



Unveiling the Therapeutic Potential of *Tripleurospermum disciforme*: Ethnobotanical, Phytochemical, and Bioactivity Studies

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Dedicatória

Ao meu querido marido e aos meus pais.

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- I. **Sheydaei P.**, Amaral M.E., Duarte A.P. Genus *Echium* L.: Phytochemical Characterization and Bioactivity Evaluation for Drug Discovery. *Plants*. 2025; 14(16), 2548. <https://doi.org/10.3390/plants14162548>
- II. **Sheydaei P.**, Duarte A.P. The Genus *Tripleurospermum* Sch. Bip. (Asteraceae): A Comprehensive Review of Its Ethnobotanical Utilizations, Pharmacology, Phytochemistry, and Toxicity. *Life*. 2023; 13(6), 1323. <https://doi.org/10.3390/life13061323>
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- I. **Sheydaei P.**, Norastehnia A. Effects of increased concentrations of chloride on the expression of Mn-SOD enzyme in tobacco. *Acta agriculturae Slovenica*, 2017; 53-59. <https://doi.org/10.14720/aas.2017.109.1.05>

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Resumo Alargado

As plantas medicinais têm sido consideradas, ao longo do tempo, como recursos terapêuticos essenciais na medicina tradicional e complementar. Nas últimas décadas, elas têm despertado crescente interesse científico devido aos seus compostos bioativos e ao seu potencial farmacêutico. O Irão, com seu clima diversificado e flora abundante, oferece oportunidades únicas para pesquisas etnobotânicas e farmacológicas, uma vez que o conhecimento tradicional sobre plantas medicinais permanece profundamente enraizado nas práticas locais de cuidado à saúde.

Os levantamentos etnobotânicos realizados nas províncias de Guilan, Alborz e Qazvin revelaram que as espécies pertencentes às famílias Asteraceae, Lamiaceae e Boraginaceae são as mais frequentemente citadas na medicina tradicional. Esses resultados destacam a relevância cultural e terapêutica dessas famílias, bem como o seu potencial para orientar pesquisas farmacológicas futuras.

Entre as espécies documentadas, *Tripleurospermum disciforme* (C.A. Mey.) Sch. Bip. e *Echium amoenum* Fisch. & C.A. Mey. foram as mais frequentemente mencionadas. A *T. disciforme* apresentou o maior Índice de Frequência Relativa de Citação (RFC = 0,42) e Valor de Uso (UV = 1,72), indicando ampla aceitação e forte consenso sobre sua eficácia terapêutica, especialmente no tratamento de distúrbios digestivos, respiratórios, cutâneos e do sistema nervoso. Esses indicadores etnobotânicos justificaram a sua seleção para investigação científica aprofundada nesta tese.

Por outro lado, embora *Echium amoenum* tenha apresentado valores ligeiramente inferiores (RFC = 0,32; UV = 1,31), continua sendo uma das espécies mais relevantes cultural e terapêuticamente dentro da família Boraginaceae, reconhecida na medicina tradicional iraniana por seu papel em condições digestivas, neurológicas, inflamatórias e infecciosas. Considerando que outros gêneros altamente citados, como *Thymus kotschyanus* e *Mentha* spp. (*Mentha pulegium* L., *Mentha arvensis* L., *Mentha aquatica* L., *Mentha piperita* L.) foram extensivamente estudados nos últimos anos, o gênero *Echium* foi selecionado para uma revisão sistemática, focada em suas propriedades fitoquímicas, etnomedicinais e farmacológicas. Embora as análises biológicas não tenham sido concluídas dentro do período desta pesquisa, estão planejadas para trabalhos futuros a fim de aprofundar a exploração de seu potencial terapêutico.

Uma revisão sistemática do gênero *Tripleurospermum* foi realizada para contextualizar sua diversidade fitoquímica e propriedades farmacológicas. Compostos como terpenos, flavonoides, taninos, compostos fenólicos e óleos essenciais foram relatados como

responsáveis pelas atividades antioxidante, antimicrobiana, anti-inflamatória e sedativa.

As análises experimentais de *T. disciforme* confirmaram a presença dos compostos fenólicos ácido neoclorogénico, ácido cafeico e acetato de p-hidroxifeniletíl, identificados por UHPLC–timsTOF–MS. A fração do extrato de acetato de etila apresentou a maior capacidade antioxidante no ensaio de eliminação do radical DPPH ($IC_{50} = 12,49 \mu\text{g/mL}$) e atividade antimicrobiana significativa contra *Bacillus cereus* ($MIC = 312 \mu\text{g/mL}$). Em relação à citotoxicidade, a fração do extrato hexânico demonstrou efeitos citotóxicos marcantes contra a linhagem de adenocarcinoma mamário humano (MCF-7), com menor toxicidade para fibroblastos normais, sugerindo um potencial anticancerígeno seletivo.

A revisão sistemática do gênero *Echium* L., fundamentada em sua relevância etnobotânica observada no estudo de campo, compilou dados sobre sua composição fitoquímica, incluindo compostos fenólicos, naftoquinonas, shikoninas, esteróis, ácidos gordos e alcaloides pirrolizidínicos, e sintetizou suas atividades antioxidante, antimicrobiana e citotóxica relatadas. Contudo, a presença de alcaloides tóxicos reforça a necessidade de investigações pré-clínicas e clínicas adicionais antes de sua aplicação terapêutica.

Ao integrar abordagens etnobotânicas, fitoquímicas e farmacológicas, esta tese oferece uma visão abrangente sobre o potencial terapêutico de *Tripleurospermum disciforme* e das espécies de *Echium*. Os resultados reafirmam sua importância na medicina tradicional iraniana e destacam seu valor como candidatas promissoras para o desenvolvimento de novos agentes terapêuticos de origem vegetal.

Palavras-chave

Tripleurospermum disciforme, *Echium*, L., etnobotânica, fitoquímica, atividade antioxidante, atividade antimicrobiana, citotoxicidade

Abstract

Medicinal plants have long been regarded as essential therapeutic resources in traditional and complementary medicine. In recent decades, they have attracted increasing scientific interest due to their bioactive compounds and pharmaceutical potential. Iran, with its diverse climate and abundant flora, provides unique opportunities for ethnobotanical and pharmacological research, as traditional knowledge of medicinal plants remains deeply embedded in local healthcare practices. Ethnobotanical surveys conducted in the provinces of Guilan, Alborz, and Qazvin revealed that plant species belonging to the Asteraceae, Lamiaceae, and Boraginaceae families are the most frequently cited in traditional medicine. These findings highlight both the cultural and therapeutic significance of these families, as well as their potential for guiding pharmacological research.

Among the documented species, *Tripleurospermum disciforme* (C.A. Mey.) Sch. Bip. and *Echium amoenum* Fisch. & C.A. Mey. were among the most frequently cited medicinal plants. *T. disciforme* recorded the highest Relative Frequency of Citation (RFC = 0.42) and Use Value (UV = 1.72), indicating widespread reliance and strong consensus regarding its therapeutic effectiveness, particularly in treating digestive, respiratory, skin, and nervous system disorders. These ethnobotanical indicators justified its selection for further scientific investigation in this thesis.

In contrast, although *Echium amoenum* presented a slightly lower frequency (RFC = 0.32, UV = 1.31), it remains one of the most culturally and therapeutically relevant species of the Boraginaceae family, known in Iranian folk medicine for its role in digestive, neurological, inflammatory, and infectious conditions. Considering that other highly cited genera, such as *Thymus kotschyanus* and *Mentha* spp. (*Mentha pulegium* L., *Mentha arvensis* L., *Mentha aquatica* L., *Mentha piperita* L.) have been extensively studied in recent years, *Echium* spp., was selected for a systematic review, focusing on its phytochemical, ethnomedicinal, and pharmacological properties. Although biological analyses could not be completed within the present timeframe, they are planned for future work to further explore its therapeutic potential.

A systematic review of the genus *Tripleurospermum* was conducted to contextualize its phytochemical diversity and pharmacological properties. Compounds such as terpenes, flavonoids, tannins, phenolic compounds, and essential oils were reported to contribute to antioxidant, antimicrobial, anti-inflammatory, and sedative activities.

