherichia coli* e *Pseudomonas aeruginosa*). O método de microdiluiçao é um teste bastante simples e de fácil reprodução. O aumento ou a diminuição da atividade metabólica de fibroblastos 3T3 e dos macrófagos RAW provenientes da pele de ratinhos foi determinado através do método colorimétrico, MTT após 24 horas dos estímulos com os extratos de *H.lupulus*. Foram também avaliadas as propriedades anti-inflamatórias dos quatro extratos de *H.lupulus*, avaliando assim o impacto destas na produção de NO através do método de Griess e na produção de ROS através de moléculas fluorescentes como o H_2DCFDA (molécula fluorescente citosólico) e o Hoechst (molécula controlo fluorescente nuclear) com emissão de luz de diferentes comprimentos de onda. A avaliação da inibição/indução da expressão da COX-2, agente interveniente nos processos de inflamação após ativação dos macrófagos pelo LPS foi feita por *Western blot*. A identificação da proteína foi feita com anticorpos primários e secundários de coelho anti-cox-2 e como controlo foi utilizada a proteína gliceraldeido-3-fosfato desidrogenase (GAPDH).

Dos resultados obtidos demonstrou-se que o hidrolato da flor do *H.lupulus* foi o que apresentou maior atividade antibacteriana, especialmente contra as bactérias Gram-positivas (*Staphylococcus aureus* e *Staphylococcus epidermidis*), antioxidante (diminuindo significativamente a produção de ROS) e anti-inflamatória, reduzindo significativamente a produção de NO e a expressão da COX-2. Em pequenas concentrações (0.78%-6.25%) o hidrolato da flor diminui significativamente a atividade metabólica da linha celular de macrófagos RAW, contudo apenas em concentrações elevadas (25%-50%) consegue diminuir significativamente a atividade metabólica de fibroblastos 3T3. As restantes substâncias (extrato aquoso da flor, extrato aquoso do *mix* e hidrolato do *mix*) apresentaram propriedades anti-inflamatórias e antioxidantes, mas menos evidentes que o hidrolato da flor. O hidrolato do *mix* e o extrato aquoso do *mix* demonstraram um baixo efeito antibacteriano para algumas estirpes estudadas e não demonstraram citotoxicidade em concentrações baixas. Porém, em concentrações muito elevadas diminuem a atividade metabólica das células.

Demonstrou-se no presente trabalho que o *H.lupulus*, principalmente a inflorescência da planta possui propriedades medicinais bastante interessantes podendo vir a ser utilizada na formulação de produtos farmacêuticos destinados ao tratamento de doenças da pele nomeadamente as associadas com processos de inflamação severa como a dermatite, a acne ou mesmo a psoríase. Futuramente, pretende realizar-se uma abordagem mais detalhada dos extratos de *H.lupulus* e dos seus componentes químicos de modo a que se conheça a concentração mínima efetiva não toxica para as células e microrganismos que compõem a pele, e assim melhor definir a sua aplicabilidade.

Abstract

The skin is the largest organ of the human body and is responsible for numerous vital functions as being a physical protective barrier against the invasion of pathogenic microorganisms, penetration of harmful chemicals, and of ultraviolet rays, while having also important roles as in body fluid homeostasis and the elaboration of immune and sensitive responses. The cooperation between the various constituting elements of the skin (keratinocytes, fibroblasts, immune system cells, motor and sensitive nerve fibres, glands, blood and lymphatic vessels) together with the microorganisms residing on its surface is important to keep the body healthy. Numerous common skin disorders such as atopic dermatitis, psoriasis and acne can be associated with an individual's genetic predisposition, but also with an imbalance in the microflora of the skin or even to an uncontrolled activation of responses of the innate and adaptive immune system due often to over-expression of proteins such as cyclooxygenase-2 (COX-2), responsible for the genesis of chronic inflammatory processes and oxidative stress that lead to the production of molecules such as nitric oxide (NO) and reactive oxygen species (ROS) that, when in excess, become toxic and harmful to the cells.

Humulus lupulus Linnaeus, belonging to the *Cannabaceae* family, is popularly used in beer making and in traditional medicine for its relaxing therapeutic properties, such as the treatment of insomnia and anxiety. The inflorescence of *H.lupulus* is the part of the plant mostly used because it is where the lupulin gland is located, an organ harbouring mainly 15-30% of resins (hard and soft resins), essential oils, polyphenols, among other minority compounds responsible for the medicinal character of the plant. These secondary metabolites of *H.lupulus* are known to have a high anti-inflammatory, anxiolytic, antidepressant, antioxidant and antimicrobial potential.

In this dissertation, the capacity of aqueous extracts of the flower, the mix aqueous extract (mixture of stems, leaves and flowers), the flower hydrolate and the mix hydrolate of *H.lupulus* was studied to decrease multiplication of Gram-positive (*Staphylococcus aureus*, *Staphylococcus epidermidis* and *Cutibacterium acnes*) and Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) bacteria by the microdilution method and in the alteration of the metabolic activity of 3T3 fibroblast cells and RAW macrophages from mouse skin by the colorimetric method, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The impact of these extracts on the production of NO was also evaluated by the Griess method, production of ROS by fluorescent

molecules such as H2DCFDA, and on the expression of COX-2 an agent involved in the inflammation processes after activation of macrophages by lipopolysaccharide (LPS) by Western blot.

The results show that *H.lupulus* flower hydrolate presents higher antibacterial capacity (especially against Gram-positive bacteria), strong antioxidant capacity, because it significantly reduces the production of ROS reduces inflammation, decreasing the production of NO and decreasing the expression of COX-2 by LPS activated macrophages. The remaining extracts also presented anti-inflammatory and antioxidant effects, but less evident than the flower hydrolate. Mix hydrolate and aqueous extract showed a low antibacterial effect for some of the strains under study and did not significantly alter the metabolic activity of 3T3 fibroblasts and RAW macrophages.

This study demonstrated that *H.lupulus*, mainly the inflorescence of the plant (flowers), has chemical elements with anti-inflammatory, antioxidant and antibacterial capacities supporting the concept of being *H.lupulus* products very interesting to be used in treatment and prevention of diseases of the skin while respecting its physiological constitution . However, further studies are necessary in order to better understand the profile of the different chemical compounds of *H.lupulus* and to establish the best effective concentration yet non-toxic for the cells and microbiota of skin.

Keywords

Cyclooxygenase-2, Fibroblasts, *Humulus lupulus L.*, Macrophages, Metabolism, Nitric Oxide, Reactive oxygen species.

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List of Abbreviations

AMPs	Antimicrobial peptides
BCPO	β-caryophyllene oxide
BHI	Brain Heart Infusion Broth
BCA	Bicinchoninic acid assay
Ca^{2+}	Calcium
CH_2Cl_2	Dichloromethane
CO_2	Carbon dioxide
COX-2	Cyclooxygenase-2
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
EMA	European Medicines Agency
ECL	Enhanced Chemiluminescence
EtOH	Ethanol
FTIR	Fourier-transform infrared spectroscopy
FBS	Foetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC-MS	Gas chromatography-mass spectrometry
HBSS	Hanks's balanced salt solution
H_2O	Dihydrogen monoxide
H2DCFDA	2',7'-dichlorodihydrofluorescein diacetate
HPLC	Hight-performance liquid chromatography
HPV	Human papillomavirus
INF-ϒ	Interferon gamma
IL-1	Interleukin-1
IL-10	Interleukin-10
IL-17-A	Interleukin-17-A
INFARMED	National Authority for Medicament and Health Products
LPS	Lipopolysacharides
MIC	Minimum Inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MHB	Mueller-Hinton Agar
MYR	Myrcene

MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaNO2	Sodium nitrite
NO	Nitric oxide
NOS2	Nitric oxide synthase 2
O_2	Dioxygen
PGE2	Prostaglandin E2
PVDF	Polyvinylidene difluoride
pH	Hydrogen potential
RNA	Ribonucleic acid
REACH	Registration, Evaluation, Authorization and Restriction of Chemicals
RIPA	Radioimmunoprecipitation assay buffer
ROS	Reactive oxygen species

I. Introduction

1.1. General description of skin anatomy and physiology

The skin is the largest organ of the human body occupying an area of approximately 2 m^2, corresponding to 20% of total adult body weight (1). It performs an important function of physical barrier, protecting against harmful pathogens from external environment, mechanical impacts, temperatures changes, ultraviolet radiation (UV), chemical irritants. It performs multiple thermoregulatory functions and is an important thermal insulator thanks to the fatty tissue. It participates too in the regulation of body's water, corporal fluid elimination, is responsible for the production of important metabolites such as vitamin D and antimicrobial peptides, and possesses others functions like sensory, endocrine and immune (2). It is a stratified epithelial layer connected to an underlying connective tissue, the dermis. As the deepest layer of the skin, the hypodermis can be found, consisting essentially of fat cells, which are a source of energy (1) (Figure 1). The skin is formed by juxtaposition of two major embryonic elements, the ectoderm and the mesoderm. The ectoderm is at the origin of the epidermis, and the mesoderm will contribute to the appearance of the dermis and is also responsible for the differentiation of attached epidermal structures such as the hair follicle (3). The skin is composed of different cells and microorganisms that cooperate with each other in order to maintain the physical and immune integrity of the barrier and to assume a rapid and effective response in situations of stress and disease (4).

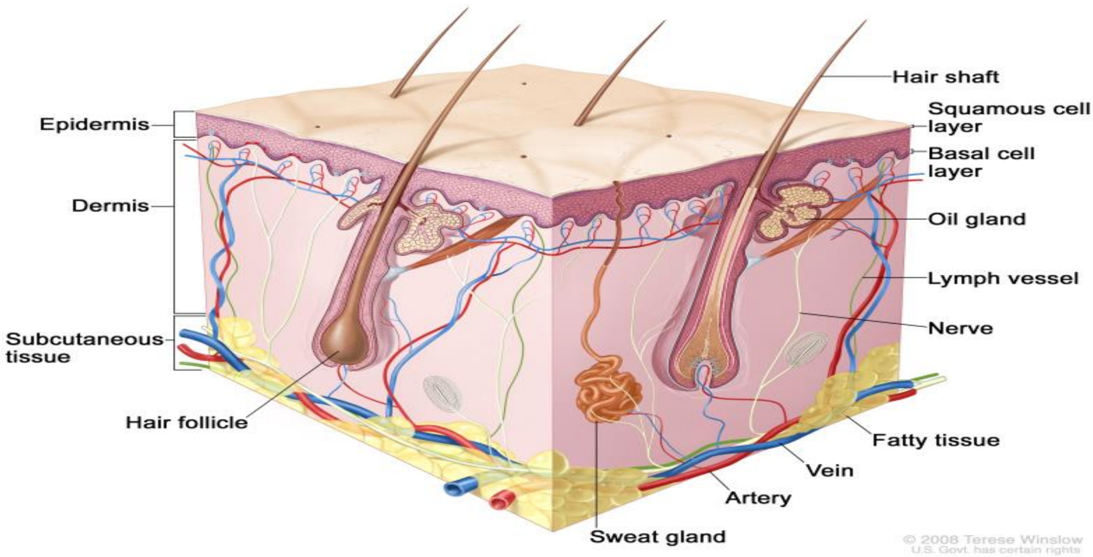

Figure 1. Anatomical organization of the skin (From (5)**).** The skin is organized essentially in three layers. The epidermis, the outermost layer, forms the physical barrier of the skin composed by squamous cells. The dermis intermediate layer has several cellular structures such as blood vessels, glands, nerve fibres among others. The hypodermis rich in adipocytes is responsible for the storage of energy and nutrients.

1.1.1. Cellular constitution

The epidermis is composed by five layers, the outermost layer being called *stratum corneum*, then the *stratum lucidum* which is present only in some places on the body such as fingertips, palm of hands and sole of feet, the *stratum granulosum*, the *stratum spinosum* and the *stratum basale* which is the innermost layer (1). The outermost layer, *stratum corneum*, is a thick layer consisting essentially of dead cells called corneocytes. These cells are surrounded by a lipid layer thus forming the physical barrier of the skin. The *stratum basale*, the inner most layer, consists of epidermal stem cells (1). The epidermis is made up of 95% of keratinocytes (originating from dividing cells from the basal *stratum*, which progress and differentiate along the layers) (3). These keratinocytes are responsible for the waterproofing of the physical barrier (2). Besides the keratinocytes there are also Merkel cells, that operate as mechanoreceptors, melanocytes, that are cells specialized in production of pigments which define skin color, Langerhans's cells, which are antigen-presenting cells of the skin immune system (2), (3).

The epidermis and dermis are separated by a basal membrane. This epidermal-dermal junction forms invaginations, rete ridges, which provides elasticity and serves as a physical barrier for the passage of cells and macromolecules between the two layers (3).

The dermis layer is a thick connective tissue. At the base of the dermis, there is a support matrix consisting essentially of polysaccharides and proteins connected to each other in order to form important macromolecules with the ability to retain water. Associated with this extracellular matrix there are collagen, the largest constituent of the dermis, elastin fibres and reticular fibres, which are produced mainly by the dermal fibroblasts. Besides the fibroblasts, other cells, like mast cells and monocytes/macrophages, can be found. Blood and lymphatic vessels are present too in this compartment and they are responsible for supplying nutrients. The dermis contains afferent sensory nervous fibres and efferent motor nervous fibres that connect the skin to the central nervous system and makes possible to perceive the environment around the body (2,3). In the dermis there are pilosebaceous units, each composed by hair follicles, sebaceous and sweat glands (3). These glands produce sebum secretion to the surface of the skin, and sweat, which is rich in salt, antibacterial molecules such as free fatty acids and antibacterial peptides (AMPs) that serve as natural antibiotics against bacteria and are responsible for the characteristic dry and cold skin (3).

The hypodermis or subcutaneous layer is the deepest layer of skin and consists of loose connective tissue and elastin. It consists essentially of adipocyte cells, fibroblasts, macrophages, blood vessels and nerve endings. It provides insulation from cold temperature, has shock adsorbent capacity and represents a nutrient and energy storage reservoir (1),(2). Hypodermis decreases in thickness along aging. Its thickness is highest in the soles of the feet, palms of the hand and buttocks (1).

There are important junctions between cells (desmosomes, adherent junctions, gap junctions and tight junctions), in order to promote the connection and communication with adjacent underlying cells. This is basically a biochemical communication where molecules pass through the membrane of these cells (3).

The skin is a dynamic structure, and its cells are constantly replaced by new ones (3-4 weeks). The stem cells existing in the basal layer have the ability to self-renew while originating daughter cells that differentiate themselves along the layers until they reach the *stratum corneum*, where they progress to finally being eliminated by skin scaling and replaced by other cells (3).

1.1.2. Microbiome

The complexity and heterogeneity of those microorganisms that live in humans is reflected in the different microbiomes that compose the skin, the respiratory tract, the gastrointestinal tract and other sites (6). Thus, the diversity of the human microbiome can diverge according to the physiological conditions of the skin, its anatomical sites and sexual maturity (6). The skin allows the colonization by commensal microorganisms on its surface while preventing the penetration of other so-called pathogens (those cause disease) (7).

The symbiosis that exists between the bacterial flora and its host (human) is beneficial and essential for the good functioning of both. On the surface of the skin and in the annexes, such as hair follicles and sebaceous glands, a multitude of microorganisms (bacteria, fungi, viruses, archaea) can be found in harmony, turning them the natural flora of the skin (4,7). There are approximately 1 million bacteria/cm^2 residing on the skin surface, where one can find more than 1000 bacterial species divided into 19 different phyla (4,6). The number of microorganisms (fungi, bacteria, viruses and archaea) (Table 1), residing on the skin flora varies according to the specific conditions of the niche, such as temperature, pH, humidity, sweat production, topography, inter/intrapersonal and

temporal factors (7,8). Certain physiological conditions such as dryness, acidity, humidity and/or oiliness predispose preferentially to the multiplication of certain strains in detriment of others and prevent/difficult the colonization by of pathogens (4,7), (9).
Species of *Staphylococcus*, *Corynebacterium*, *Propionebacterium*, *Malassezia*, and eukaryotic DNA viruses are the ones found in greater quantity among the flora of the skin. The kingdom bacteria is the most populous in relation to the others (Figure 2) (4). The bacteria's family prevalence in decreasing order is as follows: *Propionibacteria spp.* (*P.acnes, P.avidum, P.granulosum, P.lymphophilum, P.propionicum, P.freudenreichii, P.jensenii, P.thoenii, and P. acidipropionici)* 22.8-38.1%, *Actinobacteria families* (example *Corynebacterium* spp.) 53.8-66.5%, Firmicutes (*Staphylococcus* spp., *Lactobacillales*, *Clostridiales*) 23.9-28.3%, Proteobacteria (Β-Proteobacteria, ϒ-Proteobacteria, α-Proteobacteria) is 5.8-12% and Bacteroidetes (Flavobacterales) 2.1-2.9% (8–10).

The oily sites of the skin are mostly colonized by lipophilic species of *Propionibacterium*, while in sites where the humidity reigns (elbow and foot curves for example) there are more *Staphylococcus* and *Corynebacterium* species. As far as fungi are concerned, they are distributed throughout the body independently of the physiological characteristics of the skin. Fungi of the species *Malassezia* are mostly found in the central regions of the body and on the arms, however in regions like the feet they exist in combination with *Aspergillus spp., Cryptococcus spp., Rhodotorula spp., Epicoccum spp.* and others. The viruses (DNA and RNA viruses) also compose the microbiome of the skin, however their prevalence varies from individual to individual independently of the anatomical region (7). Considering the physiological conditions of the skin and the low abundance of nutrients, the commensal bacteria have adapted themselves and use the nutrients (proteins and lipids) that come from sweat that are found in tallow and the *stratum corneum* as a source of food, thus ensuring their survival (7).

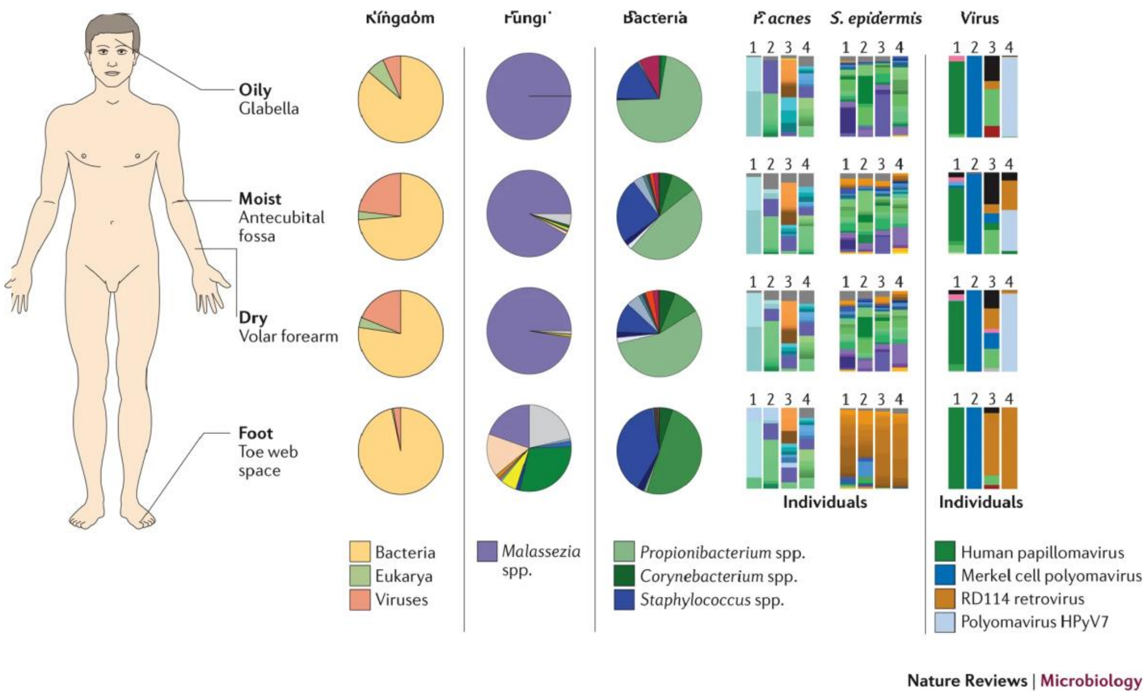

Figure 2. Distribution of flora according to physiological characteristics of skin (From (7)**).** The kingdom of bacteria is the largest kingdom found in skin flora. Individuals characteristics and certain anatomical regions predispose mostly to the colonisation of some microorganisms in relation to other parts of the body. Parts of the body where there is more oil production predispose mostly to the development of *Propionibacteria spp*. However, in this region other species can be found such as *Staphylococci spp*., *Corynebacteria spp*., *β-Proteobacteria*. In dry regions of the skin mostly *β-Proteobacteria* and others like *Corynebacteria spp*. and *Propionibacteria spp*. Are found while in moist areas *Corynebacteria spp*. are predominant coexisting with *Staphylococci spp*. and *β-Proteobacteria*. The largest population of fungi found in skin flora is represented by *Malassezia* spp and colonizes predominantly the feet.

Changes in lifestyle, hygiene practices and exposure to antibiotics can considerably modify the microbiome of the skin leading to the appearance of skin diseases such as atopic dermatitis (8).

Table 1. Ten examples of diversity of microorganism's population in symbiosis with the skin (Adapted from (7)).

Bacteria	**Eucarya**	**Viruses**
Cutibacterium acnes (previously known as *Propionibacterium acnes*)	*Malassezia restricta*	*Molluscum contagiosum* virus
Corynebacterium tuberculostearicum	*Malassezia globosa*	*Propionibacterium* phage
Corynebacterium simulans	*Malassezia sympodialis*	Merkel cell polyomavirus
Streptococcus mitis	*Candida parapsilosis*	*Pseudomonas* phage
Staphylococcus epidermidis	*Pyramimonas parkeae*	*Staphylococcus* phage
Staphylococcus capitis	*Tilletia walkeri*	*Polyomavirus* HPyV7
Staphylococcus aureus	*Parachlorella kessleri*	*Human papillomavirus* (β, ϒ, μ)
Staphylococcus hominis	*Zymoseptoria tritici*	*Stenotrophomonas* phage
Micrococcus lineus	*Aspergillus tubingensis*	*Acheta domestica* densovirus
Veillonella parvula	*Nephroselmis olivacea*	*Polyomavirus* HPyV6

1.2. Skin disorders – some considerations

The immune system of the skin assumes rapid and effective response to pathogens invasion. In childhood a rapid maturation of the immune system is observed because it is at this stage that contact with different allergens and pathogens starts in the child's environment. It is in this phase that we observe how the immune system interacts and reacts with the microorganisms that inhabit the organism (6,11). The symbiosis that exists between the immune system and the microflora is very important to maintain both the health of the individual and its responses to invasion of pathogens, however the mechanisms with which this interaction occurs still require further investigation and may be the key to the development of future strategies for the treatment of diseases associated to inflammation (6).

In the skin, immune system cells such as resident memory T lymphocytes (TRM), circulating T cells, dendritic cells, keratinocytes and monocytes/macrophages are found, that together play an important role in the body reaction against invasion by foreign bodies. Memory T lymphocytes (TRM) are strongly involved in inflammatory skin disorders (12). Macrophages are the main cells responsible for the initiation, maintenance and resolution of the inflammatory process. They are skin cells that originate in the bone marrow. They are the major components of the mononuclear phagocytic system that also included monocytes found in the blood circulation. The monocytes migrate to the tissues where they differentiate into macrophages (13). In the presence of pathogenic molecules such as LPS and IFN-ϒ, macrophages are activated and begin to secrete pro-inflammatory molecules and cytokines in order to expel the pathogenic organism. For the identification of foreign microorganisms and for the elaboration of a more effective immune response, macrophages integrate different levels of signals that imply the intervention and combination of different receptors such as the Toll-like receptor 4 (TLR-4) and the IFN-ϒ receptor (14). Thus, three main functions of macrophages can be distinguished: presentation of antigens, phagocytosis and immunomodulation which is characterized by the synthesis of cytokines and growth factors. Macrophages are deactivated by anti-inflammatory cytokines (Interleukin-10 and the transformer growth factor β) and by antagonistic cytokines that they do also produce. When there is an imbalance between the signals that lead to the initiation of the inflammatory response and the signals that establish its end, the inflammation is uncontrolled, thus damaging the cells and often giving rise to pathological conditions (13,14).
Skin disorders are triggered when the physical barrier of the skin is broken or appear when there is an imbalance between commensals and pathogens (4). Certain skin diseases

are more prone, particularly in some places depending on physiological characteristics (dryness, oiliness or humidity) (7).

The skin has an innate and adaptive immune system that is very efficient and perceptive, resulting from the combination of epidermal cells, lymphocytes and antigen-presenting cells that reside in the epidermis and dermis and the microbiota that, although it is not directly involved, is responsible for the production of important inflammatory mediators (4). In more severe situations of skin disorders, molecules (ligands) are released that by activating the keratinocytes trigger an inflammatory response by releasing its mediators (4). The resident bacterial flora also plays a fundamental role that consists in the control of the expression of innate immunity factors such as antimicrobial peptides (AMPs) that are capable of killing and/or inactivating invading pathogens (Gram-negative and positive bacteria, parasites, fungi and viruses). The AMPs of the skin belong to two families of proteins called catecholycidines and β-defensins (4). Microbiota also acts by increasing the expression of other factors of the complementary system, modulating the activity of resident cells, thus controlling the expression of important cytokines responsible for the initiation and amplification of immune system responses. Some cytokines that are involved in the defense of the host and in inflammatory diseases can be highlighted such as interleukin-1 (IL-1), responsible for the initiation and amplification of immune system responses, the IL-17-A and the IFN-ϒ. Many of the known skin pathologies such as psoriasis, atopic dermatitis and acne are characterized by the induction of inflammatory processes being a consequence of an imbalance of the bacterial flora (dysbiosis) present on the skin surface (4). Many of the most common skin diseases are associated with chronic inflammatory processes, oxidative stress, invasion of pathogenic microorganisms such as *Staphylococcus* or even fungal invasions (Table 2).

Table 2. Most common skin diseases and their characteristics

Skin Diseases	Causes	Characteristics
Atopic dermatitis (Eczema)	-Inflammation disorder linked to *Staphylococcus aureus* bacteria	-Red and scaly skin rash that is usually very pruritic. Can be hereditary and common in families with a history of asthma and allergies such as hay fever or allergies from irritating products of the environment (4,15,16)

Psoriasis	-Hyperproliferation of keratinocytes that leads to a chronic activation of T lymphocytes (namely Th17) thus leading to an overexpression of cytokines such as IL-1 and IL-17 (example: IL-17-A). Others causes: genetic predisposition, *Steptococcus* bacteria;	-Reddish, scaly rash which can cause itching, can occur in any region of the body, but classically appears in the areas of the scalp, elbows and knees and can also cause the nails to lose their natural colour, become pitted and fragile; (4,9,15,16).
Acne:	-Increased sebaceous glands activity and plugging	-Establishment of comedones, pustules and in more severe cases nodes and cysts (16)
Acne Vulgaris	-Inflammation caused by released mediators. Can be due to the alteration in the keratinization process that leads to obstruction of the pores of the hair follicle or an increase and alteration in the production of sebum under the control of androgens or colonization of the follicles by the *C.acnes* bacteria.	-Sebaceous hyperplasia and release of lipids in the follicular lumen causing obstruction of the pores (4,10).
Skin rashes:	-Irritation, allergy, infection, another disease, defects in the skin's structuring elements such as malfunction of the glands or obstruction of the pores	-Red regional, appearance of inflammation or groups of individual spots on the skin (17,18).
Fungal infections:	-Fungi infections	-Affect the scalp, skin, nail (17,19)
Warts	-Viral infection caused by the human papilloma virus (HPV)	-*Verruca vulgaris*: hands, fingers and knees and in places where some injury has occurred. They are generally not painful. *Verruca plantaris*: soles of the feet and can be quite painful (17,20).
Tinea pedis and Tinea unguium	-Onychomycosis, caused by dermatophyte fungi	-*Tinea pedis* is a reddish and scaly skin rash. *Tinea unguium,* essentially affects the nails and is manifested by the discolouration of the nail plate and accumulation of infection products (17).
Allergies	-Allergens and irritant chemicals	-Itching, redness, blisters (4).
Tumours and cancer	-90% of cases of tumours and skin cancer are caused by excessive sun exposure	-Very rapid and abnormal growth of skin cells (17).
Impetigo	-Strains of *Streptococcus pyogenes, Staphylococcus* aureus and *Methicillin-resistant Staphylococcus aureus (MRSA)*	-Appearance of blisters followed by scabs on the skin of the face and body (21).
Scarlet fever	-*Streptococcus pyogenes*	-Fever and rashes (21)
Leprosy (Hansen's Disease):	-*Mycobacterium leprae*	-Affects not only the skin but also other systems (21).
Candidosis:	-Species of Candida (e.g. *Candida albicans*)	-Inflammation of the genital area, itching, and fluid discharge (21)

1.3. Use of medicinal plants in the treatment of skin disorders

Since the dawn of mankind, it has been found that plants had the power to treat and prevent diseases. Great ancient civilizations such as Egypt, India and China have left records of the use of these plants as medicines, being used even today in traditional medicine (22). The plants or their derivatives, due to their unique properties (flagrancy, taste and therapeutic properties), have long been used both in traditional medicine and in cooking and cosmetic products (perfumes, soap). With the discovery and use of synthetic drugs there was a reduction in the use of plants in medicine (mainly in Western medicine) for the treatment of pathologies. However, nowadays this tends to change, due to the high consumer demand for natural products, with fewer side effects and less likely to jeopardize health. This new context increases the cultivation and marketing of medicinal plants worldwide, which reflects in the economy of the country (23).

According to data from the World Health Organization (WHO), 70-95% of the world's population uses plants and their derivatives in primary health care. In their constitution, medicinal plants have bioactive molecules (active substances) holding therapeutic activity, holding the medicinal properties to the plant (23). The therapeutic activity of the plants, is often related to the capacity of these substances to treat, diagnose and prevent physical and mental diseases (24). The secondary metabolites synthesized by the plants are a source of interest for the current pharmaceutical industry (20).

In more remote regions of the globe, mainly in regions inhabited by native populations (indigenous population), an ancestral knowledge, which is transmitted from generation to generation, still prevails over the use of the plants of their habitat. This knowledge, often non-scientific, led many researchers to deepen their studies based on plants in order to know better their properties and mode of action, thus avoiding or reducing the risks of toxicity triggered by lack of knowledge of the compounds (21).

There are approximately 200.000 known secondary metabolites in the plant kingdom. These secondary metabolites are essentially divided into three major chemically distinct groups (terpenoids, phenylpropanoids, alkaloids) and other less represented groups (21,25). Phenylpropanoids are derived from an aromatic 6-carbon phenyl group and a 3-carbon propene tail. A vast number of specialized metabolites are found within this group, such as monolignoids, flavonoids, lignans, phenolic acids and stilbenes (25–27). The Terpenoid group is formed by a sequence of 5-carbon (C_5H_8) blocks called isoprene units. The simple association of 1 isoprene unit or complex associations of 2, 3 and 4 units gives the name to the following metabolite groups respectively: hemiterpenes, monoterpenes, sesquiterpenes and diterpenes (25,27). The Alkaloid group contain nitrogen, which have basic properties many of which are derived from amino acids (25).

In nature an immense diversity of plants exist that are quite accessible to man and have very interesting therapeutic properties. A few examples are *Millettia pinnata, Senna alata* L.*, Curcuma longa* L.*, Ricinus communis* L., *Thymus vulgaris* L., plants that are widely used in daily life and in traditional medicine to treat many diseases including skin ones.

Millettia pinnata: Belongs to *Fabaceae* family and is native to tropical and subtropical regions with a hot and humid climate (e.g. India). The plant is known for its analgesic, antiseptic and anti-inflammatory properties. The fruits, bark, seeds, seed oils, leaves, roots and flowers of the plant are widely used in Ayurvedic medicine to treat eye diseases, ascites, skin diseases, wounds, itching, ulcers and even tumors (28).

Senna alata L.*:* Belongs to *Fabaceae* family. It is found mainly in central America, more precisely in the Caribbean intertropical regions such as the French islands (Guadeloupe, Réunion, Martinique, French Guiana). Leaves and flowers are the most used parts and are used in the treatment of constipation, abdominal pain, infectious diseases, skin diseases (such as dermatitis, rashes, athlete's foot, eczema, mycoses), liver diseases, inflammation, among others (29).

Curcuma longa L.*:* Known as turmeric, it is an herbaceous plant belonging to the *Zingiberaceae* family. It is native to tropical and subtropical regions, being India and China its main producers. Curcumin, an active substance of the plant, is known to have anti-inflammatory, anticancer, antibacterial and antifungal properties, analgesic, disinfectant among other properties (30).

Ricinus communis L.: belonging to the *Euphorbiaceae* family, it is a type of flowering plant. The leaves, roots, seeds, fruits, flowers, upper parts of the plant and the whole plant are used. It is known for its antioxidant, anti-inflammatory, antibacterial, anti-diabetic properties and has been used in the treatment of skin cancer (31).

Thymus vulgaris L.: belonging to the *Lamiaceae* family, it is used both in traditional medicine and in cooking. It is native to Europe and Asia and there are approximately 100 species of *Thymus vulgaris* L. scattered around the world. The white thyme oil, a treated product, is softer on the skin than the common thyme oil. It has many known therapeutic properties and its activity is mainly due to two active substances, thymol and carvacrol. It is used in the treatment of bronchopulmonary and gastrointestinal diseases. Its essential oil is rich in thymol and has high antimicrobial activity essentially against strains of *Salmonella*, *Staphylococcus* spp. and *E.coli*. In the skin it is used to treat oily skin, acne, dermatitis, insect bites, but is used to relieve sciatica, neuralgia and rheumatic pain. (32,33).

1.4. Plant derived products

The bioactive compounds of plants are included in formulations of various products such as cosmetics and drug delivery dosages in order to enhance the curative and preventive properties of the whole products. The active substances are removed from plant in the form of extracts, essential oils or oils, using different extraction methods such as solvent extraction, distillation or pressing (34).

1.4.1. Hydrolates

Hydrolates (also called herbal water or aromatic water) are obtained from the hydro-distillation process of the plant. In this process the vapours of water and essential oil come into contact before condensation and pass through the reservoirs where they will separate in two distinct phases. As a result, part of the hydrogenated components of the essential oil become soluble in water due to their capacity to form hydrogen bonds giving rise to the hydrolate (35). The hydrolates have bioactive molecules and have a softer aroma than the essential oils which makes them widely used in cosmetic formulations. Because they have a low concentration of terpene and sesquiterpene hydrocarbons are quite tolerated by the skin since they do not cause irritation (34). Hydrolates that have antibacterial and antifungal properties do prevent food spoilage and are widely used in the food industry. Hydrolates have other utilities and can be used on the face, in therapeutic and rejuvenating products for example, in body tonics, in lotions, shaving lotion and in aromatherapy to stimulate the brain, through inhalations and massage, among other utilities (34,35).

1.4.2. Essential oils

Essential oils are extracted from flowers, bark, stems, leaves, roots, fruits and other parts of the plant using various methods. They are synthesized by the plants and accumulated in reservoirs, in glands, in specialized cells and even in the intercellular space (36). They have a good capacity to penetrate the skin and once in the blood circulation they will act and cooperate with the elements of the organism in order to re-establish the whole balance. In aromatherapy, essential oils are used against depression, headaches, insomnia, muscular pains, respiratory problems, skin disorders among others. In order to potentiate the absorption of the essential oils, they are used in baths, local application and inhalation (36). Essential oils are very concentrated and effective. They are colourless and have a usually pleasant and quite characteristic odour. They are a heterogeneous mixture of saturated and unsaturated hydrocarbons, alcohol, aldehydes, esters, ketones, phenol oxides and terpenes (36,37). Among the medicinal properties of some essential oils the following are commonly highlighted: antibacterial activity, antifungal activity, anti-inflammatory, antioxidants(36).

1.5. Concept of Cosmetics, Medicines and Phytomedicines

Cosmetic products are distinguished from medicinal products and medical devices and are subject to well-defined rules in order to ensure conformity in production and marketing ensuring they do not present risks to human health and well-being. In the European Union cosmetic products are regulated by Law (EC) No 1223/2009 of the European Parliament and Council, Resolution No 15/CD/2013, Decree Law No 189/2008, September 24 (38). Cosmetic products do not require a prior administrative authorization to be placed on the market, however the person responsible for their manufacture and marketing must ensure that their product complies with the standards described in this law. Each country is responsible for controlling the quality of these products in view of reducing risks to user’s health. In Portugal the organism assuming the supervision role is INFARMED (National Authority for Medicinal Products and Health Products, I.P.) (39).
Cosmetic products are the creams, emulsions, lotions, gels, body oils, make-up products, perfumes, colonies, hair products, soap, bath preparations (salts, gels, mousse), etc. According to Regulation 1223/2009 cosmetic products are defined as "any substance or mixture intended to be in contact with the surface parts of the human body (epidermis, hair and capillary systems, nails, lips and external genital organs) or with the teeth and mucous membranes of the mouth with the sole or mainly purpose of washing, perfuming,

changing their appearance, protecting them, keeping them in good condition or correcting body odours". (39)
More and more people tend to look for natural products to use in their daily lives. Plant-based cosmetics have taken up more and more space in the world market.

According to INFARMED medicines are "... substances or compositions of substances that possess healing or preventive properties of diseases and their symptoms, of man or animal, with a view to establishing a medical diagnosis or to restoring, correcting or modifying their functions" (40). Medicines manufactured from parts of the plant (extracts or fractions thereof, essential oil, dried grass, ...) are called phytomedicines and their marketing is gradually increasing, particularly in some European countries (e.g. Germany, Italy, France, the United Kingdom and Spain), and more recently in the United States of America (USA). Brazil, due to its great biodiversity, plays a very important role in this area because it holds more than 35.000 plant species currently catalogued, which represents a very important source for the development of new phytomedicines that are increasingly effective (22). Although there are already several phytomedicines on the market with proven efficacy, there is still a gap in the complete information of the compounds that constitute the extracts, which confers a disadvantage in relation to the synthetic drugs used until then, and in addition these herbal medicines require a deeper evaluation of their pharmacological properties, which can be quite time consuming (22). The concept of phytomedicine, its characteristics and properties require the integration of various branches of knowledge such as chemistry, biology and physics. Currently *in vitro*, *in vivo* studies, development of new technologies such as chromatography and spectrometry (HPLC), gas chromatography-mass spectrometry (GC-MS), Fourier transform infrared spectrophotometer (FTIR) allow for further analysis of the characteristics of plant extracts (41).

1.6. *Humulus lupulus* L.

1.6.1. Taxonomy and Phylogeny

Humulus lupulus L., is a dioic, climbing, perennial plant, belonging to the *Cannabaceae* family, is included in the genus *Humulus*, order of the Rosales (Figure 3). The *Cannabaceae* family includes 170 species distributed in 11 genera of which we highlight Cannabis, Humulus and Celtis (42). The genus *Humulus* is represented by three species: *Humulus lupulus* Linnaeus (popularly known as hops, and widely used in the beer industry), Japanese hops or *Humulus japonicus,* and *Humulus yunnanensis Hu* (43,44). The genus *Humulus* was classified by Small (1978) into five taxonomic varieties based on its geographical distribution and morphological characteristics, thus showing differences between species from Europe, North America and Asia (45,46).

Figure 3. *Humulus lupulus* L., plant's inflorescence (From (47))

1.6.2. Geographic distribution

Humulus lupulus Linnaeus is a plant native to North America, Europe and Southwest Asia (48). However, it is thought that the genus *Humulus* first appeared in China and then spread to Japan, then to the Americas and Europe, thus explaining its current geographical distribution (43). Although it is native to these regions, it is also cultivated in other regions of the world with a temperate climate (43). The species *Humulus yunnanensis Hu* is thought to have its origin in the mountains of Yunnan province, situated in southwest China. *H. japonicus* is native to Japan, China and neighbouring islands. In Japan it is often used as an ornamental plant (44). The *Humulus lupulus* L. is mainly used in brewing (42). In the United States of America, the largest of *H.lupulus* cultures are observed in the regions of Idaho, Oregon and Washington. In Europe the areas of greatest cultivation are Germany, Great Britain, Poland and the Czech Republic, while in Asia the biggest producers are China and Japan. In the southern hemisphere *H.lupulus* production takes place in Australia and New Zealand (49).