Experimental analyses of *T. disciforme* confirmed the presence of phenolic compounds neochlorogenic acid, caffeic acid, and p-hydroxyphenylethyl acetate identified using UHPLC–timsTOF–MS. The ethyl acetate extract fraction exhibited the strongest

antioxidant capacity in DPPH radical scavenging ($IC_{50} = 12.49 \mu\text{g/mL}$) and significant antimicrobial activity against *Bacillus cereus* ($MIC = 312 \mu\text{g/mL}$). Regarding cytotoxicity, the hexane extract fraction displayed marked cytotoxic effects against the human breast adenocarcinoma (MCF-7) cell line, while showing lower toxicity toward normal fibroblast cells, supporting its selective anticancer potential.

The systematic review of the genus *Echium* L., based on the ethnobotanical relevance identified in the field study, compiled data on its phytochemical composition including phenolic compounds, naphthoquinones, shikonins, sterols, fatty acids, and pyrrolizidine alkaloids and summarized its reported antioxidant, antimicrobial, and cytotoxic activities. However, the presence of toxic alkaloids underlines the necessity for further preclinical and clinical investigations before therapeutic application.

By integrating ethnobotanical, phytochemical, and pharmacological approaches, this thesis provides a comprehensive overview of the therapeutic potential of *Tripleurospermum disciforme* and *Echium* species. The results reaffirm their importance in Iranian traditional medicine and highlight their promise as valuable candidates for the development of new plant-based therapeutic agents.

Keywords

Tripleurospermum disciforme, *Echium*, L., ethnobotany, phytochemistry, antioxidant activity, antimicrobial activity, cytotoxicity.

Thesis Overview

The research presented in this thesis was conducted at the CICS-UBI Health Sciences Research Center, Faculty of Health Sciences, University of Beira, and at the Department of Pharmacognosy, Faculty of Pharmacy, Guilan University of Medical Sciences, Rasht. This thesis is organized into three main sections.

The first section comprises three chapters:

Chapter 1 provides a general introduction, including a literature review on the ethnobotany of the studied plants in Iranian provinces, covering historical and socio-demographic aspects, geographical characteristics, soil and climate, geographical distribution, flora and vegetation, and traditional medicinal uses.

Chapter 2 is a published systematic review of the genus *Tripleurospermum* Sch. Bip., which forms the central focus of this thesis.

Chapter 3 is a published systematic review of the genus *Echium* L., complementing the research scope of the thesis.

The second section includes Chapter 4, Materials and Methods, and Chapter 5, which presents the main experimental results obtained in this study, as reported in the associated published article.

The final section, Chapter 6, summarizes the key findings of this research and outlines future perspectives for further investigations in this field.

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List of Abbreviations and Acronyms

¹ H-NMR	Proton nuclear magnetic resonance
A549	Human lung adenocarcinoma
AAI	Antioxidant activity index
AGS	Human gastric carcinoma
AIA	Alpha Linolenic acid
AL	Analyte List
BHIA	Brain Heart infusion Agar
CHGLY	Glycerol
CLSI	Clinical Laboratory and standard institute
CLSI	Clinical laboratory and standard institute
CUPRC	Cupric ion reducing antioxidant capacity
DMEM	Dulbeccos modified eagle medium
DMSO	Dimethylsulfoxide
DPPH	2,2-Diphenyl-1-picrylhydrazyl
FAB-MS	Fast atom bombardment mass spectrometry
FBS	Fetal bovine serum
FC	Number of informants who mentioned a particular plant
FL	Fidelity Level
FRAP	Ferric-reducing activity
HCT-116	Human colon cancer cell line
HepG2	Hepatocellular carcinoma
HpG2	Human hepatoma
HPLC-ESI-MS	High performance liquid chromatography coupled with electrospray ionization mass spectrometry
HT29	Human colon carcinoma
IC ₅₀	Half maximal inhibitory concentration
ICF	Informant consensus factor
J774.1A	Murine macrophages cell line
L2OB	Murine tumor fibroblast cell line
MCF7	Human breast adenocarcinoma
MDAMB-231	Human, epithelial-like breast cancer cell line
MHA	Mudler Hinton Agar
MHB	Muller Hinton Broth
MIC	Minimum inhibitory concentration
MRC-5	Human diploid fibroblast cell line
N	The total number of participants
NADES	Natural deep eutectic solvent
NHDF	Normal human dermal fibroblasts
Np	Number of participants who cited for a particular disease
Nt	Number of distinct plant species
Nur	Total number of use reports
ORAC	Oxygen radical absorbance capacity
OSI	Oxidative stress index
PAs	Pyrrolizidine alkaloids
PASEF	Parallel Accumulation serial fragmentation
PBS	Phosphate buffered saline

PMS	Premenstrual syndrome
RFC	Relative frequency of citation
RP-HPLC	Reverse phase high performance liquid chromatography
SF	Smart Formula
SL	Spectral library matching
SW-480	Human colon adenocarcinoma cell line
TDI	Tolerable daily intake
TFC	Total flavonoid content
TOS	Total oxidant status
TPC	Total Phenolic content
UAE	Ultrasound assisted extraction
UHPLC	Ultra high performance liquid chromatography
UI	Number of different uses
UV	Use value
WEHI-164	Mouse skin fibro sarcoma
WHO	World Health Organization

Chapter 1

Introduction

1. Introduction

Iran, known for its extensive geographical expanse and diverse climatic conditions, ranks among the richest regions globally in terms of plant biodiversity. With over 8,000 identified plant species, of which 1,727 are endemic, Iran is recognized as one of the top ten countries worldwide for plant species richness [1, 2]. This exceptional botanical diversity provides a valuable resource for studying medicinal plants, bridging traditional knowledge with modern scientific inquiry. The genus *Tripleurospermum*, commonly referred to as wild chamomile, belongs to the Anthemideae tribe of the Asteraceae family—one of the largest and most diverse plant families [3]. This genus encompasses approximately 40 species, predominantly distributed across temperate regions of Europe and Asia, with additional species found in North Africa and North America [4-6]. Turkey serves as the primary center for *Tripleurospermum* species [6, 7], while Russia is home to 17 species [8]. In Iran, six species of this genus have been documented [9].

Research into *Tripleurospermum* species has demonstrated a range of pharmacological properties, including antispasmodic, emollient, anti-inflammatory, antiherpetic, antioxidant, anticholinesterase, analgesic agents, memory enhancement, cholesterol regulation, kidney stone management; treatment of sore throat, respiratory support; diabetes management; cardiac disorders; gastric pain; antiseptic properties and hair care [10-30]. Notably, studies on *T. disciforme* have revealed its therapeutic potential, showcasing antiulcer effects, antibacterial properties, antioxidant activity, antimicrobial effects, and antiseptic properties [13, 21, 31-33]. *Tripleurospermum* species are predominantly herbaceous, either annual or perennial, and have a long history of use in traditional medicine. Phytochemical investigations have highlighted their richness in bioactive compounds, including significant phenolic and flavonoid constituents such as apigenin, luteolin, quinic acid, and their derivatives. Additionally, these plants produce a broad spectrum of phytochemicals, including terpenoids, alkanes, steroids, organic acids, and aromatic compounds. Hot water extracts of *T. parthenium* and *T. disciforme* have revealed melatonin in their flowers [34]. Moreover, chloroform extracts of *T. disciforme* contain a dioxaspiro derivative, while advanced analytical techniques like nuclear magnetic resonance (NMR), electron impact mass spectrometry, and chromatography have identified triterpenoids such as taraxasterol, lupeol, and betulinic acid in its dichloromethane extract [35].

Ethnobotany plays a critical role as a bridge between traditional knowledge and modern science, systematically documenting the medicinal applications of plants within specific cultural contexts. According to the World Health Organization (WHO), approximately 80% of the global population in less developed regions relies on medicinal plants for primary healthcare [36]. In Iran, ethnobotanical studies have been instrumental in preserving and analyzing traditional knowledge [37-39]. However, rapid technological and societal changes threaten the continuity of this knowledge unless it is thoroughly documented [40].

This study begins with an ethnobotanical survey of various regions in Iran, identifying the medicinal plants most frequently cited by local communities. Based on this analysis, the research selects the most widely utilized species for further investigation. Among them, *Tripleurospermum disciforme* and *Echium amoenum* which have been extensively used in the

provinces of Guilan, Qazvin, and Karaj. *Tripleurospermum disciforme* is chosen for a comprehensive phytochemical and biological assessment. Employing an innovative quantitative ethnobotanical approach, this study evaluates its medicinal potential and investigates its antioxidant, antimicrobial, and cytotoxic activities. The findings contribute to a deeper understanding of *Tripleurospermum disciforme* supporting its conservation, sustainable utilization, and integration into both traditional and modern medicine.