1.6.3. Morphology and reproductive biology

Humulus lupulus L. grows vigorously in the periods between late April and early July in temperate regions, in areas where there is plenty of water and in conditions conducive to growth. They can reach 7-8 m in height, and the roots can reach 100 m in length. The bloom of the *H.lupulus* depends on the length of the day, and in regions further south where the days are shorter the bloom of the plant is limited, in contrast to the northernmost regions where the days are longer an optimal bloom of the plant can be seen, which explains its preferential distribution in the northern hemisphere. It is a plant that grows only in certain altitudes (38°-51°) (49). The optimal growth of the plant occurs at temperatures of 15-18°C, with enough supply of water and nutrients in deep soils (50). It is a diode plant, where the male and female reproductive organs are located in different individuals. The flowers of the female diode plants form an inflorescence called strobilus or cone responsible to produce gametes. The cone consists of membrane stipules and structures like the petals called bracts associated in zigzag around a central axis. As the plant grows, lupulin glands form at the base of the bracts or bracteoles. The lupulin glands (Figure 4), produced by female plants, are fine, yellowish resin containers that are extremely useful in brewing and in traditional medicine, as discussed onwards. It is in the lupulin glands that resins with pharmacological properties and the essential oil are synthesized and accumulated (42,49).

Figure 4. Lupulin glands in the female inflorescence of *Humulus lupulus* L. (From (51)). The Figure represents the morphology of the *H.lupulus* inflorescence with (A) lupulin glands which contain resins and essential oil, (B) bract, (C) strig.

1.6.4. Chemical and physical constitution

Various chemical compounds are found in the cones of *Humulus lupulus* L., including resins, essential oil, proteins, polyphenols, lipids, waxes, cellulose and amino acids. In the petals, proteins, carbohydrates and polyphenols are found. The lupulin glands synthesize and accumulate resins (15-30%) and essential oils (0.5-3%) that are highly appreciated in beer making (35) (Figure 5). Lupulin is the most studied part of the plant for its medicinal properties. The aroma of the flowers is attributed to the volatile oil present in a percentage of 0.3 to 1%. The constituents responsible for its medicinal properties are mostly resins such as humulone, lupulone, and its derivatives such as 2-methyl-3-butenol, tannin, flavonoids, and the essential oil consisting mostly of myrcene, α-humulene, β-caryophyllene and farnesene (41). The chemical composition of the resins and essential oil depend on the species and environmental conditions during the growth and development of the plant (42). Resins, compounds present in the inflorescence of the plant, are divided into two categories: soluble and insoluble resins (42).

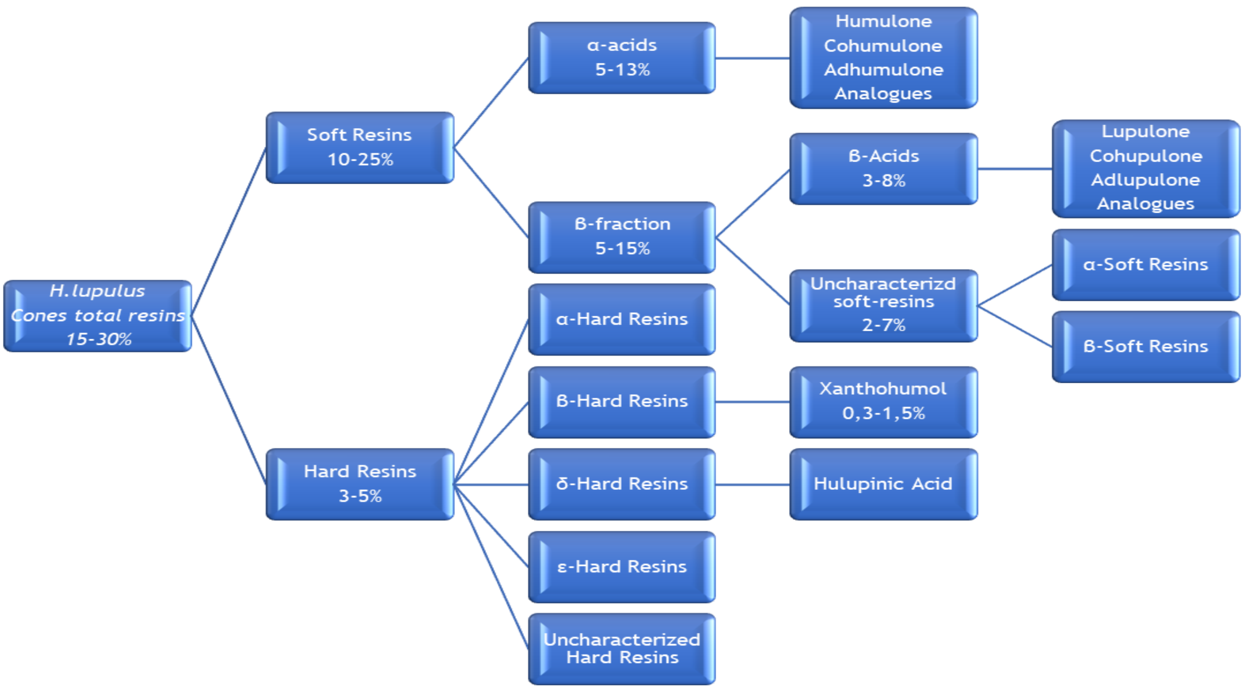

Figure 5. Classification of the resins present in the inflorescences of *Humulus lupulus* L. (From (42)). The inflorescence of *H.lupulus* is soluble in diethyl ether methanol and is where all the resins are accumulated. The resins are essentially divided into two groups, soft resins and hard resins. The soft resins are soluble in hexane while the so-called hard resins are insoluble in hexane and many of them can be soluble in water. The soft resins are present in larger quantities (10-25%) than the rest (3-5%).

The constituents that make up the essential oil are divided into 3 groups: hydrocarbons, oxygenated compounds and compounds containing sulphur. In the hydrocarbon fraction the most abundant monoterpene is β-myrcene (30-60%) and is responsible for the pungent smell of fresh *H.lupulus*. There are other monoterpenes present in smaller proportions such as ocimene, β-pinene, limonene, ρ-cymene and among others (42). Terpenes can also be found in the hydrocarbon fraction, more particularly sesquiterpenes such as α-humulene, β-caryophyllene and β-farnesene. The oxygenated compounds found in the essential oil are divided into 2 portions: the volatile portion and the non-volatile portion. The most abundant alcohols are 2-methylbutanol and linalool, while aldehydes, acids, ketones, epoxides and sterols are also the most abundant (42). Sulphur-containing compounds have a powerful aroma. The phytoconstituents present in hop essential oil are influenced by the genotype of the plant, the biogeographical origin and the biosynthetic processes that result in the accumulation of metabolites present in the mature inflorescence of the plant. For example, the volatile secondary metabolites of the plant originating in Europe are mostly rich in sesquiterpenes, particularly humulene and farnesene, while the North American one is mostly monoterpenoid (52).

Polyphenols, powerful antioxidants, account for 4% of the constituents present in *H.lupulus* inflorescences. The quantity of polyphenols also depends on the geographical area of cultivation (42) (Figure 6).

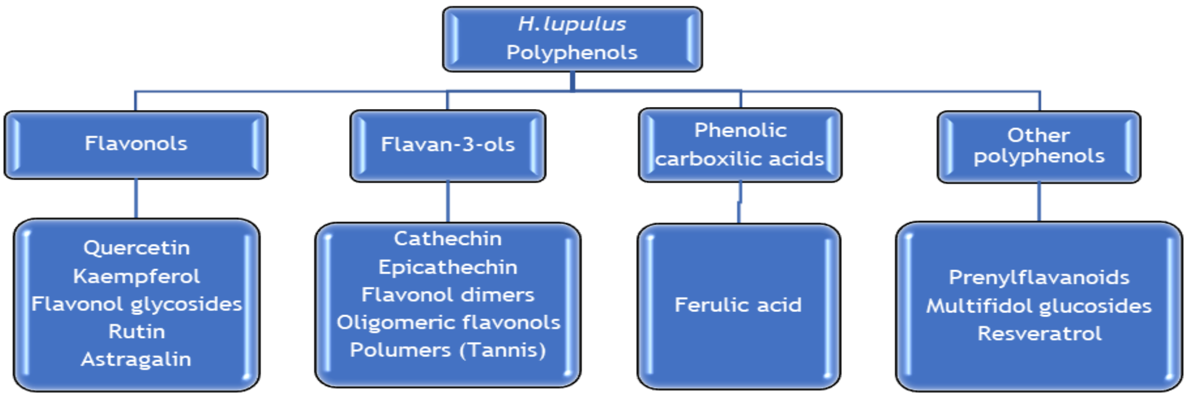

Figure 6. Classification of polyphenols in *H.lupulus* (From (42)**.** Polyphenols that are present in *H.lupulus* cones are divided essentially into four categories: flavanols, flavan-3-ols, phenolic carboxylic acids and others mineurs polyphenols.

1.6.5. Uses of *H.lupulus* in industry and traditional medicine and its effect on economy

Humulus lupulus L. is responsible for the taste and aroma of beers thanks to the bitter resins and essential oil present in the lupulin glands of the plant (53). The acids (α and β) have an inhibitory effect on bacterial growth and are used here to preserve the properties of beer (42). The lupulin glands were firstly described by Ives, where he observed that it was in this region where the bitter and aromatic substances of the plant were accumulated (42).

Although the study and the use of *H.lupulus* is mostly applied for brewing, it also has medicinal properties and can thus be applied both in cosmetic products and in the treatment of some diseases.

According to the European Medicines Agency (EMA) the inflorescence of the female *H.lupulus* is used in traditional medicine to treat symptoms of stress and insomnia (54,55) (appendix 1). *H.lupulus* contain hypnotic components which cause drowsiness (parts of the plant used: the flower, combined with other plants, the fragrance, tea and lupulin). It can also be used to treat mild pains (lupulin), used for its bitter aroma (lupulin, bitter acids, and glands), for its anti-diabetic effects (flowers and rhizome), it is beneficial for the urinary tract (flowers, rhizome, and syrups made from flowers or rhizome). It is also known for its anaphrodisiac properties (lupulin) and in the treatment of skin and against baldness (herbs, tea and hop juice) (49). The bitter acids in *H.lupulus* α and β, humulone and lupulone respectively, are efficient against gram positive bacteria. These acids work best at acid pH and in their non-dissociated form (56). The main prenylflavonoid, xanthohumol, has a high capacity in the elimination of peroxyl radicals (potent antioxidant activity) being in this case more potent than vitamin D and E (57). Xanthohumol also presents antiproliferative, anti-carcinogenic, anti-genotoxic, anti-inflammatory properties, reduces plasma glucose concentration, decreases lipid levels and the weight of white adipose tissue in diabetic mice. *H.lupulus* is also used in the treatment of diabetes because it has been reported that the substance by name isohumulene in *H.lupulus* decreases insulin resistance (58–61). It is also recommended for the treatment of intestinal disorders due to its potential to reduce symptoms associated with menopause because of its estrogenic properties (43). It has recognized antibacterial properties, thus constituting an interesting object of study in the combat of some microorganisms (54). An experimental study was performed in order to verify the antimicrobial properties of flavonoids and their derivatives from *H.lupulus* (62). In this study, seven flavonoids were used, two of which were of natural origin (α, β-dihydroxantohumol and 8-prenylnaringenin) and there was a significant activity against *Staphylococcus aureus* and

Staphylococcus epidermidis with a low MIC80 value of 0.5 µg/ml. The extracts obtained with ethyl acetate, acetone and methanol showed activity against *Fusarium oxysporum*, *F. culmorum*, and *F. semitectum* with a low MIC50 of 0.5 mg/mL (63). In another study performed to analyse the antimicrobial performances of *Humulus lupulus* L. against Gram positive and Gram negative multi-resistant bacterial species and against some yeasts (64), its extracts presented significant activity against Gram-positive bacteria, mainly against their variants resistant to methicillin and vancomycin, but were not effective activity against Gram-negative bacteria . Among all the substances tested, xanthohumol was the one that presented the greatest antimicrobial property (64).

According to the European drug agency, the use of *Humulus lupulus* L. in all its forms is contraindicated for people who have hypersensitivity to the active substance. Due to its sedative power it is consumption shall be avoided by people who are driving or working with machines (55).

In recent years there has been an increase in *H.lupulus* growing around the world due to the strong demand for artisanal beers. According to annual data (2017/2018) from the Barth-Hass group, the largest producer and supplier of *H.lupulus* and its derivate products, there has been an increase of 58.739 hectares in *H.lupulus* production areas with a *H.lupulus* production of 118.401.000 tonnes (65). The inflorescences (flowers) of the female plant are the ones with the highest commercial value being used as raw material in the beer production process and in traditional medicine due to their sedative and calming properties. Male plants are used by growers to develop new improved *H.lupulus* varieties (43,65). The large proportion of *Humulus lupulus* L. use (60%) is in the form of pellets and only 25% are used in extract form, the extract being prepared in ethanol or carbon dioxide, the remaining 15% consisting of modified products and *H.lupulus* flowers which can be marketed in either raw or powder form (49). According to the VCRP 2016 data (Voluntary Cosmetic Registration Program), *H.lupulus* extracts are used in different cosmetic products totalling their application in 362 formulations (299 in leave-one and 60 in rinse-of (hair products such as conditioners, shampoos, facial products and others) and 3 in dilutions for the bath) (48).

1.6.6. Extracts preparation

Several parts of the *H.lupulus* plant are used for various purposes. 25% are used in the form of extracts obtained with ethanol or carbon dioxide, 60% are used in the form of pellets (cut, ground or homogenised), 5% in the form of strobiles (raw, dried) and 25% are modified *H.lupulus* material (49).

The extracts are obtained after harvesting the inflorescence (cone) of the mature plant and are submitted to artificial heat drying so that the water present is reduced from 65-80% to 8-10% so that it can be stored. The extraction can be done with water, ethanol, but also steam and carbon disulphide. Alcohols, chloroform, acetone and hexane can be used as solvents to dissolve the resins (46). Methylene chloride, ethyl acetate and methanol can also be used as solvents (57). From this way the extraction is made by liquid or supercritical carbon dioxide (carbon dioxide fluid subject to critical pressure temperatures). Temperatures of 40°C and 200 bar are considered ideal conditions for the extraction of bitter and volatile compounds (43) (Table 3).

Table 3: Physical and chemical properties of *Humulus lupulus* extracts. (Table from (50)).

Physical form	Oïl, viscous liquid
Colour	Green, brown, yellow, red
Smell	Rough and bitter Citrus, tropical fruits, stone fruit, pine, cedar, floral, spicy, herbs, earth, tobacco, onion / garlic and / or grass
Density20°C	0.883-0.900
Vapoeur density mmHg	>1
Fusion point °C	40-60°C
Solubility in water	insoluble

1.6.7. *Humulus lupulus* extracts types

Different types of *H.lupulus* extract can be obtained by different ways.

Methanol *H.lupulus* extract is obtained by using methanol as solvent. In this method only *H.lupulus* cones and leaves are used. The cones and leaves are firstly air dried in a shady place, then a fraction of them (10g) is placed in a flask with 100ml of methanol (99%) and stirred for 24 hours at temperature room. Using a ultrasonicator the mixture is sonicated for 2 Hours. The mixture is filtered with a filter paper and concentrated in a rotary evaporator at 40°C. The residues are then individually resolved with a minimal volume of methanol and kept at 4 °C until used. The Methanol extract of *H.lupulus* cones and leaves showed higher antioxidant capacity and higher enzyme inhibitions (62).

Other method commonly used is hydro/alcoholic crude *H.lupulus* extract**.** It uses leaves, stems, rhizomes and female cones. This type of extract is known to contain antimicrobial and antifungal activity. To prepare hydro/alcoholic extract the entire plant is dried for ten days at room temperature and then different parts of the plants are separated, powderized and stored in the dark. Extract of different parts are obtained with addition of EtOH/ H_2O (9:1). The mixture is macerated for two hours for three successive times and full night in the dark. After that the solvent extraction evaporation is obtained to produce hydro-alcoholic extracts. To prepare aqueous extracts (liquid/liquid extraction) methylene chloride (MC) is used as solvent in proportion CH_2CL_2 / H_2O (5:5). Anhydrous sodium sulphate ($NaSO_4$) is added to organic phase, enriched in non-polar phenolic compounds to remove traces of water. Then the filtration and evaporation are performed. After filtration, MC is evaporated, and the aqueous phase is freeze-dried, to obtained two sub-extracts with different percentages yields (48).

To obtain essential oil from *H.lupulus*, the cone powder is used. This essential oil is obtained by hydrodistillation over four hours. Other way to obtain *H.lupulus* extract is by supercritical CO_2 extraction. The supercritical CO_2 is generated by pressurizing liquid CO_2 with a pump and heating it up in a heat exchanger. The solvent flows through the extractor, which is charged with biomass. The homogenous CO_2/extract mixture is separated through pressure reduction into a gaseous CO_2 and an extract phase. The extract can be removed from the process while the gaseous CO_2 is recycled again (56,62). It is possible to prioritize the extraction of a given component over others by adjusting only the temperature or the pressure of the process. The use of CO_2 at lower temperatures (down to about 10°C) and pressures is generally referred to as liquid CO_2 extraction (57).

II. OBJECTIVES

This study has as main objective to evaluate the phytochemical, cytotoxic, antioxidant, anti-inflammatory and antimicrobial profile of four extracts of *Humulus lupulus* L.: the flower hydrolate, the flower aqueous extract, the mix (mixture of leaves, stems, flowers) aqueous extract and the mix hydrolate in order to be able to include *Humulus lupulus* in pharmaceutical products destined to treat and prevent skin diseases, while respecting the characteristics and the microflora of the skin.

In addition, as part of a learning process, all the technical-laboratorial skills acquired along the way also count as objectives of the development of this dissertation.

This project is framed in a model of circular economy where it is intended to develop and invest in quality pharmaceutical products, of natural origin, biodegradable, which aims to take advantage of resources (mainly national natural resources) thus focusing on the reduction, reuse and recycling (3R policy) avoiding waste and respecting the environment (eco-friendly) and the consumer.

III. MATERIALS AND METHODS

3.1. General considerations

The four extracts in this study, flower hydrolate, flower aqueous extract, mix (mixture of leaves, steam and flowers) hydrolate and mix aqueous extract of *H.lupulus* were produced by the Plant Biotechnology Center of Beira Interior located in Quinta da Sr[a.] de Mércules, Castelo Branco. The aqueous extracts and hydrolates were obtained by hydrodistillation method, from *H.lupulus* plant parts that were kindly donated for this work from producers. The compounds used for the mix extract correspond to the surpluses that no longer had use. Throughout this work, the four extracts were submitted to the following evaluations (Table 4).

Table 4: Biologic activities analysed in *H.lupulus* extracts

Extracts	Phytochemical characterisation	Antimicrobial activity	Cytotoxic activity	Antioxidant activity	Anti-inflammatory activity
Flower hydrolate (FH)	X	X	X	X	X
Flower aqueous extract (FE)		X	X	X	X
Mix hydrolate (MH)	X	X	X	X	X
Mix aqueous extract (ME)		X	X	X	X

3.2. Phytochemical Characterisation

The purpose of this assay was to determine the chemical constituents of the *H.lupulus* extracts in order to better understand their therapeutic properties and their applications.
The phytochemical characterization of the flower hydrolate and the mix hydrolate was performed by the Polytechnic Institute of Castelo Branco (IPCB) in collaboration with the Plant Biotechnology Center of Beira Interior. Phytochemical characterizations of all compounds were performed using the GC-MS method according to the protocol (66). The GC-MS is indicated for the analysis of different types of compounds whose samples have a low molecular weight and are volatile or can be transformed into volatile compounds from derivatization. It is a quantitative, rather efficient, sensitive and reproducible method. The analysis of chemical constituents is based on the retention time of the sample as well as on the mass spectrum (specific fragmentation pattern of a compound) that is generated once the compounds are subjected to fixed electron stresses that is usually -70 eV (67,68). It is a

very advantageous method since it allows the creation of a database from the fragmentation spectra and sharing it among users (68).

The volatile hydrolate profile of the flower and that of the mix of *H.lupulus* was obtained in triplicate from GC-MS (GC-MS SCION -SQ 456 GC). Separation of all compounds was performed from a HP-5MS fused silica capillary column (Agilent J&W GC Columns), 30 m long, 0.25 mm in diameter and 0.25 µM thick using helium as a carrier gas with a flow rate of 1 ml/min. The hydrolates were injected at a concentration of 1 mg/ml (1µl), in a ratio of 1:5. The oven temperature was initially programmed at 45°C increasing gradually by 3°C/min and once it had reached 175°C the temperature was increased from 15°C/min to 300°C and remained at this temperature for the final 10 minutes. The injector and detector temperatures were maintained at 220 and 250°C respectively. Three different analytical methods were used for the identification of volatile compounds: comparison of the mass spectrometers obtained with the mass spectrometers from the NIST 17 database included in the equipment's software, comparison of the mass spectrometers with the kovats retention index (NIST 17) and the comparison of these with the experimental kovats index acquired from a series of alkanes injected according to the same methods as the sample, other compounds were identified by comparison with standards. The results for the relative amount of each compound were expressed as a percentage of the relative area of the compound peak relative to the total area of the compound peaks identified in the sample and as a percentage of the relative area of the compound peak relative to the majority compound peak area.