1.2 Ethnobotany and Ethnopharmacology

The Iranian people's deep-rooted interest in and extensive expertise with medicinal plants, combined with the nation's remarkable biodiversity and the advocacy of herbal medicine by influential medieval botanist figures like Avicenna, have been instrumental in promoting the development of this field across Iran's diverse ethnic groups [41]. Avicenna's seminal work, *The Canon of Medicine*, provided a systematic approach to herbal medicine, combining empirical observations with philosophical principles, which continues to influence traditional medicine in Iran and beyond. Ethnobotanical research conducted in various regions of Iran highlights that the most commonly used plant families are Asteraceae, Lamiaceae, and Apiaceae, with medicinal plants primarily employed to address digestive disorders and colds [2, 42, 43]. Recent studies in Europe reveal that many medicinal plants used to treat various diseases are dried plants imported from regions such as Africa, Asia, South America, and the Middle East [44, 45].

Experts have proposed several definitions for the science of ethnomedicinal plants. For instance, as many medicinal plants have historically migrated from non-Western regions to the West, this field can be understood as the study and utilization of non-Western plants in Western contexts [46]. Two notable examples of non-Western medicinal plants include the essential oils of *Lippia javanica* (Burm.f.) Spreng, widely used in South Africa for their antimicrobial properties [47], and the leaves of *Artemisia annua* L., recognized globally for their anticancer potential [48].

Ethnobotanical studies are most prevalent in countries such as Spain, Italy, and Eastern Europe [49], influential botanists of the medieval era, including Ibn Sina (Avicenna) and Ibn al-Tayar, played a significant role in preserving the Greco-Latin botanical tradition. They enriched ancient texts by documenting medicinal plants and contributing new knowledge [49].

In Europe, prominent plant families used in healthcare include Lamiaceae, Asteraceae, and Rosaceae [50]. In Spain, widely used medicinal preparations, such as rosemary oil and alcoholic tinctures, are employed to treat rheumatic and traumatic pain, as well as digestive and respiratory conditions [51]. Rosemary is also utilized in various countries for conditions like jaundice, colds, and coughs. Notably, rosemary smoke has been used historically as a protective measure against the plague [52].

Human use of plants extends to secondary metabolites such as atropine, caffeine, cocaine, nicotine, and morphine. These compounds, which plants produce to defend against predators, often possess hallucinogenic or psychoactive properties and are employed as sedatives or anti-anxiety agents [53, 54].

1.2.1 Ethnobotany as a scientific discipline

Ethnobotany holds immense value as a cultural heritage, serving as a bridge for transmitting the accumulated experiences of past generations to future ones. It encompasses traditional knowledge about plants, including their medicinal, nutritional, and ecological significance, which has been preserved and refined over centuries. This deep understanding of plant resources reflects the sustainable practices and cultural identity of various communities. By documenting and studying ethnobotanical traditions, we not only safeguard biodiversity but also ensure that valuable indigenous wisdom continues to inspire scientific advancements and sustainable development for future generations [55].

The term "ethnobotany" was first introduced in 1896 by the American botanist Johann Harshberger, defining it as the study of the interactions between anthropology ("ethno") and botany ("plant") [56, 57]. While its foundation lies in anthropology and botany, ethnobotany often integrates insights from pharmacology, chemistry, and related disciplines to enhance its applications. Ethnobotany is sometimes referred to as the "science of survival," highlighting its pivotal role in understanding the human-plant relationship and its implications for sustainability [58].

Being inherently theoretical, ethnobotany has spurred the development and exploration of several key hypotheses and frameworks. One prominent theory is the adaptation hypothesis, which posits that non-native plants were initially used for food and ornamental purposes, leading to the preservation of knowledge about their uses over time [59, 60].

The availability hypothesis, on the other hand, emphasizes the role of ecological, social, cultural, economic, and political factors in determining the accessibility and use of plants [61].

The plant use value hypothesis suggests that the importance of plants is shaped by various factors, including socio-cultural influences, demographic attributes, and the intrinsic characteristics of the plants themselves [62].

Daniel Moerman's nonrandom theory argues that the selection of medicinal plants by humans is not random but rather based on specific criteria, informed by their observed efficacy and cultural significance [63].

Another important theory, the diversity hypothesis, focuses on exotic medicinal plants, proposing that their unique bioactive compounds—distinct from native species—drive their diversification and application in treating a wide range of diseases. These exotic species often possess novel chemical structures and pharmacological properties that are absent in native

flora, making them valuable sources for new drug discovery and therapeutic innovations [64]. Indigenous communities often acquire deep knowledge of local plants and ecosystems through generations of empirical observation and practical investigation [65-67].

1.2.2 Ethnopharmacology

The World Health Organization (WHO) estimates that nearly three-quarters of the global population depends on traditional medicines derived from plants for a variety of treatments. Throughout human history, plants have served as fundamental resources, providing shelter, food, and medicinal remedies. The use of medicinal plants has been integral to the development of civilizations, including those of the Egyptians, Greeks, Arabs, Chinese, Japanese, and Western societies.

Traditional medicine, rooted in the knowledge and practices of these cultures, remains a cornerstone of healthcare in many parts of the world. Ethnopharmacology, as a discipline, focuses on the study and application of medicinal plants, offering valuable empirical insights into their pharmacological properties and therapeutic uses [68]. Historical evidence further highlights the innovative and sophisticated ways in which ancient civilizations utilized plants for healing. For instance, quinine, derived from the bark of cinchona trees, was used to treat malaria symptoms long before the disease was scientifically understood. Similarly, plant-derived compounds such as salicylic acid, the precursor to modern aspirin, were employed as pain relievers centuries before the advent of synthesized pharmaceutical formulations [69]. These examples underscore the enduring significance of traditional medicine and its contributions to both historical and contemporary healthcare practices.

1.2.3 Importance of ethnobotanical studies in drug development

Traditionally, medicinal plants were utilized in various forms, including raw powders, teas, tinctures, and poultices. By the early 19th century, approximately 1,000 plant species had been extensively used for their therapeutic properties, including *Cedrus duham*, *Commiphora myrrha* Engl., *Cupressus sempervirens* L., *Glycyrrhiza glabra* L., and *Papaver somniferum* L [70].

One of the most significant advancements in medicinal plant use was the first isolation of morphine from opium in the early 19th century. This breakthrough was followed by the extraction and identification of numerous bioactive compounds from plants, such as strychnine (*Strychnos nux-vomica* L.), quinine (*Cinchona officinalis* L.), colchicine (*Colchicum autumnale* L.), atropine (*Atropa belladonna* L.), papaverine (*Papaver somniferum* L.), and salicin. (*Salix* spp.) [71-73].

According to Gilani *et al.* (1992) plants accounted for approximately 80% of all medicines in the mid-19th century. However, the rise of synthetic drugs significantly reduced this proportion, with plant-derived medicines now constituting roughly 25% of pharmaceuticals [73]. Among

these invaluable compounds are atropine, artemisinin, colchicine, digoxin, ephedrine, morphine, physostigmine, pilocarpine, quinine, quinidine, reserpine, taxol, tubocurarine, vincristine, and vinblastine. Many of these were initially identified through the traditional knowledge of indigenous communities. In contrast, aspirin was derived from salicin, a compound found in willow bark and was semi-synthesized from salicylic acid. Additionally, taxol was discovered through a systematic screening of North American plants rather than traditional medicinal use, highlighting different approaches to drug discovery.

Among plant-derived analgesics, morphine—extracted from the opium poppy (*Papaver somniferum*) by Friedrich Sertürner in 1806—remains one of the most potent painkillers ever discovered [69].

Another notable example is digitalis, derived from *Digitalis purpurea*, which has been widely used for the treatment of heart failure due to its cardiac glycoside content [74, 75].

Reserpine, isolated from the roots of *Rauwolfia serpentina*, gained prominence between 1952 and 1958 for its potential in treating cardiovascular disorders. However, excessive use was associated with adverse effects, including depression and Parkinson-like symptoms. Despite its diminished popularity with the advent of modern antihypertensive drugs, reserpine remains in use among low-income populations. Administered in doses of 0.05 to 0.1 mg combined with low doses of thiazide diuretics and hydralazine, it has shown effectiveness in reducing blood pressure without renal complications. Other plant-derived compounds, such as muscarine and nicotine (early selective agonists for muscarinic and nicotinic receptors), cocaine (a catecholamine reuptake inhibitor), yohimbine (a selective α_2 -blocker), and himbacine (a prototype cardio-selective antimuscarinic agent), have been extensively studied for their mechanisms of action and physiological effects [76].

Dicoumarol and warfarin, two globally significant anticoagulant drugs, are both related to *Melilotus officinalis*, but warfarin was synthesized from dicoumarol rather than being extracted directly from the plant. Research has demonstrated that warfarin has lower toxicity than dicoumarol and is widely used to prevent stroke, manage cardiovascular conditions, and aid post-surgical recovery [69].

Additionally, extracts from *Dieffenbachia seguine* (Araceae) have been studied for their potential application in sterilization, including experiments on rats [77].

1.3 Ethnobotanical Studies in the provinces of Iran

To provide a comprehensive understanding of ethnobotanical studies in Iran, Table 1.1 presents data on the most frequently reported plant families, their primary applications, and common preparation methods across various provinces. The selection of these provinces was based on the richness of their medicinal plant diversity, the extent of previous ethnobotanical studies, and their geographical and ecological variety. The goal of this compilation is to highlight the regional

differences in the use of medicinal plants and contribute to the broader knowledge of traditional medicine in Iran.

Medicinal plants belonging to families such as Lamiaceae, Asteraceae, Apiaceae, Rosaceae and others have the highest prevalence in these regions. These plants have been used to treat a wide range of diseases and health issues, including digestive disorders, respiratory problems, diabetes, high blood pressure, skin diseases, and many more. The preparation and consumption methods of these medicinal plants are also diverse, including infusion, decoction, powder, poultice, oil, and both oral and topical applications.

Table 1.1 Ethnobotanical studies in the provinces of Iran

Number	Location	Most reported families	Most reported applications	Most common preparations	References
1	Guilan (Amlash, Rudsar)	Lamiaceae Rosaceae Asteraceae Malvacea Rutacea	Digestive system disorders, Blood circulation, Respiration and colds	Infusion and decoction	[78]
2	Guilan, Rudbar, Joubn	Apiaceae Lamiaceae Asteraceae	Gastrointestinal diseases, Infectious diseases and colds	In the form of food and food preparation	[42]
3	Alamout, ghazvin	Apiaceae Lamiaceae Boraginaceae	Stomachache, Renal calculus, Infections, Sedative	Infusion	[41]
4	Guilan	Rosaceae Lamiaceae Asteraceae	-	-	[79]
5	Kohghiluyeh va Boyer Ahmad province, Turkmen Sahra in north of Iran, Chaharmahal va bakhtiyari province, hormozgan province, Arjan in Fars province and Mashhad province	Rosaceae Lamiaceae Asteraceae	Hypertension disease	-	[80]

6	Turkmen Sahra region, Kerman, Fars, Hormozgan, Razavi Khorasan and Isfahan province	Asteraceae Berberidaceae	Liver disorders	-	[81]
7	Ilam, Mashhad, kerman, Lorestan, Sardast, Kazeroon, the Northeast Latrine Zone of Persian Gulf, Kohghiluyeh va Boyer Ahmad, Maraveh Tappe region, Mobarakeh (Isfahan), Khuzestan, Zilberchay Watershed of East Azerbaijan, Alamut region, Natanz region (Kashan)	Apiaceae	Digestive system disorders, Urinary duct problems, Respiratory problems, Urological troubles, Nervous system disorders, Skin problems	Infusion, decoction, Poultice	[82]
8	Sirjan, kerman	Lamiaceae	Gastrointestinal diseases, Respiratory ailments	Decoction, Powder	[83]
9	Hamedan	Asteraceae Fabaceae	Digestive disorders, Infections	Decoction, Powder	[27]
10	Hormozgan	Lamiaceae Asteraceae	Painful disorders, Gastrointestinal, Dermatological diseases	Decoction, Powder	[84]
11	Kohghiluyeh	Asteraceae Lamiaceae	Digestive system disorder Respiratory system	Decoction, Powder, infusion	[85]
12	Mobarakeh (Isfahan)	Lamiaceae Asteraceae Rosaceae	Diabetes and hyperlipidemia, palpitations, kidney stones, sinusitis, hypotension, amnesia	-	[86]
13	Lorestan	Anacardiaceae	Anti-pain agent and relieve	Poultice, Decoction	[87]

		Thymelaeaceae Caprifoliaceae Anacardiaceae Lamiaceae Rutaceae Papaveraceae Zygophyllaceae Asteraceae Cupressaceae Rosaceae Papilionacea	toothache		
14	Sarv abad, Kordistan	Asteraceae	Psychological, musculoskeletal, Digestive and respiratory disorders	Decoction	[43]
15	South of kerman	Apiaceae Asteraceae Lamiaceae	Cold-flu, Fever	Decoction, liniment and infusion	[2]
16	Saravan region, Baluchistan	Lamiaceae	Cold-flu, Fever, Blood disorders	Decoction	[88]
17	Kazeroon	Lamiaceae Asteraceae	-	-	[89]
18	Central Zagros, Lorestan	Asteraceae Lamiaceae Apiaceae Fabaceae Cruciferae	Gastrointestinal, Respiratory and skin problems	Infusion, Catapasm, Decoction	[90]
19	Arasbaran	Lamiaceae Asteraceae	Cold-flu, Cough and infection	Decoction, Infusion	[38]
20	Balengestan, khouzestan	Poaceae Asteraceae Papilionaceae Cruciferae	Colds, Low blood sugar, respiratory, Digestive diseases		[39]
21	Zangelanlo, Khorasan razavi	Lamiaceae Asteraceae Apiaceae Fabaceae	Febrifuge, Anthelmintic, Digestive problems, Respiratory ailments, Urinary diseases, Skin diseases and diabetes	Natural (no preparation)	[24]

22	Ilam	Asteraceae Lamiaceae Fabaceae Apiaceae	Gastric pain, Antiseptic, Indigestion, Laxative, Wound healing, Anti calculus, Cough, Sore throat	-	[91]
23	review	Asteraceae Lamiaceae Fabaceae	Diabetic treatment	Infusion, Decoction	[92]
24	Ardestan, esfahan	Asteraceae	Pain relief, Relief of digestive disorders, Metabolic disorders such as diabetes and blood lipid	-	[93]
25	Fars	Asteraceae	Wound healing	-	[94]
26	Sistan	Fabaceae Liliaceae Plantaginaceae Portulacaceae	Diuretic, Stomach tonic, Wound-healing, Fever treatment, Various pains, Inflammations, Expectorant	Infusion, Decoction	[95]
27	Mashhad	Polygonaceae Asteraceae Plantaginaceae Salicaceae	Jaundice	Decoction	[96]
28	Kohgiluyeh and Boyer-Ahmad Province, Basht and Gachsaran regions	Asteraceae Apiaceae	Gastrointestinal diseases	Decoction	[97]

1.3.1 The province of Guilan

1.3.1.1 Historical characterization and Sociodemographic characterization

Rasht, the provincial capital, is situated near the Caspian coast. As the largest city in the province, it serves as a significant commercial and cultural center. The city is renowned for its lively local culture and its crucial role in the trade of agricultural products, especially rice and seafood. Guilan is renowned for its verdant forests, predominantly consisting of broadleaf species such as oaks, beeches, and hornbeams. These forests blanket much of the province's hilly terrain and are part of the expansive Caspian Hyrcanian forests, a UNESCO World

Heritage site. While the province is not known for abundant mineral resources, it does have some deposits of minerals and raw materials such as limestone and clay used in local industries

Guilan Province is considered one of the key regions of the country. According to the most recent census conducted in 2016, the province consists of 16 cities and 52 towns. The 2016 Statistical Yearbook reports a population of 2,530,696 people in Guilan, with 1,603,026 individuals residing in urban areas. In addition to Rasht, other notable cities in the province include Anzali, a port city, and Lahijan, renowned for its tea plantations. The rural areas of the province are predominantly centered around agriculture and fishing, with numerous villages situated in the hills and along the riverbanks. According to the 2016 Statistical Yearbook of Guilan Province, 63.34% of the population resides in urban areas, while 927,670 individuals or 36.65%, live in the rural regions of the province.