3.3. Antimicrobial Activity

The aim was to identify the antimicrobial profile of *H. lupulus* extracts and the lowest concentration of the compound in culture medium that inhibits the growth of microorganisms (MIC) when placed in contact with bacterial strains such as *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 9027), *Escherichia coli* (ATCC 8739), *Staphylococcus epidermidis* (ATCC 178970) and *Cutibacterium acnes* (DSM1897), bacteria that are naturally present in the skin microflora and in some cases may be related to the appearance of skin disorders.

The determination of the antimicrobial activity of *H.lupulus* was carried out with the microdilution method, using microplates of 96-wells. It is a simple, effective, inexpensive test that allows us to determine in a short time the bacterial proliferation or its inhibition. The protocol was adapted from methodologies previously described (69,70). At least three independent assays were performed for each test.

For this assay, culture media Tryptic soy agar (TSA, 40g/1L, Sigma-Aldrich), Mueller-Hinton agar (MHB, 21g/1L, Sigma-Aldrich) and Brain heart infusion broth (BHI, 37g/1L, Sigma-Aldrich) were prepared in distilled water (Water Milli-Q) and sterilised by autoclave in a high temperature (121°C, 15 minutes). The bacterial strains *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli* and *Pseudomonas aeruginosa* were plated and incubated in Petri dishes containing TSA for 18 to 24 hours at a temperature of 37°C in aerobic conditions, optimal conditions to the strains growth. The *Cutibacterium acnes* strain was plated on BHI supplemented with 5% Glucose in Petri dishes and incubated for 72 hours in an anaerobic environment at 37°C. The aqueous extracts and hydrolates were prepared in culture medium (MHB or BHI for *Cutibacterium acnes*) in serial dilutions of 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 (six different concentrations of each extract under study). The different controls: negative, sample sterility and positive control were prepared using culture media, aqueous extracts/hydrolates diluted in media (1:2) and culture media in presence of cells respectively. The dilutions in test and the controls were pipetted in duplicate in a 96-well plate. Each well was inoculated (except in the negative control and sample control) with $1.5x10^8$ CFU/ml of a previously prepared bacterial suspensions in sterile NaCl 0.85% solution and that turbidity was normalized using a McFarland densitometer to 0.5 units. A first reading of the absorbance was performed before the incubation, T0 hours, in an ELISA microplate reader at an optical density of 600 nm. The plates were incubated for 24 hours or 72 hours to *C.acnes*, after the recommended time for the growth of the strains, the content of each well was resuspended and a new reading at 600 nm was made in the ELISA reader (T24 hours or 72hours for *C.acnes*). Visually all plates were validated, thus presenting the translucent negative and sterility controls of the sample and the turbid positive control due to strain growth. The antimicrobial activity and the MIC50 were analysed using GraphPad prism parameters.

3.4. Cytotoxic effect

Cell lines culture of skin fibroblasts (3T3, ATCC® CRL1634™) and macrophages (RAW 264.7, ATCC®, TIB -71TM) were obtained firstly plaiting the cells on 25 cm^2 culture T flasks containing DMEM culture medium supplemented with 25 mM of D-Glucose (Sigma-Aldrich), 17.95 mM of sodium bicarbonate for Raw cells and 44.27mM of sodium bicarbonate for 3T3 cells lines (Sigma-Aldrich), 100U/ml of penicillin and 100 Ug/ml of streptomycin (Sigma-Aldrich) and 10% of foetal bovine serum (FBS, Sigma-Aldrich). The culture medium was stabilised at a pH of 7.2 and then filtered using a 0.22 µm membrane filtration unit. Cells were kept at 37 ºC in a 95% air and 5% CO_2 humidified atmosphere. Sub culturing was performed according to ATCC recommendations. After the cells have reached the desired confluence (~80%) the number of viable cells was evaluated counting trypan-blue excluding cells, and the cells were used for the *in vitro* assays described below.

The aim of this study was to evaluate the cytotoxic potential of *H.lupulus* aqueous extracts and hydrolates in mouse 3T3 fibroblast (3T3, ATCC® CRL1634™) and mouse macrophage (RAW 264.7, ATCC®, TIB -71TM) cell lines, using the MTT (Sigma-Aldrich) colorimetric method. The protocol was adapted from methodologies previously described (71). The results will be expressed as a percentage of metabolic activity.

To determinate cellular viability, the cells lines were seeded ($1x10^4$ for 3T3 cells and $5x10^4$ for RAW cells) in microplates with 96-wells containing a mixture of DMEM media and *H.lupulus* aqueous extracts and hydrolates at a ratio of 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128. The cells were then incubated for 24 hours, in a 95% air and 5% CO_2 humidified atmosphere. The cytotoxic assay was performed using 10 µl of MTT for every 100 µl of medium in a 5 mg/ml stock solution in each well. Then the plates were incubated at 37°C for 4 h in a humidified incubator containing 5% CO_2. Thereafter, Dimethyl sulfoxide (Sigma-Aldrich) was added to each well and mixed in order to dissolve the dark blue formazan crystals. Formazan quantification was evaluated using the xMarkTM Microplate Absorbance Spectrophotometer (Bio-Rad) at 570 nm, with a reference wavelength of 620 nm, and the results were expressed as percentage of metabolic activity in relation to the group control. The viability of the cells with the concentration of the extracts 3.12% was higher than 80% and was chosen for the next stimuli.

3.5. Antioxidant activity

3.5.1. Evaluation of ROS production

The objective was to evaluate the antioxidant effect of *H.lupulus* aqueous extracts and hydrolates testing the inhibition of ROS production when the cells (macrophages) are exposed to an external source of oxidative stress.
The production of intracellular ROS was quantified through the 2′,7′-Dichlorofluorescin diacetate (H2DCFDA, Sigma-Aldrich), a cell-permeable non-fluorescent probe that is rapidly oxidized to the fluorescent 2′,7-dichlorofluorescein in the presence of intracellular ROS and the Hoechst nucleic acid stain (Hoechst, Sigma-Aldrich) which is cell-permeant nuclear and emits blue fluorescence when bound to dsDNA. The oxidative stress was induced by the introduction of LPS in the medium. The LPS present in the environment

induces an inflammatory response by the macrophage cells and thus increases the production of ROS levels, which in excess causes cell toxicity (20).

For this assay $2x10^4$ RAW Cells /well were plated in 96-well black plates with clear bottom during 24h, and then treated with 3.12% of *H.lupulus* aqueous extracts and hydrolates simultaneously with 1 ug/mL of LPS for 18h. After treatments, the cell medium was removed and replaced with sterile Hanks' Balanced Salt Solution (HBSS) containing 5 μM H2DCFDA and 0.5 g/mL Hoechst Solution for 30 min at 37ºC in the dark and in a humidified atmosphere with 5% CO_2. The solution with the dye was replaced by HBSS and the fluorescence was measured in a xMarkTM Microplate Absorbance Spectrophotometer (Bio-Rad) at excitation and emission wavelengths of 485/530 nm and 350/461, respectively. The results are expressed as percentage of control of the ratios H2DCFDA / Hoechst absorbances in each condition.

3.6. Anti-inflammatory activity

3.6.1. Evaluation of NO production

This part aims to evaluate the anti-inflammatory profile of *H.lupulus* aqueous extracts and hydrolates against the production of NO by macrophages (RAW cell line), during an inflammation process. The inflammation process was induced by inflammatory LPS. Macrophages in healthy individuals when in contact with LPS endotoxin promote an inflammatory response that translates into NO production by nitric oxide synthase enzymes (NOS).
Cells were seeded at a density of $1.5x10^5$ cells/ml in 48-well plates and exposed to the *H.lupulus* extracts and with DMEM medium in presence or absence of LPS (1 μg/mL), for 24h. Then NO production was further determined in supernatants using Griess colorimetric nitrite assay·, mixing equal volume of cell culture supernatant to an equal volume of Griess reagent (Reagent A prepared with Sulphanilamide 1% (w/v) in phosphoric acid 5% (v/v)) and Reagent B prepared with 0.1 g N-1-naphthylenediamine 0.1% (w/v)). Then the plate was placed in the dark, at room temperature (RT) for 30 minutes. The absorbance was measured at 550 nm and nitrite concentration was calculated through a regression analysis of a sodium nitrite standard curve ($NaNO_2$).

3.6.2. Evaluation of scavenging activity

This assessment should be made if there is a decrease in NO from the medium. The capacity of the extracts of *H.lupulus* to remove NO from the medium through a NO donor, *S*-Nitroso-*N*-acetylpenicillamine (SNAP, Sigma-Aldrich) was evaluated with this method. Nitrite (NO_2^-) production was then evaluated by Griess Reagent. This is a cell-less assay.
The scavenging activity was performed using a microplate with 48 wells. A stock solution of SNAP at 100 mM in DMSO was prepared and added in each well containing previously DMEM medium control and the DMEM medium with *H.lupulus* extracts. The plate without cells was Incubated for 3 hours at 37°C, in a 95% air and 5% CO_2 humidified atmosphere. After the Griess test was performed, the fluorescence was measured in a xMarkTM Microplate Absorbance Spectrophotometer (Bio-Rad). The results were presented in percentage of nitrites levels present in the medium with and without SNAP.

3.6.3. Analysis of proteins expression by Western blotting assay

The objective of this evaluation is to identify the proteins related to the inflammatory process (COX-2) in samples treated with the different types of aqueous extracts and hydrolates. The cells were seeded at a density of $7x10^4$ cells/well in 6-well plates. 1ug/ml of LPS and a concentration of 3.12% of the aqueous extracts and hydrolates under study were added to each well. After 24 hours the cells were collected for protein quantification in order to proceed with the western blot.

3.6.3.1. Total Protein Extraction

Total protein was extracted from mouse skin macrophages cells using the radioimmunoprecipitation assay buffer (RIPA buffer) (150 mM NaCl, 1 % Nonidet-P40 substitute, 0.5 % Na-deoxycholate, 0.1 % SDS, 50 nM Tris, 1 mM EDTA) supplemented with 1% protease inhibitors cocktail (AppliChem, Darmstadt, Germany) and 10 % phenylmethylsulphonyl fluoride (PMSF) (Fisher, Darmstadt, Germany). The samples were kept on ice for 30 min and occasionally mixed. Then, samples were centrifuged at 14000 rpm for 20 min at 4° C to caused rupture of the nuclear membrane and eliminate any insoluble cellular debris in order to extract total proteins. Proteins present in supernatant, were recovered to a new Eppendorf tube. Total protein concentration was assessed using BCA Protein Assay Kit (Prod#23225, Lot#SA244529, Thermo Scientific). In a 96-well plate, 1 µL of protein sample was mixed with 80 µL of recently prepared working kit reagent and 19 μL of milli-Q water to reach a total volume of 100 μL. 1 μL of RIPA buffer was added instead of 1 μL of protein sample to be used as the blank. The absorbance was measured spectrophotometrically (xMark™ Spectrophotometer, Bio-Rad, Hercules, CA, USA) at 562 nm. The calibration line for protein quantification was obtained in the same way using serial concentrations of bovine serum albumin (BSA).

3.6.3.2. Western Blot Analysis

Total protein (30 μg) of all cell lines were heat-denatured at 100° C for 5 min and resolved on 12.5 % (or 8 % in case of high molecular weight proteins) sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The electrophoresis was performed at 120V for 1 hour and 30 minutes approximately. Then, proteins were electrotransferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad) at 750 mA for 1 h and 30 min. Membranes were blocked with 5 % skimmed dried milk for 1 h and then incubated overnight at 4° C with primary antibodies: rabbit anti-COX-2 (1:10000). The membranes were washed 3 times with TBS-T buffer (mixture of tris-buffered saline and Tween 20) for 10 minutes each in order to remove any unspecific binding and then were incubated with the respective secondary antibody (anti-rabbit or anti-mouse) for 1 hour at room temperature (RT). The anti-GAPDH (1:5000, Sigma-Aldrich) antibody was used for protein loading control in all blots. At the end, membranes were washed, incubated with ECL substrate (Enhanced Chemiluminescence, Bio-Rad) for 5 min, and scanned with the Chemidoc™ MP Imaging System (Bio-Rad). Band densities were obtained by the volumetric analysis tool of Bio-Rad Image Lab 5.1 software and normalized with the respective GAPDH band density.

3.7. Statistical Analysis

Statistical analysis of all results was performed with GraphPad prism v7.03 software (GraphPad Software, San Diego, California, USA). Statistically significant differences between the groups tested were obtained by applying t-Student test and the ANOVA ONE WAY, as applicable. Differences were considered significant when P-value values were $p<0.05$ (*), $p<0.01$(**), $P<0.001$ (***) and $p<0.0001$(****). The experimental data are shown as mean ± standard deviation (SD).

IV. Results

4.1. Chemical characterisation

Humulus lupulus L. flower hydrolate and the mix hydrolate (stems, leaves and flowers) were subjected to an analysis of their chemical composition using the gas chromatography method coupled with a mass spectrophotometer (GC-MS, SCION -SQ456 GC Bruker). The separation of the compounds was performed using a silica capillary column (Agilent J&W GC columns) and the identification of the compounds was determined by comparison with the NIST 17 database and by comparison of the Kovats indices. The results were expressed as a relative percentage of the total compounds identified.

In the mix hydrolate (stems, leaves and flowers) 69.94 % of the chemical compounds were identified, the major compounds found being caryophyllene, humulene and humulene II. In the hydrolate of the flower 80.07% of the total compounds were identified, being the majority compounds cis-linalool oxide, trans-linalool oxide, linalool, ρ-Mentha-1.8-dien-7- and humulenol II (Table 5).

Table 5. Chemical characterisation of the mix and flower hydrolates of *Humulus lupulus* L. by gas chromatography-mass spectrometry (GC-MS). The relative amount of each compound expressed in percentage (%) is obtained through the relative area of each compound and the total peak area of the compounds identified in the samples.

Compounds name	Flower hydrolate	Mix hydrolate (stems, leaves and flowers)
	(Relative area in %)	(Relative area in %)
cis-Linalool oxide	11.32	-
trans-Linalool oxide	8.50	-
Linalool	10.76	1.12
Fenchol	0.55	-
endo-Borneol	2.45	-
α-Terpinol	2.55	-
Geraniol	5.05	-
p-Cymen-7-ol	0.80	-
p-Mentha-1,8-dien-7-ol	7.37	-
Geranyl acetate	0.51	-
Humulene	2.88	-
Humulene epoxide I	0.64	-

Humulenol II	20.83	46,90
tau-Cadinol	0.85	1 .23
2-Undecanol	-	0.80
Copaene	-	1.14
Caryophyllene	-	6.18
Humulene	-	18.02
ϒ-Muurolene	-	2.38
β-Selinene	-	2.26
α-Selinene	-	2.57
2-Tridecanone	-	0.93
α-Muurolene	-	0.92
ϒ-Cadinene	-	2.78
δ-Cadinene	-	4.03
Carryophyllene oxide	-	4.55
Humulene epoxide I	-	2.09
Humulene epoxide II	-	7.33
α-Cadinol	-	1.69
% Total compounds identified	**80.07%**	**69.94%**

4.2. Antimicrobial activity

The viability of bacterial strains was expressed in percentage (%) of viability in relation to the control group and it was measured in different time-points (after t24 hours and after t72 hours of incubation). From the results obtained, it was observed that the mix aqueous extract (stem, leaves and flowers) did not alter the viability of the *S.aureus* (Figure 7, A) and *C.acnes* (Figure 11, A) strains after treatment with different extract concentrations (0.78, 1.47, 3.13, 6.25, 12.50, 25 and 50%). However, specifically for *E.coli* (Figure 7, B) strains treated with concentrations between 6.25-50% and *P.aeruginosa* (Figure 7, C) treated with 25-50% of the extract a significant increase in viability was observed (%). For the *S.epidermidis* strain (Figure 7, D) it was found that after being stimulated with 1.56 and 6.25 % of the extract viability decreased significantly compared to the control group but stimulated with concentrations of 3.13, 12.50, 25 and 50% of the extract viability increased significantly. There was observed a significant decrease in viability (%) of

S.aureus strains after being treated with 1.56%, 3.13%, 6.25% and 12.50-50% of aqueous flower extract (Figure 8, A). For *E.coli*, *P.aeruginosa* strains (Figure 8, A and C) and for *C.acnes* (Figure 11, B) the aqueous flower extract had no significant effects. Antimicrobial effect was observed against *S.epidermidis* strains after being stimulated with different concentrations of the extract (0.78-50%) presenting MIC_{50} at 50% of the extract. *S.aureus*, *E.coli*, *S.epidermidis* and *C.acnes* strains did not present significant changes in viability (%) after contact with the different concentrations (%) of the mix hydrolate (Figure 9, A, B and C), but for *P.aeruginosa* strains a significant decrease in viability was observed when treated with 12.50-50% of the hydrolate. Flower hydrolate in high concentrations (25-50%) significantly decreases the viability of the *S.aureus* strain presenting MIC_{50} at 50% of the hydrolate concentration (Figure 10, A). Antimicrobial activity against *E.coli* (Figure 10, B) and *C.acnes* (Figure 11, D) was not observed after the stimulus with the different concentrations of the flower hydrolate. A significant decrease in viability of the *P.aeruginosa* strain was observed after being stimulated with a concentration of 12.50% of flower hydrolate (Figure 10, C). For the *S.epidermidis* strain after being stimulated with a low concentration (0.78%) of the flower hydrolate it was observed a significantly decreased in viability, however with the increase of concentrations (12.50-25%) viability increased but not significantly decreasing again to a concentration of 50% of hydrolate in relation to the control group presenting MIC_{50} at 50% of the flower hydrolate (Figure 10, D).

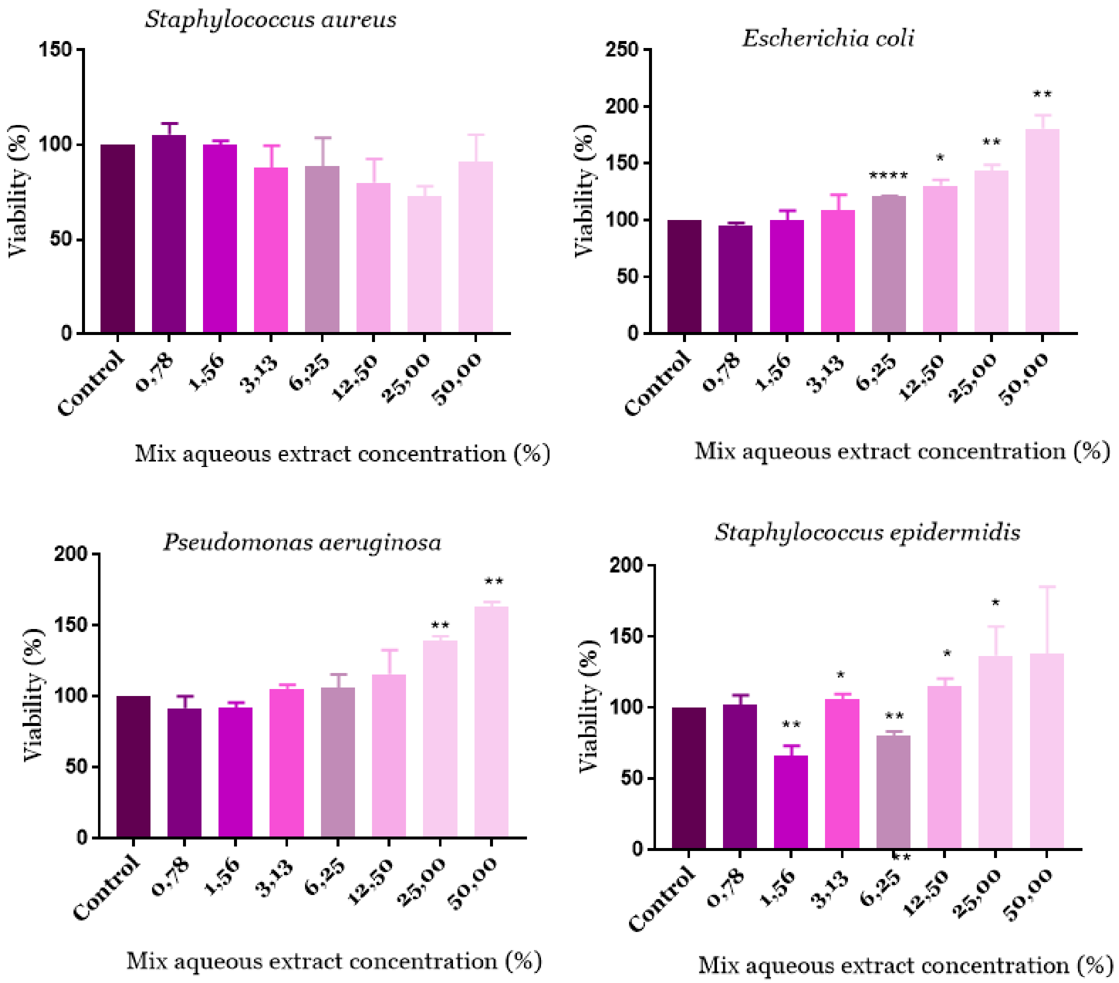

Figure 7. Viability (%) of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* after 24 hours with contact with different concentration percentile of *H.lupulus* mix aqueous extract (steams, leaves and flowers). Error bar indicate mean ± SD. Significantly P-values are expressed like * p<0.05 ** p<0.01 and ***** p<0,0001 in relation to the control group.

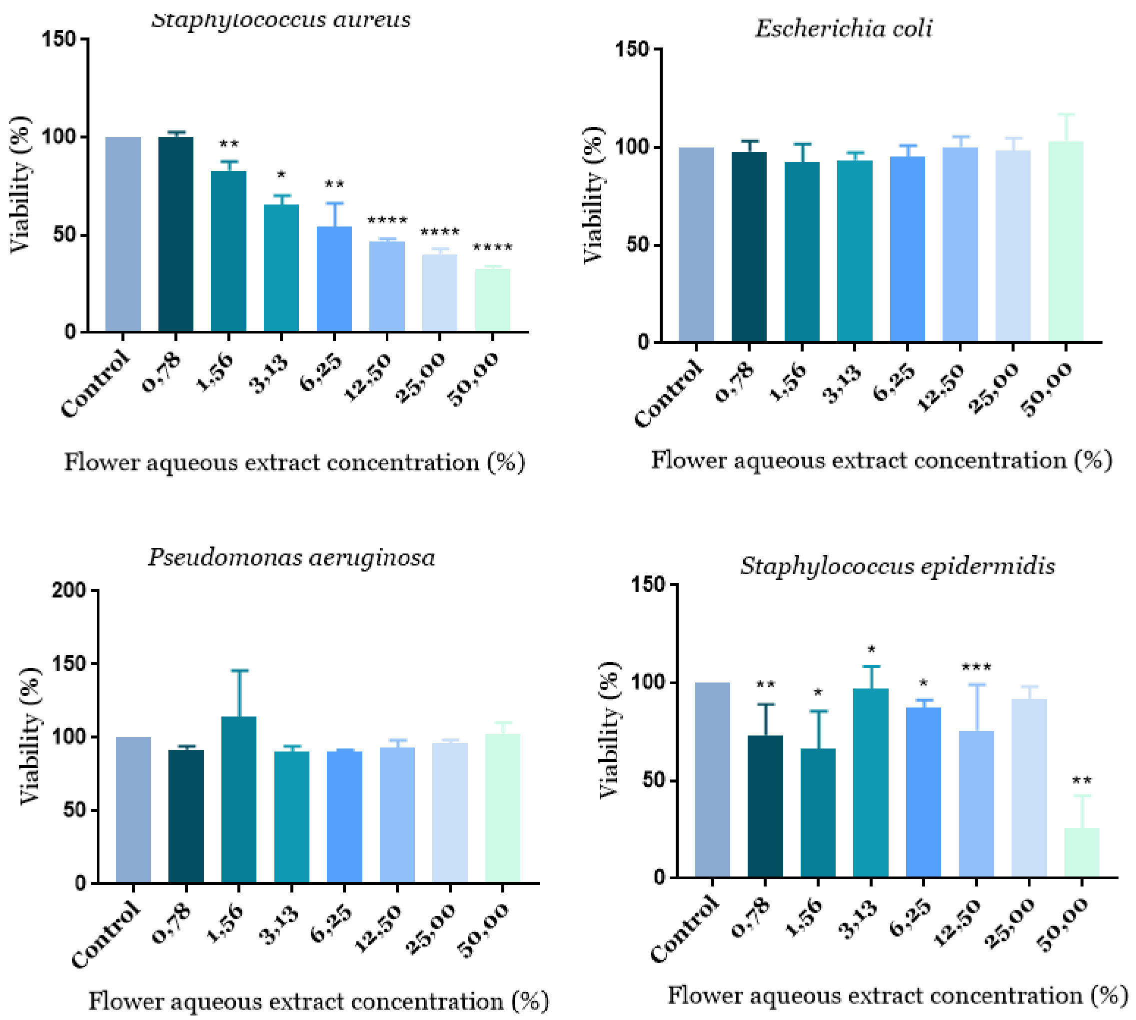

Figure 8. Viability (%) of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Stahylococcus epidermidis* after 24 hours with contact with different concentration percentile of flower aqueous extract. The minimum inhibitory concentration (MIC_{50}) for *Staphylococcus aureus* is above 12.50-50% and for *Staphyloccocus epidermidis* is above 50% of the extract concentration. Error bar indicate mean ± SD. Significantly P-values are expressed like * p<0.05, ** p<0.01), *** p<0.001 and **** p<0,0001 in relation to the control group.

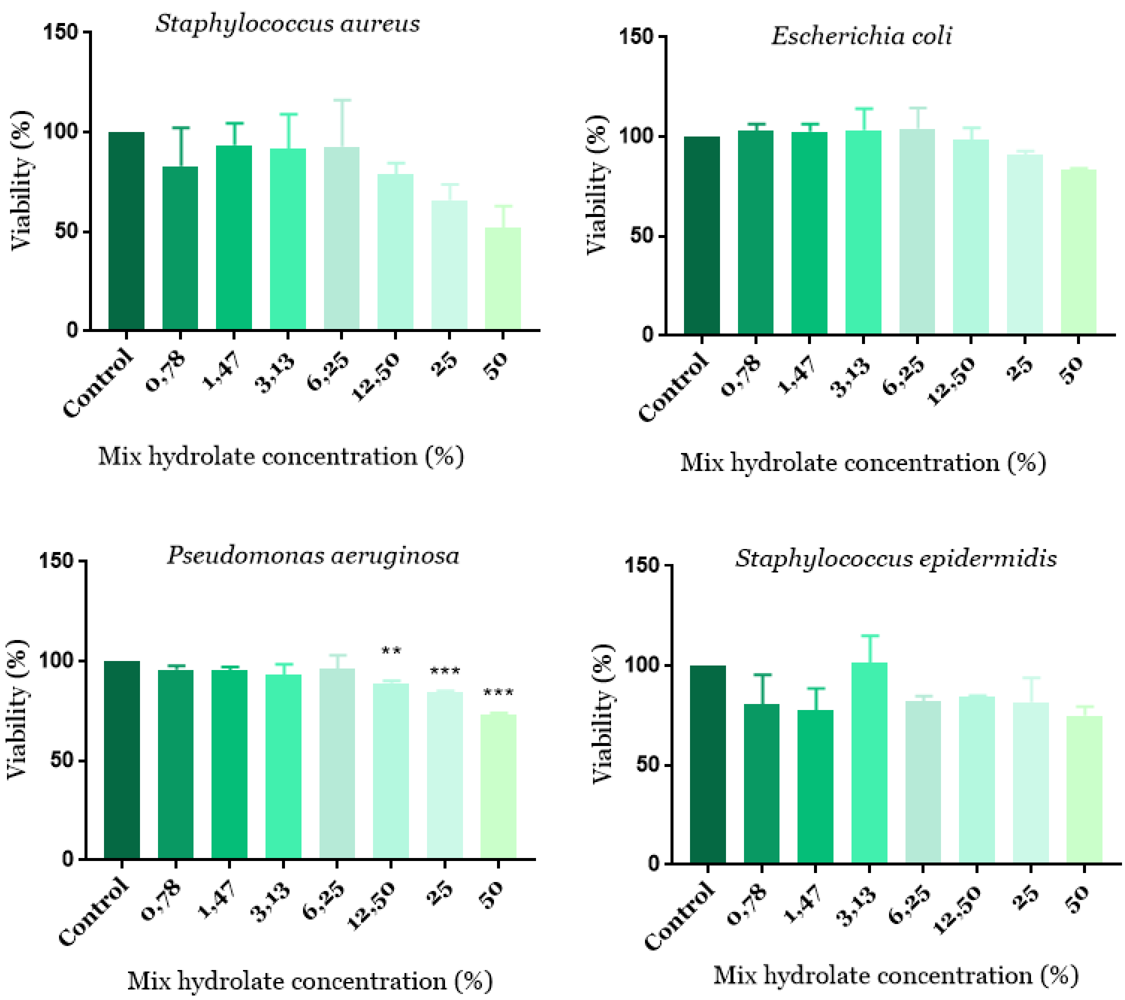

Figure 9. Viability (%) of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* after 24 hours with contact with different concentration percentile of mix hydrolate. Error bar indicate mean ± SD. Significantly P-values are expressed like ** p<0.01, *** p<0.001 in relation to the control group.

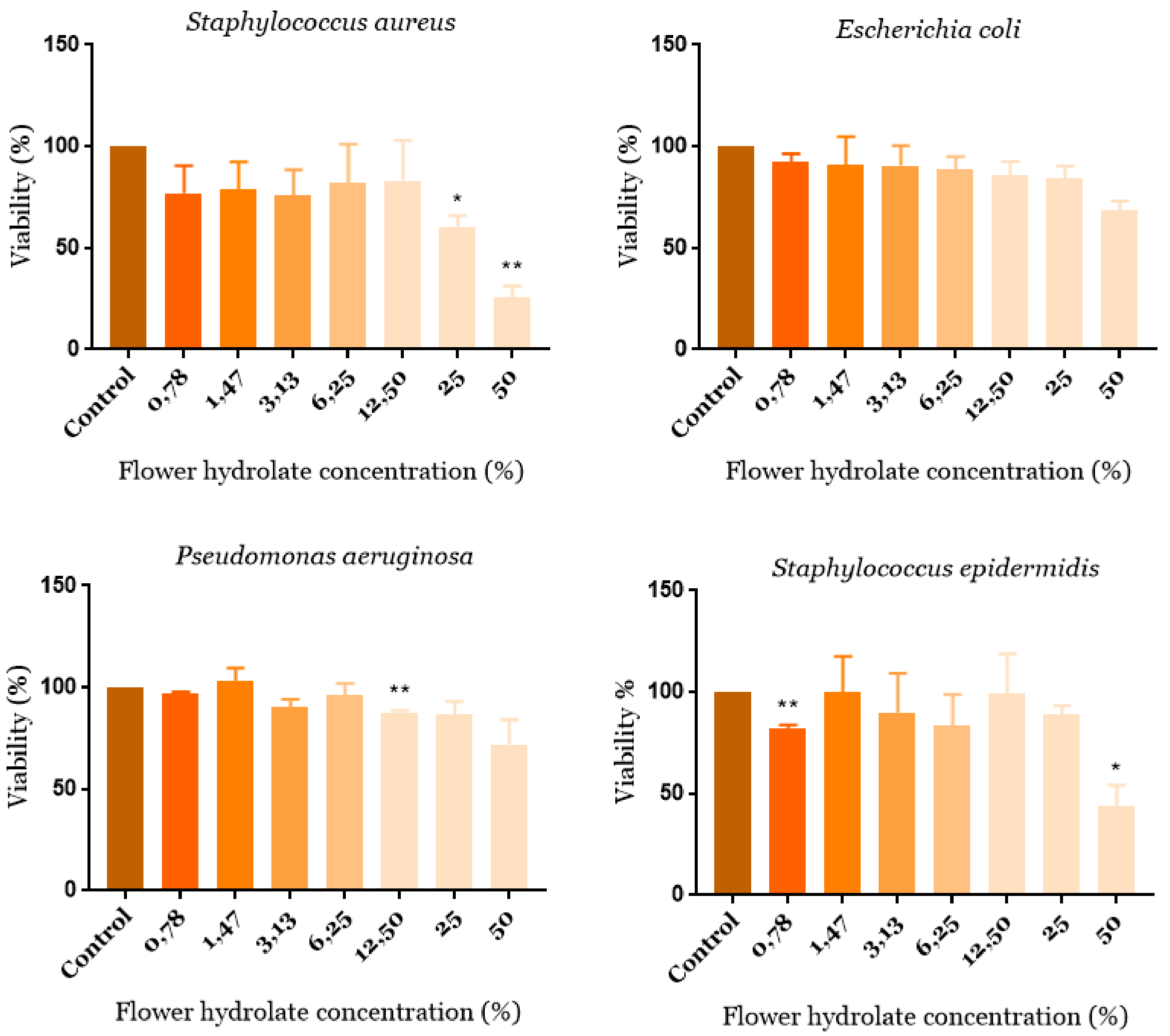

Figure 10. Viability (%) of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Stahylococcus epidermidis* after 24 hours with contact with different concentration percentile of *H.lupulus* flower hydrolate. The minimum inhibitory concentration of flower hydrolate for *Staphylococcus aureus* and *Staphylococcus epidermidis* are 50% for both. Error bar indicate mean ± SD. Significantly P-values are expressed like * p<0.05 and ** p<0.01 in relation to the control group.

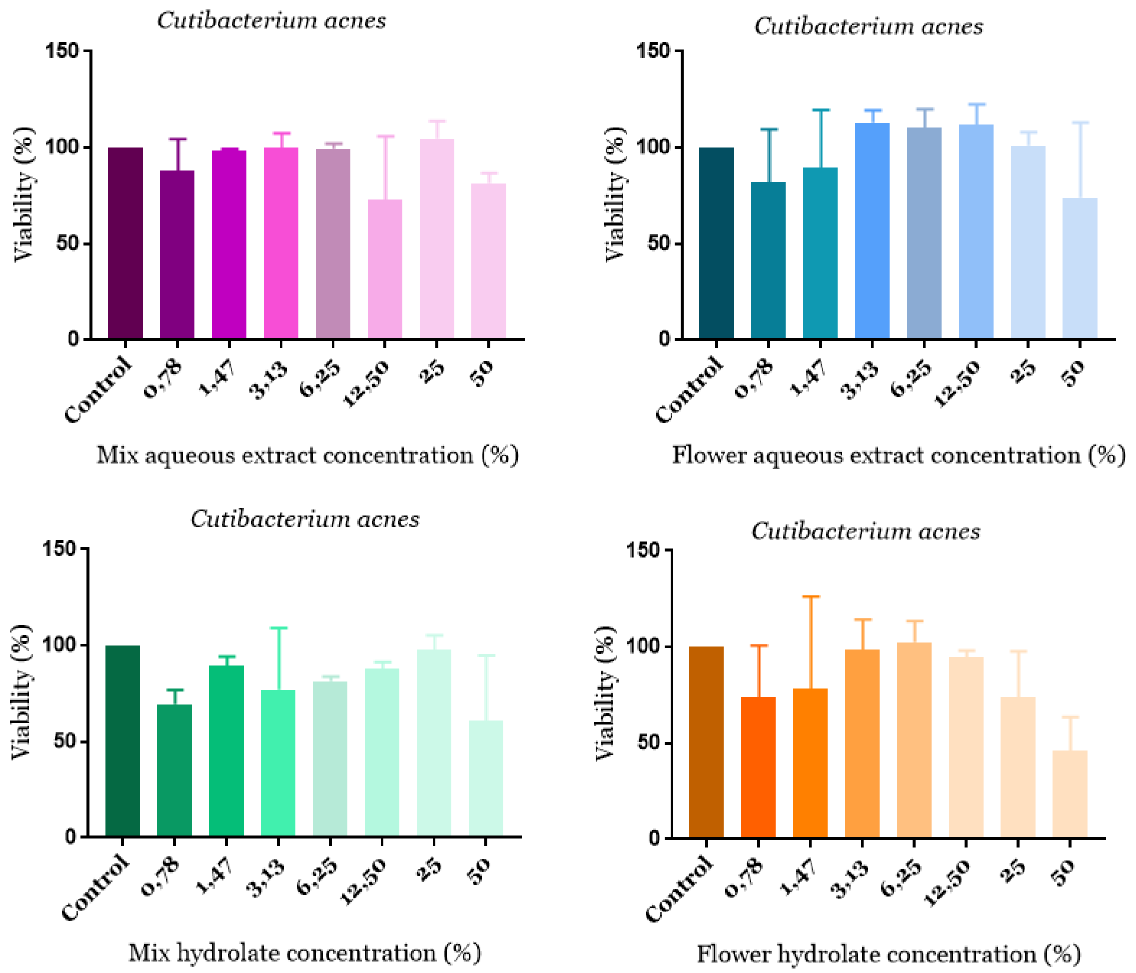

Figure 11. Viability (%) of *Cutibacterium acnes* assay with *H.lupulus* mix aqueous extract, flower aqueous extract, mix hydrolate and flower hydrolate after 72 hours of incubation in 37°C. Error bar indicate mean ± SD.

4.3. Cytotoxic activity

Our results demonstrated that a significant increase in the metabolic activity (%) of 3T3 and RAW cell lines were observed when stimulated with small doses of mix aqueous extract (0.78%-0.625%). With higher concentrations a decrease in the metabolic activity was observed, although not statistically significant (Figure 12, A and B). The metabolic activity of the stimulated RAW cells with different concentrations of flower aqueous extract decreased significantly compared to the control group. In 3T3 cells a significant increase in their metabolic activity was observed when stimulated with high concentrations (50%) of the aqueous flower extract (Figure 13, A and B). In the Raw and 3T3 cells stimulated with high concentrations of the mix hydrolate (25-50%), a significant decrease in their cellular metabolic activity was observed (Figure 14, A and B). With the flower hydrolate it was observed that also in high doses the activity of RAW (6.25%-50%)

and ET3 (25%-50%) decreased significantly (Figure 15, A and B). Of notice that the extract of the flower seems to have a stronger cytotoxic profile than the others (Figure 13, A and B).

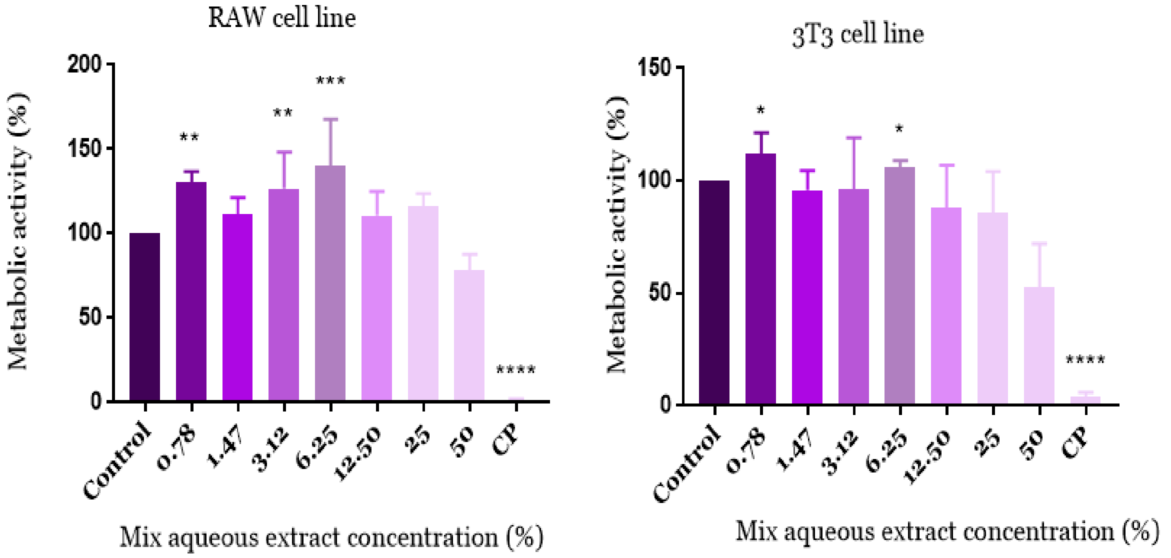

Figure 12. Metabolic activity of mouse skin macrophages RAW cell line and mouse skin fibroblasts 3T3 cell line after 24 hours of *H.lupulus* mix extract stimuli. Cell lines were stimulated with different concentrations of *H.lupulus* extract (0.78%, 1.47%, 3.12%, 6.25%, 12.50%, 25% and 50%), with DMEM medium as negative control and with SDS at 1% as positive control (cell death). Error bar indicate mean ± SD. Significantly P-values are expressed like * p<0.05, ** p<0.01 and *** p<0.001 and ***** p<0.0001 in relation to the control group.