1.3.1.2 Geographic characterization

Iran is a country located in southwestern Asia, while Guilan Province, situated in the northern part of the country (Figure 1.1), and is recognized as the fourth smallest provinces in Iran. It boasts unique geographical features, encompassing a diverse landscape that seamlessly blends coastal, mountainous, and forested regions. Guilan is situated along the southern shore of the Caspian Sea, which forms its northern border. This region covers an area of approximately 14,044 km² [98]. The province is part of the northern region of Iran and is bordered by the provinces of Mazandaran to the east, Zanzan to the west, and Ardabil to the northwest. The province's long coastline (about 300 km) along the Caspian Sea provides it with a strategic position for trade and cultural exchanges with other countries around the Caspian region. The southern part of the province is dominated by the rugged Alborz Mountain Range, which stretches from the northwest to the southeast of Iran. These mountains create a sharp contrast between the humid northern region and the drier central and southern areas of Iran. The highest peak in the province is Mount Siahkuh, which reaches elevations above 3,000 meters. In summary, Guilan Province is geographically diverse, with coastal plains, fertile agricultural areas, lush forests, and rugged mountains. This combination of natural resources, climate, and topography makes it one of Iran's most important regions both economically and ecologically. This study selected Guilan Province (northern Iran) which is a coastal province located between 48° 32' N to 50° 36' N and 36° 33' E to 38° 27' E

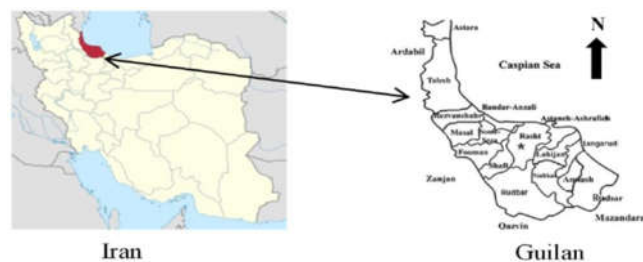


Figure 1.1 Geographical location of Guilan province in Iran

1.3.1.3 Soil and climate characterization

In Guilan Province, Manjil County is characterized by saline soils, where salt-tolerant species such as *Suaeda dendroides*, *Kaviria tomentosa*, *Salsola tomentosa*, *Spach*, and *Halimocnemis rarifolia* thrive on chalky hills. Similarly, in the vicinity of Manjil, Rudbar County features sand dunes near the Sefid Rud River, providing a habitat for desert species such as *Calligonum persicum* [99], *Heliotropium aucheri*, and *Corispermum aralocaspicum* [100].

The plains and lowlands of Guilan, especially near the Caspian Sea, are dominated by alluvial soils. These nutrient-rich soils are deposited by rivers such as the Sefid River, which flows from the Alborz Mountains and drains into the Caspian Sea. The ongoing movement of water continually replenishes the soil, ensuring its fertility by bringing in fresh layers of nutrient-rich sediments.

The South Caspian region plays a significant role in the acclimatization of agricultural trees and shrubs [101]. Its cultivation zone is vital to Iran's agriculture, with the fertile alluvial soils of the South Caspian lowlands supporting diverse agricultural activities. These include the cultivation of rice, wheat, colza, citrus fruits, kiwi, peaches, strawberries, and tea. Olive production is also a key agricultural activity, particularly in the Sefid Rud Valley and Gorgan, where the local Mediterranean bioclimatic conditions favor its growth [100].

Hydromorphic soils, adapted to wet conditions, are prevalent in the province's wetlands and coastal areas. These soils, which are waterlogged for much of the year, are typically found in lowland regions or areas with high rainfall. They are ideal for growing water-intensive crops, such as rice [102]. Clay content is a key soil attribute and is often used as a soil quality indicator for paddy fields [103]. Clayey soils are abundant in Guilan, particularly in the plains, where expansive (smectite) and moderate (vermiculite) clays are the dominant minerals. These soils retain water well, which is beneficial given the area's high rainfall, although they can pose drainage issues, particularly in flood-prone regions [104]. Loamy soils, a balanced mixture of sand, silt, and clay, are also found throughout the region. These soils are ideal for rice cultivation due to their excellent water retention and drainage properties [105].

The soils in Guilan are generally slightly acidic, a characteristic typical of regions with high rainfall and dense vegetation. Lime is commonly used to neutralize soil acidity. Despite this, the soils are highly fertile, making Guilan a vital agricultural region in Iran, particularly for the cultivation of rice, tea, and citrus fruits.

Climate of Guilan Province: Guilan's climate is heavily influenced by its proximity to the Caspian Sea and its location between the Alborz Mountains and the sea. This unique combination results in a humid subtropical climate with several defining features.

Guilan is one of the wettest regions in Iran, receiving significant rainfall year-round. Annual precipitation can exceed 2,000 millimeters (79 inches) in certain parts of the province,

particularly along the coast and in the foothills of the Alborz Mountains. The mean annual precipitation and pan evaporation are 1,506 mm and 1,306 mm, respectively [98].

Precipitation patterns vary across the Hyrcanian region, with distinct seasonal variations between the western and eastern areas. The higher rainfall in the western part of the Hyrcanian region during the autumn months is due to the influence of north-easterly winds originating from the Siberian anticyclone or polar front. These winds sweep across the Caspian Sea, bringing moisture to the South Caspian region before meeting the hot and dry air masses descending from the Iranian high plateau [106].

The substantial precipitation is driven by moisture-laden winds from the Caspian Sea, which are forced upwards by the Alborz Mountains, causing condensation and rainfall. Western disturbances, which bring moisture from the North Atlantic, Mediterranean, and Black Seas, contribute to the autumn, winter, and spring rains in the southern Caspian region [106, 107].

The Siberian anticyclone, which forms over Northern Eurasia and occasionally extends into Central Asia, obstructs the eastward penetration of these disturbances during the colder months but leads to high rainfall during the summer and autumn in the Caspian coastal areas [106].

Relative air humidity is exceptionally high in some stations, such as Astara, Rasht, and Ramsar, where it often exceeds 80%, creating persistent fogs in the higher altitudes [100].

1.3.1.4 Flora and vegetation

Iran holds significant global importance in terms of biodiversity. The northern provinces of the country—Guilan, Mazandaran, Golestan, as well as the Talesh region in Azerbaijan—are home to 3,234 vascular plant species, encompassing 856 genera and 148 families. Of these, 475 species of medicinal plants have been identified in the forests of Guilan Province [100]. The Hyrcanian forests, which closely resemble the forests of Central Europe, are situated in the northern provinces of Iran Guilan, Mazandaran, and Golestan. Stretching 800 kilometers along the western Caspian Sea and the northern Alborz Mountain Range, these forests cover an area of 1.84 million hectares and are 110 kilometers wide [108].

Research has indicated that gymnosperms, which include 5 genera and 11 species, exhibit the lowest diversity among seed plants when compared to ferns and angiosperms. In Iran, the largest monocot family is Poaceae, with 246 species commonly known as grasses, with around 780 genera and around 12,000 species [109], while the largest dicot families include Asteraceae with 404 species (such as *Senecio othonnae* M. B., *Senecio vernalis* Waldst. & Kit., *Achillea vermicularis* Trin., *Artemisia absinthium* L., *Artemisia vulgaris* L., *Artemisia annua* L., *Tanacetum parthenium* (L.) Schultz – Bip., *Tripleurospermum disciforme* L., *Tragopogon bupthalmoides* (DC.) Boiss., *Cichorium intybus* L., *Scorzonera laciniata* L., *Inula helenium* L., *Arctium lappa* L., *Silybum marianum* (L.) Gaertn., *Bellis perennis* L., *Calendula officinalis* L., *Centaurea gilanica* Bornm., Fabaceae with 326 (such as *Cassia angustifolia*, *Glycyrrhiza glabra*

L, *Medicago sativa* L, *Trigonella foenumgraecum* L, Cruciferae with 157, Rosaceae with 155 (*Rosa* spp.), Lamiaceae with 153 (such as *Mentha longifolia*, *Salvia officinalis*, *Thymus vulgaris*, *Lavandula angustifolia*, *Stachys lavandulifolia*, *Ziziphora persica* Bunge), Apiaceae with 146 (such as *Foeniculum vulgare*, *Eryngium planum*, *Anethum graveolens* L, *Bunium persicum* B. Fedtsch. *Cuminum cyminum* L.) and Boraginaceae (*Echium amoenum*, *Echium italicum*) 102 species [100].