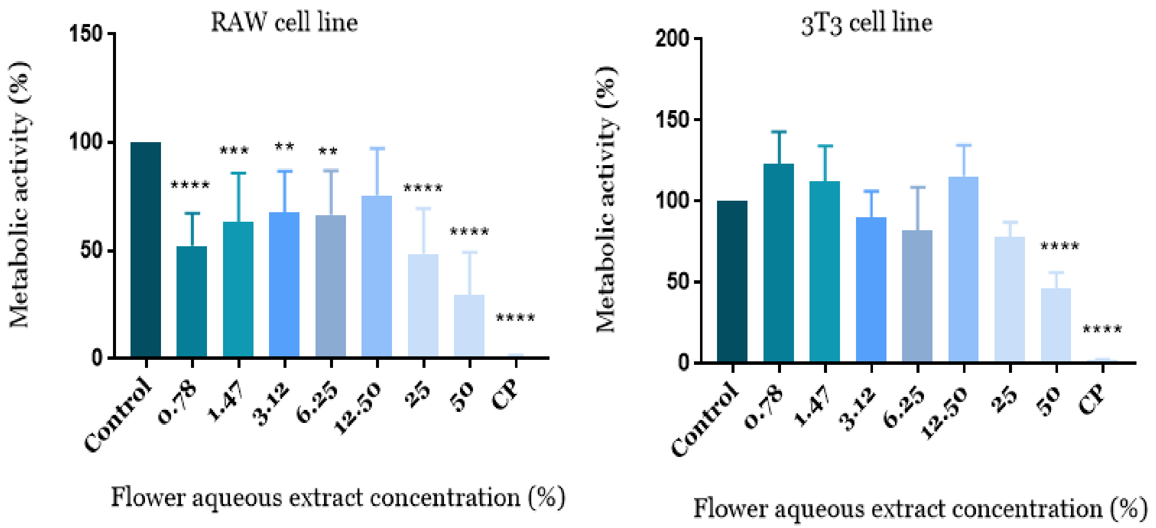

Figure 13. Metabolic activity of mouse skin macrophages RAW cell line and mouse skin fibroblasts 3T3 cell line after 24 hours of *H.lupulus* flower extract stimuli. Cell lines were stimulated with different concentrations of *H.lupulus* extract (0.78%, 1.47%, 3.12%, 6.25%, 12.50%, 25% and 50%), with DMEM medium as negative control and with SDS at 1% as positive control (cell death). Error bar indicate mean ± SD. Significantly P-values are expressed like ** p<0.01 and *** p<0.001 and ***** p<0.0001 in relation to the control group.

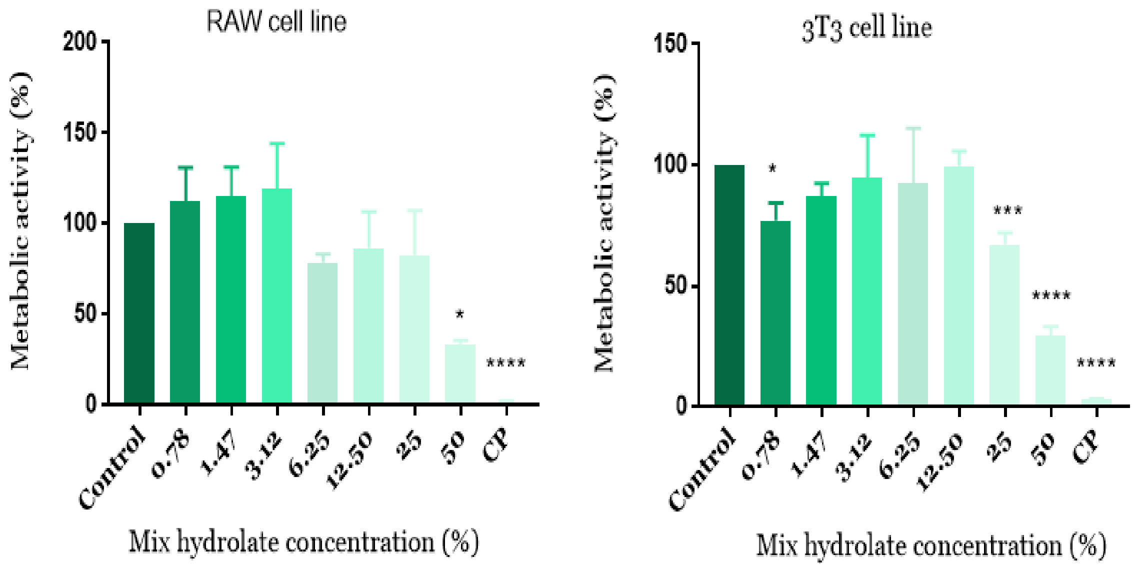

Figure 14. Metabolic activity of mouse skin macrophages RAW cell line and mouse skin fibroblasts 3T3 cell line after 24 hours of *H.lupulus* mix hydrolate stimuli. Cell lines were stimulated with different concentrations of *H.lupulus* extract (0.78%, 1.47%, 3.12%, 6.25%, 12.50%, 25% and 50%), with DMEM medium as negative control and with SDS at 1% as positive control (cell death). Error bar indicate mean ± SD. Significantly P-values are expressed like * p<0.05, *** p<0.001 and **** p<0.0001 in relation to the control group.

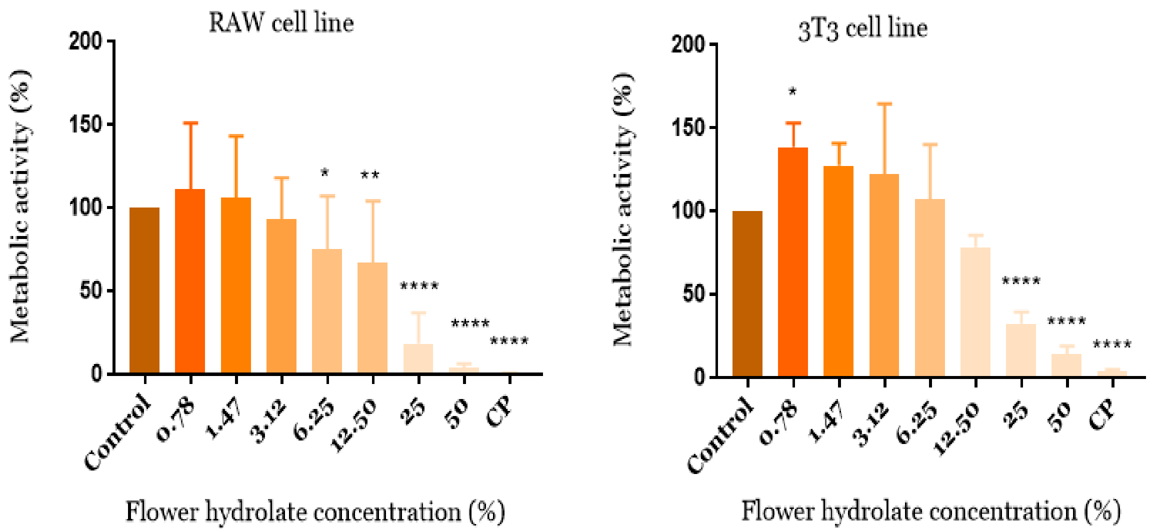

Figure 15. Metabolic activity of mouse skin macrophages RAW cell line and mouse skin fibroblasts 3T3 cell line after 24 hours of *H.lupulus* flower hydrolate stimuli. cell lines were stimulated with different concentrations of *H.lupulus* hydrolate (0.78%, 1.47%, 3.12%, 6.25%, 12.50%, 25% and 50%), with DMEM medium as negative control and with SDS at 1% as positive control (cell death). Error bar indicate mean ± SD. Significantly P-values are expressed like * P<0.05), ** p<0.01 and ***** p<0.0001 in relation to the control group.

4.4. Antioxidant effect

From the results obtained (Figure 16) we observed that the cells treated with the aqueous extract of the mix (Figure 16, A) and with the hydrolate of the mix (Figure 16, C) in the presence of LPS endotoxin there was a significant increase in the levels of fluorescence emitted by the fluorochrome H2DCFDA compared to the respective LPS-free (&&& $p<0.001$) control groups, which means that there was an increase in the synthesis of ROS. In cells treated with the aqueous flower extract (Figure 16, B) in the absence of LPS a significant increase in fluorescence levels emitted was observed compared to the control group (CTR) without LPS (*** $p<0.001$). In the presence of LPS it was observed that in the control group there was a significant increase in fluorescence levels compared to the control group without LPS (&&& $p<0.001$) however it was found that in the group treated with aqueous flower extract and LPS the fluorescence levels were not as high as in the control group (CTR) with LPS (# $p<0.05$) which means that flower extract decreased ROS production during the inflammatory process (Figure 16, B). In the absence of LPS the group treated with flower hydrolate and with only medium maintained the same levels of fluorescence emitted by H2DCFDA however with LPS a significant increase in fluorescence levels was observed in cells treated with only medium (control) in relation to their respective control without the addition of LPS (&&& $p<0.001$). In the group containing flower hydrolate plus LPS a significant decrease in fluorescence levels was observed in relation to the control group (CTR) with LPS (### $p<0.001$), resulting in an inhibition of ROS production by macrophages treated with hydrolate during an inflammatory process (Figure 16, C).

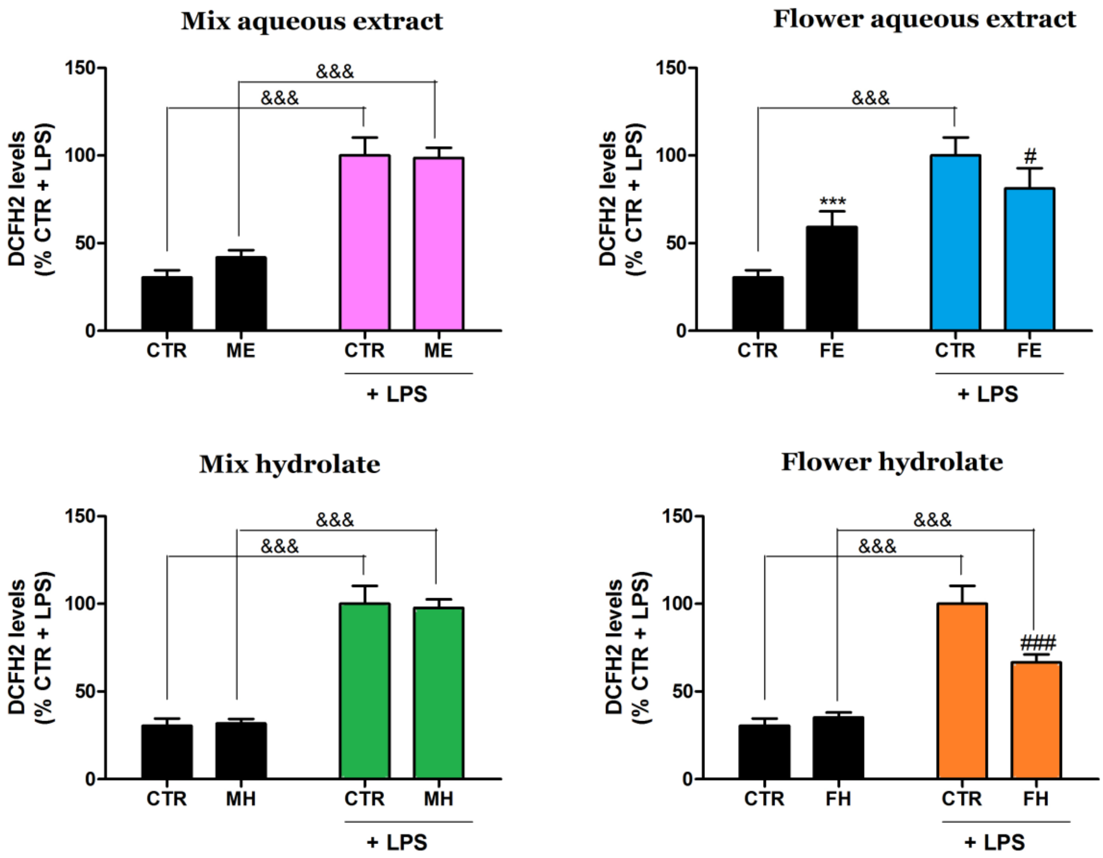

Figure 16. Levels of reactive oxygen species production by RAW cells in the presence and absence of lipopolysaccharide (LPS) with *H.lupulus* substances stimuli by a fluorescence method. Error bar indicate mean ±SD. Significantly P-values indicates &&& p<0.001 when compared with the respective groups without LPS endotoxin. ***, p<0.001 when compared with control group without LPS. ### show that p<0.001 and # p<0.05 when compared to control group with LPS. CTR: cells treated with medium; ME: Cells treated with medium and mix extract (A); FE: cells treated with medium and flower hydrolate (B); MH: cells treated with medium and mix hydrolate (C); FH: cells treated with medium and flower hydrolate (D).

5.5. Anti-inflammatory activity

5.5.1. NO production

The analysis of NO production by the RAW cells was performed by the Griess method. NO produced by macrophages plays important role in many inflammatory responses (72). Our results showed that under normal conditions, there was a significant increase (*** p<0.001) in NO production in the cells treated with the aqueous extract of the mix (stems, leaves and flowers) compared to the control group without endotoxin LPS (Figure 17, A). After the LPS stimulus, there was a significant increase (&&& p<0.001) in the production of NO by the cells treated with the medium and the aqueous extract of the mixture when compared to their respective controls without LPS (Figure 17, A). Cells stimulated with the aqueous extract of the mix and with LPS significantly decreased (# p<0.05) the NO production compared to the control group (CTR) with LPS. (Figure 17, A). Cells that were treated with the aqueous flower extract in the absence of LPS showed a significant increase in NO production compared to the control group without LPS (*** p<0.001) (Figure 17, B). When cells treated with medium and flower extract were stimulated with LPS endotoxin, a significant increase in NO levels was observed in the medium compared to the control group without LPS (&&& p<0.001), however in the medium containing flower aqueous extract no significant change in NO levels was observed compared to the control group without LPS. (Figure 17, B). In the group treated with flower extract and LPS a significant decrease in nitric oxide production levels was observed in relation to the group treated with medium and LPS only (# p<0.05) (Figure 17, B). In cells treated with medium and mix hydrolate and stimulated with LPS, a significant increase in NO production was observed in relation to their respective groups (CRT and HM) without LPS (&&& p<0.001). (Figure 17, C). However, cells stimulated with mix hydrolate and with LPS decreased NO production relatively to the control group with LPS (# p<0.005). (Figure 17, C). In cells treated with only medium and with flower hydrolate the levels of nitric oxide production remained low and similar, however after LPS stimulation it was observed a strong increase in nitric oxide production in cells treated with only medium and with LPS relative to the control group without LPS (&&& p<0.001) and a slight increase in nitric oxide levels produced by the cells that were treated with the flower hydrolate together with LPS in relation to their respective group not stimulated with LPS (& p<0.005). (Figure 17, D). In macrophages stimulated with flower hydrolate and LPS the NO production decreased strongly in relation to the control group with LPS (### P<0.001) indicating a strong anti-inflammatory capacity of flower hydrolate (Figure 17, C).

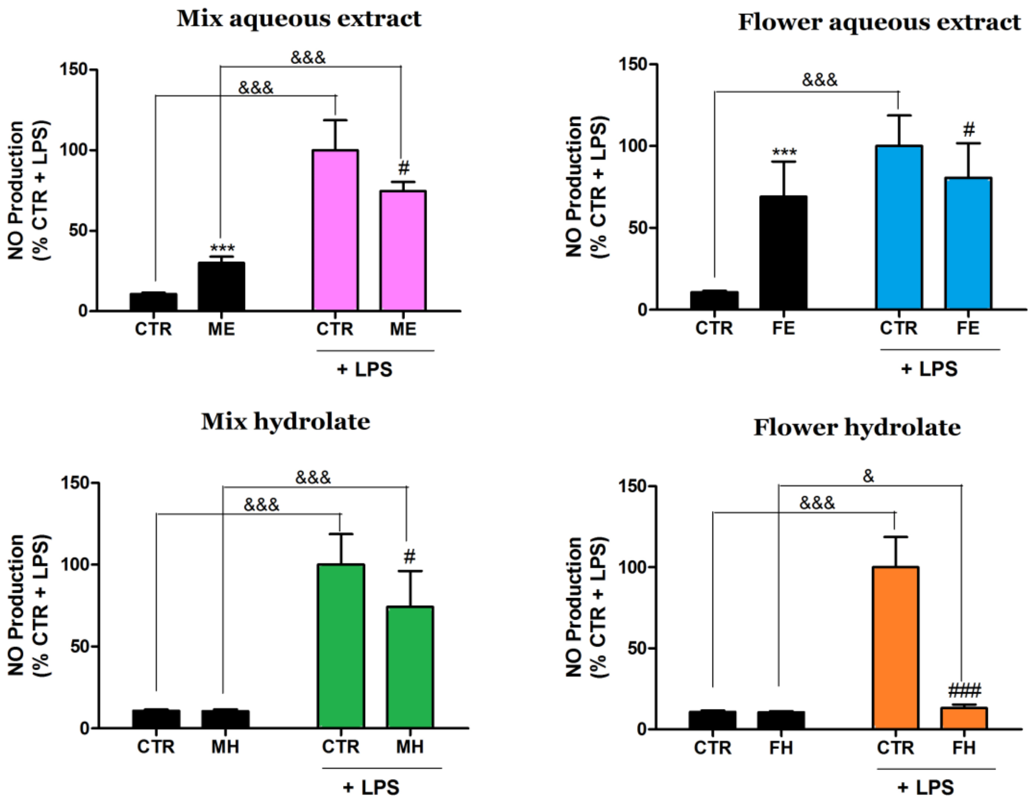

Figure 17. Evaluation of levels of nitric oxide (NO) production by the macrophages RAW cell line after stimuli with aqueous extracts A and B (EM, EF), hydrolates C and D (MH, FH) and with lipopolysaccharide (LPS) by Griess method. Error bars indicate mean ± SD. ***, indicates that p<0.001 when compared with control group without LPS. Significantly P-values indicates &&& p<0.001 when compared with respective groups (CRT/extract/hydrolate) without LPS. ### p<0.001 and # p<0.05 when compared with the control group with LPS.

5.5.2. Scavenging activity

The scavenging activity was performed in the medium with or without the extracts without cells and using a NO donor, the SNAP, and analysed by the Griess method. From the results obtained (Figure 18), it is observed that in the medium containing aqueous extract from the mix (stems, leaves and flowers) without SNAP there was a significant increase in nitrate levels compared to the control group without SNAP *** p<0.001 (Figure 18-A). After stimulation with SNAP, there was a significant increase in nitrate levels in the medium (CTR) and in the medium with the aqueous mix extract (ME) compared to the respective control groups without SNAP, &&& p<0.001 (Figure 18-A). It was also observed that the levels of nitrate present in the control group treated with medium only and the control group as medium and extract remained similar in both. With SNAP stimulation, there was a significant increase in nitrate levels in wells containing only medium and in wells containing medium and flower extract compared to controls without SNAP &&&

p<0.001 (Figure 18, B) Flower extract stimulated with SNAP significantly decreased nitrate levels compared to the control group with SNAP, ### p<0.001 (Figure 18, B). In the wells containing mix hydrolate and flower hydrolate (Figure 18, C-D) it was observed that in the absence of stimulation with the NO donor, the nitrate levels in the wells with medium and with medium plus hydrolates remained similar. After stimulation there was a significant increase in nitrate levels in relation to their respective control groups without SNAP, &&& p<0.001.

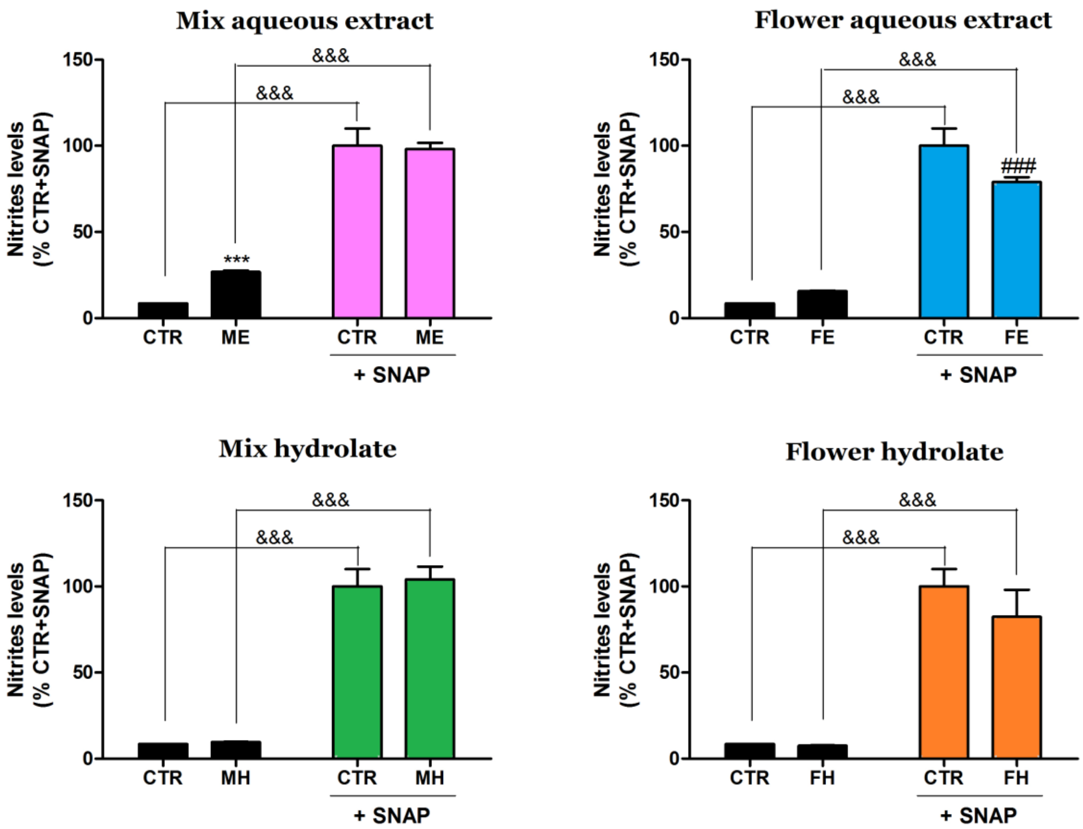

Figure 18. Nitrites levels production by the NO donor, SNAP and profile of mix extract (ME), mix hydrolate (MH), flower extract (FE) and flower hydrolate (FH) to reduce nitrites in the medium and analysis using the Griess method. Error bars indicate mean ± SD. Significantly P-values indicates *** p<0.001 when compared with the control group without SNAP, &&& p<0.001 when compared with the respective group (CRT or extract/hydrolate) without SNAP and ### p<0.001 when compared with the control group with SNAP.

5.5.3. Expression of COX-2 protein

The analysis of the COX-2 protein expression after treatment of Raw cells with LPS-induced inflammatory response and the extracts, was performed by the western blot method. Results were standardized with GAPDH control protein. In response to the LPS-induced inflammatory process there was no difference on the expression of the COX-2 inflammatory protein in both the control group and the group treated with the *H.lupulus* mix hydrolate (Figure 19, A). The results obtained after treatment of the cells with flower hydrolate show that in response to LPS there is a significantly decrease on the expression of COX-2 when compared with the control with LPS treated group. (Figure 19, B).

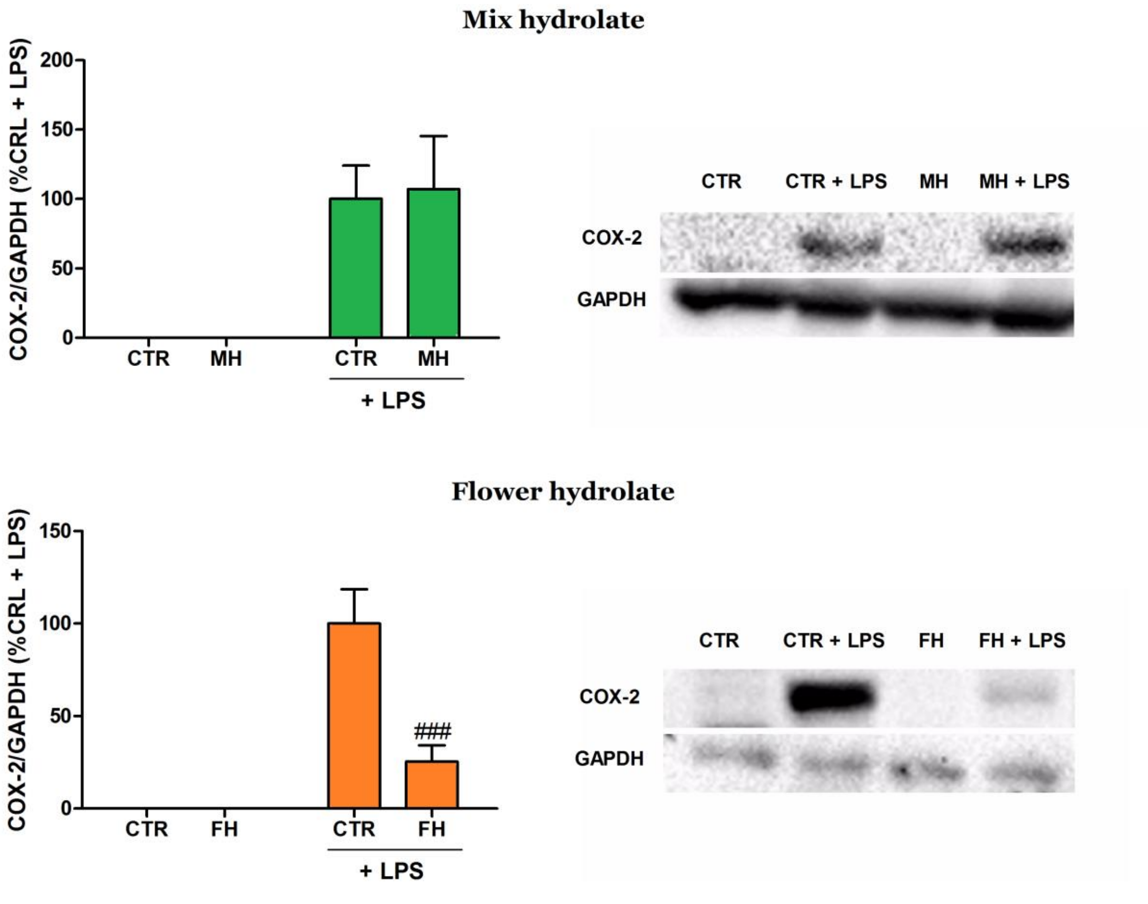

Figure 19. Expression of COX-2 protein in Raw cells treated with LPS and with mix hydrolate (MH) or flower hydrolate (FH) extracts during 24h. The analysis was performed by Western blotting assay. The results were standardised using GAPDH control protein, which means that the same amount of protein was loaded into each well at the time of electrophoresis. Representative immunoblots are showed for the respective graph. Results are expressed as percentage comparatively to control with LPS. Errors bars indicate mean ± SD. Significantly P-values indicates ### $p<0.001$ when compared with the control with LPS.

V. DISCUSSION

The skin, the largest organ in the human body, is the main line of defence against the penetration of pathogenic microorganisms and harmful chemical compounds from the environment into the body. Some risk factors such as genetic predisposition, microflora imbalance (bacteria, viruses, fungi), deficiencies in the functioning and elaboration of immune system responses, environmental factors (air pollution, lifestyle, hygiene and food habits) make the barrier to become inefficient leading to the emergence of skin diseases. The penetration of foreign bodies inside the skin activates the macrophages that reside there, the main cells of the immune system responsible for innate immunity, thus generating an inflammatory response. Inflammation is the first response generated by the body to repair damaged tissues and in defense against the invasion of pathogens. However, when this inflammation becomes very severe, it leads to the destruction of tissues and the organs dysfunctions, leading to the appearance of diseases such as atopic dermatitis, acnes and psoriasis, which are skin diseases associated with severe inflammation (14). Atopic dermatitis (atopic eczema) affects 20% of the world's youth population (children and adolescents). Psoriasis is more common with advancing age and reaches 0.53 to 11.43% of adults while the prevalence of acnes is higher in adolescents and young adults and affects approximately 40 to 90% of the world's young population (73–75).

Humulus lupulus is a well-known plant in both brewing and traditional medicine and is used for example in the treatment of anxiety and insomnia. Some of its secondary metabolites such as resins (lupulone, humulone, xanthohumol, β-caryophyllene), essential oils (terpenes), polyphenols, have been subjected to several clinical tests *in vitro* and *in vivo* so that the mode of action of *H.lupulus* and its medicinal properties were better known (64,76).

In order to better understand and identify the action of *Humulus lupulus* in the treatment of skin-associated diseases, this thesis investigated the effect of four extracts derived from *H.lupulus* (aqueous flower extract, aqueous extract from the mixture of stems, leaves and flowers, flower hydrolate and mix hydrolate) on Gram-positive and negative bacterial strains and on cell lines of 3T3 fibroblasts and RAW macrophages from mouse skin. At the beginning of this research we hypothesized that the extracts of *H.lupulus* had an antibacterial and anti-inflammatory profile and did not present toxicity to the cells and bacterial strains under study.

For this purpose, we first analysed by gas chromatography and mass spectrometry (GC-MS) the compounds which constitute the flower hydrolate and the mix hydrolate (stems, leaves and flowers) obtained by hydrodistillation. Our results show that the flower hydrolate consists mostly in cis-linalool oxide (11.32%), trans-linalool oxide (8.50%), linalool (10.76%), p-mentha-1.8-dien-7-ol (7.37%) and humulenol II (20.83%). The mix hydrolate showed as major compounds (%) caryophyllene (6.18%), humulene (18.02%), humulene epoxide II (7.33%) and humulenol II (8.45%). Characterizations of the plant were performed in some studies by the GC coupled HPLC method where the upper parts (leaves and flowers) of *H.lupulus* were analysed and these showed that the most important compounds found were β-Myrcene, β-caryophyllene and α-humulene (64) and hydra/alcoholic extracts (leaves, stems, flowers, rhizomes) and the essential oil consisted mostly of chalcones (xanthohumol and desmethylxanthohumol), α-acids (co-humulone and humulone) and β-acids (co-lupulone and lupulone), myrcene, linalool, trans-caryophyllene and α-humulene (51). Some of these components were also found in our extracts and it can explain their properties like antimicrobial, antioxidant and cytotoxic activity that were found in it.

In this work the antibacterial activity of *H.lupulus* extracts were determined by the microdilution method against three Gram-positive bacterial strains (*Staphylococcus aureus, Staphylococcus epidermidis* and *Cutibacterium acnes*) and two Gram-negative bacterial strains (*Escherichia coli* and *Pseudomonas aeruginosa*). It was shown that the *H.lupulus* mix aqueous extract (stems, leaves and flowers) does not present antibacterial activity against any of the five studied strains. However, the increase in concentration (%) of the extract had a positive effect on the proliferation of *E.coli* (B), *P.aeruginosa* (C) and *S.epidermidis* (D) strains. *H.lupulus* flower aqueous extract decreased the multiplication of *S.aureus* and *S.epidermidis* species significantly ($p<0.05$) with the increase in concentration (dose dependent) showing a MIC50 between 12.50-50% of the extract concentration. For the remaining strains there was no relevant inhibition of growth. The hydrolate of the mix does not seem to affect significantly the viability of *Staphylococcus aureus, Staphylococcus epidermidis* and *Cutibacterium acnes* strains which leads to say that it has no antibacterial effect against these strains. However, for strains of *Pseudomonas aeruginosa* in very high concentrations this extract decreases significantly its viability. From the results obtained, the flower hydrolate does not seem to affect the viability of Gram-negative bacterial strains (*E.coli* and *Pseudomonas aeruginosa*), but it does significantly reduce ($p<0.05$) the viability of Gram-positive bacteria (*S.aureus* and *S.epidermidis*) when exposed to concentrations between 25-50% of the extract presenting a MIC50 at 50% of the compound concentration. Flower hydrolate appears to decrease significantly the viability of *S.epidermidis* strain in relation to the group control.

A study conducted in 2015 by Evrendileck and his collaborators tested the antibacterial and antioxidant properties of the essential oil of *H.lupulus* L. Ten bacterial strains were used, being four Gram-positive *(Listeria innocua*, coagulase-negative *Staphylococci*, *Staphylococcus aureus*, and *Bacillus subtilis*) and six other Gram-negative strains (*Yersinia enterocolitica*, *Salmonella enteritidis*, *Salmonella typhimurium*, *Proteus mirabilis*, *Escherichia coli O157:H7* and *Klebsiella oxytoca*) (77). The antibacterial potential of the essential oil was determined by the method of agar diffusion or disk diffusion test where after incubation at 35°C ± 2°C for 24 to 48 hours, the inhibition zone was analyzed and expressed in mm, by a Vernier micrometer (77). The results of the phytochemical characterization obtained in this study by the GC-MS method corresponding to the essential oil of *H.lupulus* showed that α-Myrcene (16%), α-copene (3.5%), β-cubebene (2.9%), β-caryophyllene (14.7%), β-farnesene (6.8%), α-humulene (32.6%), β-selenene (10.5%) were the major compounds found in the *H.lupulus* essential oil from the upper parts of the plant and seeds. The essential oil of *H.lupulus* showed strong inhibition (mm) of Gram-positive bacteria (coagulase negative *Staphylococcus, Bacillus subtilis*) and Gram-negative (*Proteus mirabilis, Escherichia coli* O157:H7) in relation to the control group (essential oil of *Anise*) (63). In a study carried out by Naoto Yamaguchi and collaborators to determine the antibacterial and antioxidant activities of *H.lupulus* extracts in five bacterial strains (*Cutibacterium acnes*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Kocuria rhizophila* and *Staphylococcus pyogenes),* related to primary and secondary skin and soft tissue infections, The antibacterial activity was performed by the broth dilution method. Extracts with xanthohumol, lupulone and humulone showed a strong inhibition of bacterial activity of all strains under study. The lowest results corresponding to MICs against *C.acnes* and *S.pyogens* were observed for lupulones and the values obtained were 0.1 and 0.3 mg/ml, respectively. The lowest MICs values against *S.epidermidis*, *K.rhizophila*, and *S.aureus* strains were observed for lupulones and xanthohumol, and the value reached 1 mg/ml (57). A recent study developed by Katerina Bogdanova and her team aimed to determine the antibacterial activity of humulone, lupulone, xanthohumol extracts and a commercial CO_2 extract of *H.lupulus* (a mixture of α and β acids) in Gram positive multi-resistant bacteria. Commercial CO_2 extract of *H.lupulus* has been shown to be most effective in inhibiting the activity of Methicillin-resistant *S.aureus* (MRSA) (7.5mg/ml) and *Staphylococcus haemolyticus* (MIC 15mg/ml). Lower efficacy against Gram positive bacteria was observed against *Enterococcus faecalis* and *Enterococcus faecium (*vancomycin resistant

Enterococcus VRE, MIC=60 and 30 mg/L respectively). However, this study concluded that *H.lupulus* has good antibacterial properties against Gram-positive bacteria in general, but mainly against their resistant MRSA and VRE variants (58). All these studies showed that *H.lupulus* primarily inflorescence has metabolites, for example, humulene, lupulone, xanthohumol and others that selectively inhibit the multiplication of some bacterial strains, particularly Gram-positive strains. These studies are in agreement with our results where it was observed that flower aqueous extract and flower hydrolate (inflorescence of the plant) have an antibacterial effect in high concentrations (12.50%-50%) against the strains of *S.aureus* and *S.epidermidis* (Gram-positive bacteria). Moreover, and most interestingly, we also observed that the extracts obtained from the mix have a positive effect on the proliferation of some strains, having therefore potential to be compatible with skin commensals, preserving the natural skin microbiome.

The metabolic activity of 3T3 mouse fibroblast cells and RAW mouse macrophages in response to the extracts treatment was evaluated by the MTT colorimetric method. These cells were exposed to different concentrations (%) of *H.lupulus* aqueous extracts and hydrolates, DMEM medium and with 1% of SDS for negative and positive control respectively for 24 hours. From the results obtained, it was observed that the mix extract significantly increases the metabolic activity of RAW cells ($p<0.0001$) with the increase in concentration. It was observed that at a concentration of 12.50 % of the extract the metabolic activity begins to decrease but this decrease is not significant relatively to the control group. With 3T3 the same scenario occurs, however at a concentration of 50% the metabolic activity seems to decrease. Then it can be said that the mix aqueous extract in very high concentrations may decrease the metabolic activity but in low and/or moderate concentrations does not change the metabolic activity of the cells. From the results obtained it was found that the flower aqueous extract significantly decreases the metabolic activity of RAW cells. For 3T3 the flower extract seems to alter cell activity only in very high concentrations (50%). The hydrolate of the mix as the flower aqueous extract also seems to alter the activity of RAW and 3T3 cells only in very high concentrations (25-50%). Flower hydrolate at higher doses (25-50%) significantly decreases the metabolic activity of both RAW and 3T3 cells. Cytotoxicity of *H.lupulus* seed extract was previously evaluated in tumor lines (MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), HeLa (cervical carcinoma), and HepG2 (hepatocellular carcinoma) (78). The seeds were transformed into powder and subjected to solid-liquid extraction where the solvent used was methanol in water (80/20, v/v). These seeds extracts were found to be rich in (+) cathechins and (-)-epicatechins, important polyphenols for several industrial sectors such as the food industry. *H.lupulus* seed extract was cytotoxic for all tumor lines, with IC_{50} values lower or equal to 278+/-4 μg/ml. This cytotoxic result may be related to the high catechin content found in the extract (78). Extracts of resins obtained from the extraction in ethanol showed cytotoxicity against PC3 and HT29 cancer cell lines. In this study it was demonstrated that β-acids found in the resins are more effective in inhibiting cell growth than α-acids (79). Overall those results are in accordance with ours because all *H.lupulus* constituents studied that in very high concentrations (25-50% of the compound) diminished the metabolic activity and consequently the viability of fibroblasts and macrophages.

For the antioxidant and anti-inflammatory studies, we selected a concentration (3.12%) that does not change significantly the viability of the cells.

In situations of oxidative stress or severe inflammation the levels of oxygen free radicals (ROS) become high and toxic to the body which compromises its proper functioning. Reactive oxygen species are unstable molecules which are highly reactive, often formed from oxygen in reactions of oxide-reduction that occur in mitochondria. Excess ROS are associated with diseases, such as early aging of the skin, cancer, lung diseases, atherosclerosis, among others (80).

In this study we evaluated the production of ROS using two fluorescent molecules H2DCFDA (cytosolic fluorochrome) and Hoechst (nuclear) with different levels of excitation. The tests were performed in macrophages, in the presence and absence of LPS concomitantly with the extracts. In the absence of LPS, ROS normally remain at low levels. In the presence of LPS, an increase in ROS production and release was observed in both the control and treated with mix extract and hydrolate groups. In the cells treated with flower aqueous extract and hydrolate in the presence of LPS, although there was an increase in ROS production compared to cases without LPS, this increase is significantly lower than that in the control group with LPS, which leads to the claim that the extract and hydrolate of the flower inhibited the production of ROS in the macrophage cells during oxidative stress stimuli. An *in vitro* study (57) tested the fractions of *H.lupulus* in order to determine its antioxidant effect. Green tea catechins (*Polyphenon* 60) were used as a higher value control of oxygen free radical absorption capacity among edible plants. Vitamin C and E were also used as controls for water and liposoluble molecules, respectively. The antioxidant capacity of xanthohumol from *H.lupulus* fractions was higher than the antioxidant action of vitamin C and E and showed antioxidant activity similar to fat-soluble and water-soluble solutions. By the results obtained, xanthohumol is the compound that has greater antioxidant capacity compared to humulene or lupulone

(13). In our study the flower extract and the flower hydrolate seem to have clear antioxidant activity, linked to xanthohumol or even to humulene or lupulone.