An ethnobotanical study conducted in Joben village, located in Gilan Province, identified 34 plant species belonging to 18 different families, categorized into medicinal and edible groups. Among these, the most represented families were Apiaceae, comprising 7 species (*ferulago galbanifera*, *Heracleum persicum*, *Eryngium planum* L, *Foeniculum vulgare* Mill. *Coriandrum sativum* L, *Anisosciadium orientale* DC and *Ammi visnaga* (L) Lam, (21%), followed by Lamiaceae (*Mentha pulegium* L, *Melissa officinalis* L, *Origanum vulgare*, *Mentha arvensis* L and *Veronica persica* Poir) and Asteraceae, each with 5 species (*Taraxacum officinale*, *Matricaria chamomilla*, *Tragopogon buphthalmoides* (DC.) Boiss, *Silybum marianum* Gaertn and *Cichorium intybus* L) (15%). In terms of growth forms, herbaceous plants constituted the majority, with 27 species (79%), while shrubs and trees accounted for 5 species (15%) and 2 species, respectively. Furthermore, Borginaceae family includes *Echium amoenuem* [42].

An ethnobotanical study in the Guilan province, Amlash and Rudbar counties, led to the identification of 91 species of medicinal plants native to this region, most of which were in the Lamiaceae family with 15 species (*Stachys lavandulifolia*, *Melissa officinalis* L., *Lamium alba* L *Teucrium chamaedrys* L, *Salvia sclarea* L , *Stachys bisantina* L , *Ocimum basilicum* L. *Lamium amplexicaule* L. , *Marrubium vulgare* L, *Lycopus europaeus* L, *Mentha piperita* L, *Mentha pulegium* L., *Mentha aquatica* L, *Origanum vulgare* L, *Thymus vulgaris* L), Rosaceae with 12 species (*Lauruscerasus officinale* L. , *Rubus hirtus* L, *Cydonia oblonga* Mill, *Geum urbanum* L, *Alchemilla xanthochlora*, *Mespilus germanica* L, *Rosa* spp , *Potentilla anserine* L , *Crataegus microphyla*, *Fragaria vesca* L, *Sorbus orientalis* Schönbn, *Pyrus spinosa* L. , *Agrimonia eupatoria* L) and Asteraceae with 7 species (*Cichorium intybus* L, *Achillea millefolium*, *Artemisia annua* L, *Centaurea cyanus* L, *Taraxacum officinale*, *Artemesia dracunculus* L and *Arctium lappa* L). Furthermore Known medicinal plants of Gilan Province from the Rosaceae family, 23 species include (*Rosa* spp, *Mespilus germanica* L, *Cydonia oblonga* Mill, *Rubus hirtus* L, *Fragaria vesca* L) [78].

The Hyrcanian forests are also rich in moss and fungal flora. Of the 437 moss species found throughout Iran, 338 are native to the southern Caspian provinces, which include northern Iran [110].

The vegetation of the Caspian's shrubs and forests is attributed to three primary origins: transitional, edaphic, and successional [111]. Many of the Hyrcanian Forest beds are rocky with a thin soil layer, which leads to the growth of dry shrub species such as *Carpinus orientalis* and *Crataegus*. Extensive research has been conducted on the flora of Iran's four main geographical regions: Irano-Turanian, Euro-Siberian, Sahara-Saudi Arabia, and Sudan [112-116].

The Hyrcanian forests span a latitudinal range from below sea level to elevations of up to 2,700 meters, fostering the development of diverse ecological formations within these forests, which accommodate species that are both cold-resistant and cold-sensitive [117, 118].

1.3.2 The province of Alborz

1.3.2.1 Historical characterization and Sociodemographic characterization

Numerous historical and contemporary earthquakes have been associated with the activity of faults originating in the Alborz province [119]. This province is characterized by a significant production of roses, each year, approximately one million rose stems are exported from Alborz to Tajikistan, Georgia, and Iraq.

Alborz province has a diverse demographic composition and has a high migration potential. This province hosts various ethnic and cultural groups from all over the country. This province includes the six cities of Karaj, Ashtard, Savjablak, Taleghan, Nazarabad and Fardis (Figure 1.2).

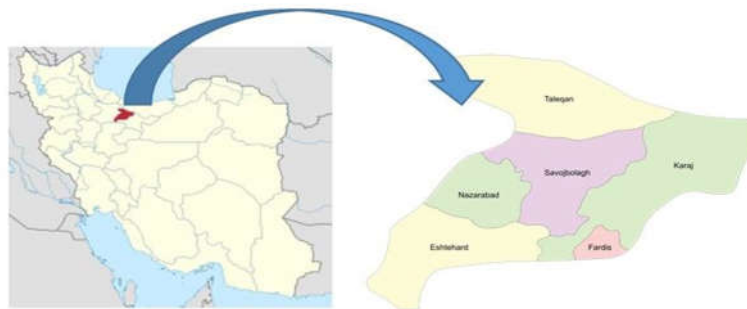


Figure 1.2. Geographical location of Alborz province in Iran

1.3.2.2 Geographic characterization

Alborz Province, covering 519,391.5 hectares, constitutes approximately 0.3% of Iran's total land area. Geographically, it lies between 50° to 51.30° east longitude and 30.35° to 30.36° north latitude, positioned along the southern slopes of the Alborz Mountain range. The province exhibits diverse topographical characteristics. Its northern areas ascend to altitudes of 4,104 meters, forming a continuous mountain range stretching from east to west. Meanwhile, the central region consists primarily of plains, sitting at an average altitude of approximately 1,500 meters. Overall, most of the province lies at elevations ranging from 1,500 to 2,000 meters [107].

The Alborz Mountain range is part of two major mountain chains running along Iran's northern border. These mountains connect the Hindu Kush and Himalayas to the east with the Anatolian and Caucasus ranges to the west [120]. This transitional zone has significant historical, evolutionary, geographical, botanical, and biogeographical importance [121]

1.3.2.3 Soil and climate characterization

Two major Iran rivers, the Karaj River and the Dorvan River, flow through Alborz Province. The region experiences distinct seasonal precipitation patterns, with rainy winters and dry summers. The southern and central cities, located in flat plains, typically receive less than 300 mm of annual rainfall, reflecting a dry and semi-arid climate. However, pockets near the high peaks in the northern and eastern regions enjoy more humid conditions, with annual precipitation reaching 500–600 mm. At higher altitudes, such as in Savojbolagh and Taleghan, rainfall is more abundant. However, steep slopes in these areas result in poorly developed soils unsuitable for agriculture [122].

Summers in Alborz are arid, hot, and sunny, with intense solar radiation. Snowmelt during summer provides the primary water supply, crucial for vegetation in the alpine zones. Snowmelt timing significantly influences vegetation patterns and the distribution of plant communities [121]. The province experiences substantial temperature fluctuations. Absolute minimum and maximum temperatures recorded are -18°C and 42°C , respectively, with an average annual temperature of 15.1°C .

Alborz Province is marked by prominent Quaternary faults, with landslide risks predominantly in the northern and northeastern regions, coinciding with elevated terrains. Conversely, the central and southwestern areas show minimal landslide risks. According to the USDA Soil Taxonomy, the region's soils fall into three primary groups: Aridisols, Inceptisols, and Entisols [123].

These characteristics highlight the unique geographical and climatic diversity of Alborz Province, as well as the challenges and opportunities posed by its topography and natural resources.

1.3.2.4 Flora and vegetation

The flora and vegetation of Alborz Province are exceptionally diverse, forming part of the Irano-Anatolian biodiversity hotspot, renowned for its high concentration of endemic and rare plant species. Approximately 70% of the province's land demonstrates ecological potential for agriculture [122]. Of this, 33.9% is deemed suitable for irrigated farming, while 36.2% supports rainfed agriculture. Furthermore, 35.3% of the land is suitable for gardens and orchards, categorized as first and second degree in suitability. However, 23.5% of the province's land lacks ecological potential for agriculture, with 12.1% specifically unsuitable due to factors such as poor soil texture and low salinity [122]. Strategic crops, including wheat and barley, can be cultivated under semi-arid conditions in soils characterized by salinity, sodicity, and high calcium carbonate content [122, 124, 125].

Alborz Province holds significant potential in horticulture, with 43% of its land being highly favorable for this purpose.

Initial assessments of the vascular flora in Iran's alpine zones reveal 682 species, spanning 193 genera and 39 families [121]. Among these, Asteraceae is the most prevalent angiosperm family with 32 genera and 111 species; stringent montane genera encompass *Myopordon*, comprise four species [126], *Jurinella* comprise with three species followed by Fabaceae (6 genera, 106 species), Caryophyllaceae (11 genera, 52 species), Poaceae (21 genera, 50 species) include Graminoid clad, Lamiaceae (16 genera, 50 species), Cruciferae (19 genera, 38 species); include genres *Elburzia*, *Physoptychis* (one species in Iran), *Didymophysa* (one species in Iran), *Zerdana* (an endemic alpine species in Zagros) and *Anchonium* (one species in Alborz.) Rosaceae (7 genera, 38 species), Apiaceae (16 genera, 30 species); genera encompass *Diplotaenia*, comprise two alpin species, *Sclerochorton* [121].

The largest alpine genera in Iran include *Astragalus* (78 species), *Nepeta* (21 species), *Cousinia* (20 species), *Potentilla* (19 species), *Silene* (18 species), and *Oxytropis* (14 species) [127].

Notably, 22.5% of Iran's endemic plant species are confined to alpine life zones. Research supports the hypothesis that the proportion of endemic species increases with altitude [128]. Of these, 32% are restricted to the Alborz Mountain range, while 46% are endemic to the Zagros range. The taxonomic relationships of these species suggest that the alpine flora of Iran originates primarily from the Irano-Turanian region [129]. These findings underscore the ecological and botanical significance of Alborz Province, highlighting its critical role in preserving biodiversity within the Irano-Anatolian hotspot.

1.3.3 The province of Qazvin

1.3.3.1 Historical characterization and Sociodemographic Characterization

Qazvin Province is one of Iran's most historically significant regions, home to a wealth of ancient monuments. Remarkably, 11% of the nation's registered historical landmarks are located within this province, placing it first in the country in this regard.

Recently, Iran's oldest settlement was uncovered within a 10,000-year-old Kurd fortress located in Auj, in the southern Zagros Mountain range. Additionally, in the Qazvin Plain, Zoroastrian tombs from the Median and Sasanian eras have been discovered, further underscoring the province's historical importance.

The village of Alamut, named after the Alamut mountains, is another notable area within Qazvin Province. Renowned for its high-quality medicinal plants and its numerous ancient castles, Alamut attracts a significant number of tourists annually. Furthermore, the inhabitants of Alamut have a longstanding tradition of cultivating and exporting medicinal plants, contributing to the region's cultural and economic heritage.

As of 2016, the population of Qazvin Province was approximately 1.3 million, with 600,000 residing in Qazvin City. Despite comprising only 1% of Iran's total land area, the province plays a disproportionately large role in the nation's economy, accounting for nearly 10% of its production output. The province includes several cities, such as Qazvin, Abyek, Auj, Alborz, Buin Zahra, and Takestan, each contributing uniquely to the region's economic and cultural landscape.

1.3.3.2 Geographic characterization

The study area is situated between latitudes 36°1' and 36°9' N, and longitudes 50°21' and 50°14' E. It spans approximately 16,630 hectares within the Abyek region of Qazvin Province, Iran. These historical, demographic, and geographic attributes highlight the province's cultural and strategic importance in Iran's national framework (Figure 1.3).



Figure 1.3. Geographical location of Qazvin province in Iran

1.3.3.3 Soil and climate characterization

The mean annual precipitation and temperature at the site are 284 mm and 14°C and the coldest and hottest months are December and July, respectively. Soil moisture and temperature regimes are dry xeric, weak aridic, and thermic, respectively, according to [130].

1.3.3.4 Flora and vegetation

The flora of Qazvin Province is highly diverse due to its varied topography and climate, ranging from 300 meters above sea level in Tarom to 4,140 meters in the Alborz Mountains. About 61% of the province consists of mountainous regions. Notable areas like Alamut are renowned for medicinal plants, with species such as *Echium amoenum* from Boraginaceae family. *Echium amoenum* is traditionally cultivated in certain villages of Alamut. This perennial plant produces striking purple flowers and remains viable for harvesting over a span of four to five years. Once

collected, the plant is carefully dried in the shade to preserve its properties before being sold in the medicinal plant market [131].

Some medicinal species have limited habitats includes, *Valeriana officinalis*, *Papaver bracteatum* and *Ziziphus vulgaris* while medicinal species such *Achillea millefolium*, *Rheum ribes* and *Thymus kotschyanus* have spread across a wide area of the Qazvin highlands [131]. Over 250 medicinal plant species have been identified, with many used in traditional medicine and local markets [132]. Lamiaceae family includes species: *Salvia syriaca*, *Salvia verticillata*, *Salvia virgata*, *Salvia reuteratha*, *Malva neglecta*, *Malva sylvestris*, *Menta longifolia*, *Stachys lavandulifolia*, *Thymus kotschyanus*, *Ziziphora clinopodoides* Asteraceae family includes: *Achillea bibersteinii*, *Achillea millefolium*, *Achillea santolina*, *Achillea tenuifolia*, *Achillea vermiculata*, *Achillea wilhelmsii*, *Ranunculaceae* include *Nigella arvensis*, *Rocaceae* include *Rosa damascena*, *Urticaceae* include *Urtica urens*, *Boraginaceae* include *Echium amoenum*, *Adiantaceae* include *Adiantum capillus* [131]. The 13 important medicinal plants are traditionally used in herbal medicine and have research applications in pharmacology and medicine from Qazvin province includes; *melissa officinalis* L, *bunium persicum* B.F., *Agrimonia eupatoria* L, *Echium amoenum* F.M., *Biebersteinia multifida*, *Nepta pogonosperma*, *Thymus kotschyanus*, *Papaver bracteatum*, *Ziziphus vulgaris*, *Viola odorata*, *Ziziphora clinopodoides*, *Heracleum percicum* and *Salvia hydrangea* [131].

The medicinal plant species of Qazvin province are distributed as follows: 17% are in the form of shrubs and small trees, 66% are herbaceous perennials, and 17% are annual herbaceous plants. The majority of the collected species originate from elevations ranging between 1,000 and 2,500 meters above sea level, while the least number of species are found at altitudes below 1,000 meters or above 2,500 meters[131].

The current study aims to provide a comprehensive ethnobotanical and ethnopharmacological assessment of *Triplerospermum disciforme* (C.A.Mey) Sch.Bip., a medicinal plant use in the provinces of Guilan, Qazvin, and Alborz. Specifically, it seeks to document and systematically analyze indigenous knowledge related to local flora by integrating traditional practices with quantitative ethnobotanical methodologies. Furthermore, the research intends to evaluate the chemical composition of bioactive secondary metabolites extracted from selected flowering specimens using advanced analytical techniques, namely UHPLC–timsTOF–MS. In parallel, it aims to assess the antioxidant and antimicrobial properties of these extracts' fractions. Through this multidisciplinary approach, the study aspires to establish a scientific foundation that contributes to both regional and global understanding of medicinal plants and supports the validation and potential application of traditional herbal knowledge.

The Genus *Tripleurospermum* Sch. Bip. (Asteraceae): A Comprehensive Review of Its Ethnobotanical Utilizations, Pharmacology, Phytochemistry, and Toxicity

This chapter corresponds to a published manuscript with the following reference:

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Abstract

This review provides a comprehensive overview of the botany, traditional uses, phytochemistry, pharmacology, and toxicity of the genus *Tripleurospermum*. *Tripleurospermum*, a prominent genus within the family Asteraceae, is recognized for its therapeutic potential in treating various ailments, including skin, digestive, and respiratory diseases; cancer; muscular pain; and stress and as a sedative. Through extensive phytochemical studies regarding the *Tripleurospermum* species, numerous chemical compounds have been identified and classified into distinct classes, predominantly encompassing terpenes, hydrocarbons, steroids, hydrocarbons, oxygenated compounds, flavonoids, tannins, alcohols, acids, melatonin, and fragrant compounds. The findings from this review highlight the presence of bioactive compounds within the *Tripleurospermum* species that possess significant medicinal properties.