The anti-inflammatory activity of the aqueous extracts of the mix (stems, leaves and flowers) and the flower of *H.lupulus* as well as the hydrolates of the mix and flower in macrophages RAW, was analysed after treatment of the cells with LPS. Macrophages are one of the main cells responsible for the innate immune response. Their activation by LPS from Gram-negative bacteria is important in order to control infections. However, an uncontrolled activation of macrophages can lead to a severe inflammatory state (64). During this study macrophage activation was induced by LPS (1μg/ml). NO is normally present in the blood stream in low concentrations and is an important mediator of the inflammatory response, however in situations of oxidative stress or severe inflammation the concentration of NO becomes quite toxic for the organism (64). NO from macrophages is synthesized by a homodimeric enzyme, NOS2 (also called iNOS), in macrophages activated by an external pathogenic agent such as LPS. (81). For the evaluation of the anti-inflammatory activity of extracts of *H.lupulus,* the production of NO was determined by the Griess method in macrophages (RAW) cells treated or not with LPS.

A significant increase in NO levels of the LPS-treated control group compared to the LPS-free control group was observed , a result which was expected since under normal conditions NO is present in a low concentration and since LPS is derived from a pathogen that induces an inflammatory response this translates into an increase of NO. In the groups treated with mix and flower aqueous extract in the absence of LPS a strong increase was observed in levels of NO compared to the control group. This leads us to say that these extracts may induce an inflammatory response without an inflammatory stimulus, as LPS. Interestingly, after induction of inflammation of the cells with LPS, both of extracts, mix and flower, seem to reduce the inflammatory response, diminishing the production of NO compared with the control LPS-treated group. In the cells treated with *H.lupulus* mix hydrolate in the absence of LPS, NO levels are low as in the control group. In the presence of LPS, there is an inflammatory process and the NO levels increase considerably in the control group but in the group treated with the hydrolate of the mix there is a significant reduction on the production of NO comparatively with the control LPS treated group which leads us to infer that the hydrolate of the mix has an anti-inflammatory effect reducing the NO levels in inflammatory conditions. In cells treated with flower hydrolate, in the absence of an inflammatory response, NO levels remain low as in the control group without LPS, which indicate that this extract does not induce inflammation. During an inflammatory response induced by LPS, the levels of NO increase significantly in the control group however in the groups treated with flower hydrolate the levels of NO remain low as in the cells without LPS, which leads us to say that there was a complete inhibition of NO production by macrophages when in contact with flower hydrolate. These results show that the aqueous extract of the mix, the aqueous extract of the flower, the hydrolate of the mix present some anti-inflammatory action when the cells are in the presence of an inflammatory stimuli, however the hydrolate of the *H.lupulus* flower has a greater anti-inflammatory capacity in relation to the others because it decreases considerably the levels of NO in the medium. In order to reach a conclusion about the cause of the decrease in nitric oxide levels, a scavenging test was performed using a NO donor, SNAP, which allows to verify whether the decrease observed in nitric oxide levels was really due to the fact that these substances inhibited the inflammatory process performed by the LPS in the cells, decreasing NO production, or whether they had the capacity to remove NO produced from the cells to the medium. This assay was performed in the medium with respective extracts and with the NO donor SNAP. From the results obtained with the mix aqueous extract, in the absence of SNAP the nitrite levels of the medium increase significantly in relation to the respective control group, which indicates that the mix extract has in their constitution some NO levels, and for this reason we also observed an increase on NO levels in the macrophages treated with this extract without LPS. These results suggest that the mix extract does not increase the production of NO in RAW treated cells, the levels of NO are already present at the extract. All the other extracts studied seem do not have NO. In the presence of SNAP, only the flower extract seems to have a scavenging effect because in relation to the control group treated with SNAP a significant decrease in nitrite levels is observed. With this result we can speculate that the decrease on the levels of NO in the medium of RAW cells treated with LPS and flower extract result from the capacity of the extract to remove the NO levels from the medium, and not from the capacity of the extract to decrease the production of NO in the cells. Relatively to the other extracts, mainly with the flower hydrolate treatment, the reduction on the production of NO in LPS-induced macrophages when compared with the control LPS-treated group, may be associated with the capacity of the extract to have anti-inflammatory properties in the cells, reducing the production of NO to the medium probably by influencing the expression of the iNOS protein. In fact, the substances derived from the *H.lupulus* flower present important constituents in their lupulin gland, known for their anti-inflammatory properties such as xanthohumol, which demonstrated their effects on macrophages (RAW cells) activated by LPS and Interferon ϒ, inhibiting the expression of iNOS which translates into a reduction in NO production by macrophages (13). In another study, an inhibition of the iNOS synthase enzyme and consequently reduction in NO production by macrophages was observed after treatment of the cells with an ethyl acetate solution of *H.lupulus* (72). Five chalcones were isolated from this soluble humulene fraction, one of them being xanthohumol, 2,2-di-(3-methyl-2-butyleyl)-4,5-dihydoxy-cyclopent-4-en-1,3-dione,lupulone. These compounds significantly inhibited NO production by suppressing the expression of iNOS (72). Our mix and flower hydrolates are rich in humulene (100% and 13,85 respectively). This substance may be responsible for the anti-inflammatory action of our substances that leads to the decreases of NO production.

COX-2 is an inflammatory isoenzyme associated with pain in inflammation and that belongs to the COX family. It allows the synthesis of prostaglandins E2 (PGE2) and thromboxanes from arachidonic acid (AA). Its activity is activated thanks to pro-inflammatory factors such as cytokines and interferons (82). We also evaluated the protein expression of COX-2 in RAW cells treated with the mix and flower hydrolates after an LPS stimuli, since both of these extracts have an influence on reducing NO production by the macrophages It was observed that in the absence of LPS the expression of COX-2 is inhibited both in the control group (culture medium and macrophage cells) and in the group treated with the hydrolate of the mix and with the hydrolate of the *H.lupulus* flower. The activation of macrophages by LPS causes COX-2 expression in the control group. However, it was observed that in the group treated with LPS and with the flower hydrolate there is a decrease in the expression of the isoenzyme COX-2 which reinforces the idea that the flower hydrolate has a strong anti-inflammatory capacity exerting a negative effect on the production of NO as well as exerting a negative effect on the expression of COX-2. Of notice, the over-expression of COX-2 and NO can be quite harmful to the body and its inhibition is very important to maintain the health of cells and an entire system. An in vitro study used CO_2 extract of *H.lupulus* in human monocytes in order to analyse the effects of *H.lupulus* in the production of PGE2 and consequently in the selective expression of COX-1/COX-2 (82). To evaluate *in vivo* the effect of *H.lupulus* extract, this was administered orally in C5BL/6 mice whose right joint was exposed to inflammation induced by intra-aortic Zymosan injection. The results showed that *H.lupulus* extracts inhibited the production of PGE2 in macrophages stimulated by LPS without compromising the metabolic activity of the cells. A decline in production of PGE2 in COX-2 blood samples was shown while in COX-1 whole blood (WBA) there was no inhibition in production of PGE2. This demonstrated that *H.lupulus* extracts exert a negative effect on the expression of COX-2. However, oral administration of 1.25 mg daily of *H.lupulus* showed no effect on the healing of the joint (82). These studies show that some preparations of *H.lupulus* have important compounds capable of interfering with the cells by changing their metabolism, thus modulating inflammatory and oxidative stress processes.

Overall, and integrating all results, in this study we found that the flower hydrolate of all the extracts studied was the one that presented stronger antibacterial activity (mainly against Gram-positive strains), antioxidant capacity (because it decreased significantly the production of oxygen free radicals) and anti-inflammatory properties (decreasing NO production and COX-2 expression). The extract of the mix and the hydrolate of the mix, besides not altering the metabolic activity of the cells also did not present antibacterial activity for the strains under study, on the contrary, they seem to favour the growth of some of these strains, which could be interesting for the development of prebiotic products, for example. These extracts, mix hydrolate and aqueous extract, demonstrated anti-inflammatory capacity compared to the control group treated with LPS (# $p<0.05$) but did not demonstrate antioxidant capacity compared to the control group with LPS. The flower extract demonstrated anti-inflammatory, antioxidant, scavenging activity in relation to the control group with SNAP, antibacterial activity against Gram-positive bacterial strains and demonstrated toxicity to RAW cells at low concentrations and toxicity to 3T3 cells at high concentrations.

VI. Conclusion and Future Perspectives

The skin, the largest organ in the human body, assumes important functions in human body such as a physical barrier protecting against the penetration of pathogenic microorganisms, harmful chemical substances, against the excessive incidence of ultraviolet rays but also acts on the homeostasis, protects the internal organs from shocks from the external environment in addition to presenting immune and cognitive functions. Changes in lifestyles (hygiene, diet, stress) and in the environment (pollution, use of non-degradable products) increase the incidence of skin-related diseases such as atopic dermatitis, psoriasis, acnes, skin cancer, among others.

Currently there has been a considerable increase in demand for products of natural origin, mainly products of plant origin, thus encouraging the pharmaceutical industry to invest more in plant-based products and limiting the use and sacrifice of animals in the design and formulation of these products. *Humulus lupulus* L., despite being widely used in the beer industry, has also been the target of numerous studies due to its therapeutic properties as anti-inflammatory, antioxidant, anti-bacterial, antifungicide.

In this work the effects of aqueous extracts of the flower, aqueous extracts of the mix (mixture of stems, leaves and flowers), hydrolates of the flower and hydrolate of the mix of *H.lupulus* in fibroblasts and macrophages from the skin were evaluated. The results obtained using the four extracts studied showed them to present very interesting characteristics that could be used in the future for the formulation of products (cosmetics or drugs) to be applied on the skin as prevention and/or treatment of diseases. The flower hydrolate of all the substances studied was the one that presented cumulatively better antibacterial capacities mainly against Gram-positive strains, antioxidants capacity (by decreasing significantly the production of oxygen free radicals and anti-inflammatory ability (decreasing the NO production and inhibition of COX-2 expression). The extract of the mix and the hydrolate of the mix, besides not altering the metabolic activity of the cells (macrophages and fibroblasts) and also for showing low antibacterial activity for the strains under study, on the contrary, they seem to favour the growth of some of these strains, which could be interesting if they were used in (prebiotic) products destined to favour the growth of beneficial and indispensable microorganisms to maintain the skin microflora. They have demonstrated moderate anti-inflammatory capacity and have not demonstrated antioxidant capacity. The flower aqueous extract demonstrated moderate anti-inflammatory, antioxidant, scavenging and antibacterial activity against Gram-positive strains of bacteria and demonstrated toxicity to RAW cells in low concentrations and toxicity to 3T3 cells in high concentrations.

In the future it would be important to perform more tests with these extracts and hydrolates of *H.lupulus* in order to establish a concentration where we can observe more therapeutic benefits. It would also be interesting to study alone the majority compounds located preferably in the inflorescence of the plant (the main organ that holds important bioactive molecules) so that it would be possible to relate each biomolecule to its action in the organism, thus allowing the conception of more effective pharmaceutical products in the treatment of skin diseases.

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APPENDIX

1. Community herbal monograph of *Humulus lupulus* L.

Keywords	*Humulus lupulus* L., composition of herb, pharmacological use, traditional use, community herbal monograph

1. Name of de medicinal product and general information

- Scientific name : *Humulus lupulus* Linnaeus
- Family : Cannabaceae
- Genus : *Humulus*
- Species : *Humulus lupulus* Linnaeus, *Humulus japonicus* and *Humulus yunnanensis Hu*
- Geographic distribution: Asia (China, Japan), North America and Europe
- Common name in all EU official languages:

BG (bălgarski): Хмел, съцветие	HR (Croatian): cvijet uzgojenog hmelja
CS (čeština): chmelová šištice	IT (italiano): Luppolo fiore
DA (dansk): Humlekopper	LV (latviešu valoda): Apiņa ziedi
DE (Deutsch): Hopfenzapfen	MT (malti): Fjura tal-Lupulu
EL (elliniká): Στρόβιλος λυκίσκου- άνθος λυκίσκου	NL (nederlands): Hopbellen
EN (English): Hop Strobile	PL (polski): Szyszka chmielu
ES (espanol): Lúpulo, flor de	PT (português): Lúpulo, cone
ET (eesti keel): humalakäbi	RO (română): conuri de hamei
FI (suomi):humala, kukka FR (français): Houblon (cône de)	SK (slovenčina): Chmeľový kvet
FR (French) : Houblon	SL (slovenščina): cvet navadnega hmelja
HU (magyar): Komlótoboz	SV (svenska): Humlekotte
LT (lietuvių kalba): Apynių spurgai	NO (norsk): Humle

*Others: perennial climbing plant that grows every spring from the rhizomes; Presenting differences between species from Europe with those from North

America and Asia because of their geographical distribution and their morphological characteristics.

2. Qualitative and quantitative composition

Well established-used	**Traditional use**
	With regard to the registration application of Article 16d (1) of Directive 2001/83/EC as amended ***Humulus lupulus L.*** **a) Herbal substance** Not applicable **b) Parts of the plant** i. Hop cones ii. Lupulin glands iii. Rhizomes iv. Herbal substance v. Stems vi. Leaves **C) Herbal preparation/ extraction** D) Comminuted herbal substance E) Powdered herbal substance F) Dried with artificial heat G) supercritical carbon dioxide supercritical CO2 is generated by pressurizing liquid CO2 with a pump and heating it up in a heat exchanger. The supercritical solvent flows through the extractor, which is charged with biomass. The homogenous CO_2/extract mixture is separated through pressure reduction into a gaseous CO_2 and an extract phase. The extract can be removed from the process while the gaseous CO_2 is recycled again H) liquid CO_2 The use of CO_2 at lower temperatures (down to about 10°C) and pressures is generally referred to as liquid CO_2 extraction I) Solvents extraction: alcohols, chloroform, acetone, hexane, methylene chloride, ethyl acetate, methanol, Water J) Liquid extract (DER 1:1) extraction solvent ethanol 45% v/v K) liquid/liquid extraction with methylene chloride (MC) in proportion CH_2CL_2 / H_2O (5:5). Anhydrous sodium sulfate ($NaSO_4$) was added to organic phase, enriched in non-polar phenolic compounds to remove traces of water. Filtration and evaporation. After filtration, MC was evaporated, and the aqueous phase was freeze-dried, to obtained two sub-extracts

	with different percentages yields L) Liquid extract (DER 1:10) extraction solvent sweet wine M) Hydro/alcoholic crude Hops extracts The entire plant was dried for 10 days at room temperature. Then different parts of the plants are separated, powderized and stored in the dark. Extract of different part are obtained with addition of **EtOH/ H_2O** (9:1). Macerations for 2 h for 3 successive times and full night in the dark. Solvent extraction evaporation to obtain hydro-alcoholic extracts; N) Tincture (ratio of herbal substance to extraction solvent 1:5) extraction solvent ethanol 60% v/v O) Dry extract (DER 4-5:1) extraction solvent methanol 50% v/v P) Essential oil from the cone powder, using a Clavenger apparatus. Hydro-distillation of 4 h allowed to obtain an optimum amount of the essential oil.

3. Description composition and physical form

Well established-used	**Traditional use**
	i. Herbal composition a) Terpenes: Y-Myrcene (MYR), β-caryophyllene (BCP), caryophyllene oxide (BCPO), Humulene (HUM), α-Pinene, β-Pinene, Linalool, Limonene, Perillyl alcohol, Terpinolene, Y-Terpinene, α-terpinene, Terpineols, Geraniol, Nerolidol, Borneol, α-Bisabolol, Bisabolenes, β-elemene, Fenchone, Pulegone, α-Pellandrene, β-eudesmol. <u>Others:</u> Isopulegol, isoborneol, sabinene, 3-carene, Δ-cadinene, selinene, farnesol, Lynalyl acetate, (-) - Guaiol, p-Cymene. b) Bitter acids (humulone and lupulone) c) Chalcones d) Glicosydic flavonoids (Kaempferol, quercetin, quercetrin, rutin). e) Catechins (galate catechin, galate epicatechin) ii. Physical information a) Physical form: Oil, viscous liquid b) Colour: Brownish-yellowish-reddish brown Yellow / orange for brown / green c) Fragrance: Rough and bitter Citrus, tropical fruits, stone fruits, pine, cedar, floral, spicy, herbs, earth, tobacco, onion / garlic and / or grass d) Density 20°C: 0.883-0.900 e) Vapor density mmHg: >1 f) Melting temperature °C: 40-60°C g) Water solubility: Insoluble

4. Pharmaceutical form:

Well-established used	Traditional use
	According to VCRP data from 2016 (Voluntary Cosmetic Registration Program), hop extracts are used in different cosmetic products totaling its application in 362 formulations (299 in leave-one and 60 in rinse-off products such as conditioners, shampoos, facial products, etc.) and 3 in dilutions to the bath). Comminuted herbal substance as herbal tea for oral use. Herbal preparations in solid or liquid dosage forms for oral use. The large percentage of hop use (60%) is in its pellet form and only 25% of the hop is used as an extract, the extract being prepared in ethanol or carbon dioxide, the remaining 15% consists of products and hop flowers which may be marketed in their raw or powder form.

5. Traditional medicinal use

Therapeutic indications	Comments
a) insomnia, depressive symptoms, irritation, tension, anxiety and digestive disorders b) Brewing industry c) Microbial activity	The acids (α and β) present in hops exert an inhibitory effect on bacterial growth and are used in this case to preserve the properties of beer. An experimental study was carried out to verify the properties of flavonoids and their derivatives derived from hops in microbial activity. In this study, 7 flavonoids were used, 2 of which were of natural origin (α and β-dihydroxanthohumol and 8-prenylnaringenin) and there was a significant activity against Staphylococcus aureus and Staphylococcus epidermidis with a low MIC80 value of 0.5ug / ml. The extracts obtained with ethyl acetate, acetone and methanol showed activity against *Fusarium oxysporum, F. culmorum*, and *F. semitectum* with the low MIC 50 of 0.5mg / ml, while the extract obtained with methylene chloride presented antifungal activity against Botrytis cinerea with an MIC50 of 1mg / ml. Another pilot study was carried out in order to analyze the antimicrobial properties of *Humulus lupulus L.* against multi-resistant Gram positive and Gram-negative microbial species and against some yeasts. The results of this study demonstrated that extracts of the plant showed a significant activity against Gram-positive bacteria, but mainly against its variants resistant to methicillin and vancomycin, but no effective activity was observed against Gram-

	negative bacteria. Among all the substances tested, xanthohumol was the one with the highest antimicrobial properties. Traditionally hops are indicated for the treatment of insomnia containing hypnotic components that cause drowsiness (parts of the plant used: the hop flower, combined with other plants, the fragrance of hops, tea and lupulin). It can also be used in the treatment of mild pains (lupulin), used for its bitter aroma (lupulin, bitter acids, and glands), for its anti-diabetic effects (flowers and rhizome), is beneficial to the urinary tract (Flowers, rhizome, and syrups made from flowers or rhizome). It is also known for its anaphrodisiac properties (lupulin) and in the treatment of the skin and against baldness (hop herbs, tea and hop juice). Bitter acids of hops α and β, humulone and lupulone respectively are effective against gram positive bacteria. These acids work best at acid pH and in their non-dissociated form. The main prenylflavonoid, xanthohumol, has an eluated ability to eliminate the peroxyl radicals (potent antioxidant activity) being in this case more potent than vitamin D and E. Xanthohumol also has anti-proliferative, anti-carcinogenic, anti-genotoxic, anti-inflammatory properties, lowers plasma glucose concentration, lowers lipid levels and white adipose tissue weight in diabetic mice. Lupulus is also used in the treatment of diabetes because it has been reported that the substance by the name of isohumulene present in hops decreases insulin resistance.

5.1. Contraindications

Well-established used	Traditional use
	Hypersensitivity to the active substance.

5.2. Fertility, pregnancy, lactation

Well-established used	Traditional use
	Safety during pregnancy and lactation has not been established. In the absence of enough data, the use during pregnancy and lactation is not recommended. No fertility data are available

5.3. Posology and method of administration to treat stress and insomnia

Stress	a) Herbal tea: 500 mg of comminuted herbal substance in 150-200 ml of boiling water as a herbal infusion, up to 4 times daily. b) Powdered herbal substance: 400 mg two times daily for adults and 200 mg two times daily for adolescents. c) Liquid extract (1:1): 0.5-2.0 ml, up to 3 times daily. d) Liquid extract (1:10): 19 g, 2-3 times daily. e) Tincture (1:5): 1-2 ml, up to 3 times daily. f) Dry extract (4-5:1): 125 mg, 2-3 times daily
Insomnia	a) Herbal tea: 500-1000 mg of comminuted herbal substance in 150-200 ml of boiling water as an herbal infusion 30 - 60 min before bedtime. b) Powdered herbal substance: 800–2000 mg, 30-60 minutes before bedtime. c) Dry extract (4-5:1): 125-250 mg, 60 min before bedtime.
Duration of use	If the symptoms persist longer than two weeks during the use of the medicinal product, search a qualified health care.
Method of administration	Oral use

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