Keywords: *Tripleurospermum*; phytochemistry; antioxidant activity; antimicrobial activity; cytotoxicity; anti-inflammatory and analgesic effects

2.1. Introduction

In recent years, there has been a growing trend in the utilization of natural medicines to treat illnesses due to their reduced adverse effects, cost-effectiveness, and wide availability [9]. Although several articles have already explored the medicinal potential of active compounds, namely active molecules, derived from the genus *Tripleurospermum* in various therapeutic areas [133-137], it is imperative to conduct further investigations into the therapeutic and toxicological properties of this plant genus. The aim of this review was to comprehensively analyze the botanical characteristics, traditional uses, phytochemistry, pharmacology, and toxicity profiles of different *Tripleurospermum* species. Moreover, we intended to facilitate and guide future research endeavors by documenting the ethnopharmacological applications of *Tripleurospermum*. Noteworthy scientific contributions have been made on diverse facets of the genus *Tripleurospermum*, such as its pharmacology [133], toxicity [133], biochemical properties, the pharmaceutical potential of proteins and peptides derived from this genus [138-143], as well as, its chemical constituents [133, 144, 145]. Lastly, this review provides a concise summary and outlines future research directions in the field of the *Tripleurospermum* genus. In the subsequent sections, an overview of the taxonomy, stomatal characteristics, achene, as well as the anatomical features of leaves and stems observed in various species of *Tripleurospermum* is presented. The genus *Tripleurospermum* is a member of the tribe Anthemideae, in the family Asteraceae, comprising nearly 40 species. It is closely related to the genus *Matricaria* L. which consists of approximately seven well-defined taxa [4]. In the geographical region of Turkey, *Tripleurospermum* is reported to encompass 40 species and 32 taxa [5, 134, 146-150], of which 12 exhibit distinct leaf and achene/cypsela anatomical characteristics [151]. Recent taxonomic research by Tekşen et al. [152] identified *Tripleurospermum eskilensis* as a new species, thus

expanding the number of *Tripleurospermum* taxa in Turkey to 33. Notably, Turkey has been recognized as the primary source of the cultivated variety of *Tripleurospermum* [6, 7]. In Russia, the genus *Tripleurospermum* comprises 17 species [8], while Iran is home to 6 species of *Tripleurospermum* [144, 153]. The taxonomic classification of the genus *Tripleurospermum* remains a subject of contention, particularly due to its species' distinct characteristics. Initially, the genus *Tripleurospermum* was classified under the genus *Matricaria* L. but was later reclassified as a separate genus based on two distinguishing factors: the unique shape of its achene and the prevalence of tetrasporic embryo sacs [135]. However, it should be noted that Refs. [136, 137] have erroneously reported *Matricaria* L. as part of the *Tripleurospermum* genus. Subsequent investigations confirmed the association of *Tripleurospermum* with *Anthemis* L. rather than with *Matricaria* L. [154]. In addition, limited chromosomal information is available for both *Tripleurospermum* and *Anthemis* L. The ploidy levels observed in *Tripleurospermum* range from $2n = 2x = 18$ to $2n = 3x = 27$ to $2n = 4x = 36$. There are four ploidy levels (2x, 3x, 4x, and 5x) in all *Tripleurospermum* species [155]. In contrast, *Matricaria* L. was found to have a single ploidy level (2x) [139]. Polyploids have been proposed as a potential mechanism contributing to the diversification and divergence of the genus *Tripleurospermum* [6, 134]. Furthermore, the outermost layer of this genus' leaves has been identified as a significant factor in taxonomic differentiation and to play a role in the development of stomatal complexes, including those found in achenes [156, 157]. It is worth noting that anatomical features such as stomatal length, vascular bundle size, and palisade sclerenchyma thickness are important for distinguishing between *Tripleurospermum* species and for establishing a correlation between ploidy level and anatomical structures across different *Tripleurospermum* species [151, 158]. The stomata of *Tripleurospermum* species are distributed on both the upper and lower surfaces of their leaves, with equal stomatal density reported [151]. Stomatal characteristics such as stomatal frequency, guard cell length, and stomata plastids' diversity have been used as morphological indicators for assessing ploidy levels in different plant species [159-162]. Studies have demonstrated that polyploid plants exhibit longer stomata than diploid plants [163, 164]. Furthermore, a positive correlation was observed between ploidy level, stomatal size, and altitude in *Tripleurospermum* species [134]. Taxonomically, stomatal and vascular bundle sizes are associated with the degree of polyploidy, which holds significance in distinguishing different *Tripleurospermum* species, particularly within the Turkish endemic species [156]. In all species of the genus *Tripleurospermum*, achenes exhibit a general similarity, but their anatomical structure, i.e., their pericarp, seed coat, endosperm, and seed lobe, display distinct variations among species [151], which aligns with previous findings reported by Inceer et al. [156]. The morphology and anatomy of achenes are known to contribute to the systematic and phylogenetic understanding of the Anthemideae tribe within the Asteraceae family [4, 15, 165-169]. Furthermore, achene morphology, such as size, shape, number of ribs, slime formation, and pericarp shape, are crucial in the classification of *Tripleurospermum* taxa [157, 165, 169, 170]. The leaf anatomy of *Tripleurospermum* species bears similarities to that of *Matricaria* L. [151]. The leaf anatomy of Turkish *Tripleurospermum* taxa typically consists of lower and upper epidermis, parenchymatous mesophyll, and a vascular

bundle [171]. The epidermal cells are isodiametric, resulting in nearly straight walls in both the lower and upper epidermis [156]. The leaf surface are covered with nonglandular uniseriate and multicellular trichomes, although *T. corymbosum* has been reported to be glabrous [151, 156]. The mesophyll blades primarily comprise dorsiventral and palisade parenchyma cells, similar to the mesophyll structure observed in the Asteraceae family [151, 172, 173]. In addition, all taxa of *Tripleurospermum* exhibit either one major vascular bundle or two medium-sized vascular bundles with a secondary vascular bundle [151]. It is worth noting that the large vascular bundle, which constitutes 50% of the mesophyll's area, holds taxonomic significance in the delimitation of the taxa [151, 156, 172]. Regarding stem anatomy, *Tripleurospermum* stems are usually rounded, but some species, such as *T. caucasicum*, *T. melanolepis*, *T. rosellum* var. *album*, *T. parviflorum*, *T. sevanense*, *T. transcaucasicum*, and *T. monticola* [158], were reported to have slightly ridged stems with seven to nine ribs [158]. The stem anatomy of the studied *Tripleurospermum* species, including *M. chamomilla* [174], *T. baytopianum*, *T. caucasicum*, *T. monticola*, and *T. transcaucasicum* [175, 176], generally exhibits similarities. However, the stem anatomy of *Matricaria* L. and *Tripleurospermum* is considered of minor importance for taxa delimitation [158].

This review was conducted using a structured and integrative approach to provide a comprehensive overview of the genus *Tripleurospermum*. A systematic literature search was performed using major scientific databases, including PubMed, Scopus, and Web of Science, as well as Google Scholar. Relevant articles were identified using combinations of keywords such as “*Tripleurospermum*”, “ethnobotany”, “phytochemistry”, “pharmacology”, and “toxicity”. Inclusion criteria comprised studies published in English, with a focus on peer-reviewed articles reporting ethnobotanical data, chemical composition, and biological activities (in vitro and in vivo). Studies with insufficient data or lacking scientific rigor were excluded. The selected articles were critically evaluated based on their methodology and reliability. Data were then extracted and categorized into four main areas: ethnobotanical uses, phytochemical constituents, pharmacological activities, and toxicity. This integrative approach enabled a comprehensive comparison of findings and facilitated the identification of existing research gaps.

2.2. Habitat, Distribution, and Ecology

The Asteraceae family represents the largest and most prominent family of plants [177]. The related extensive research has focused on the remarkable diversity of the species and genera, global distribution, and effective plant species [168]. *Tripleurospermum* Sch. Bip., a genus within the family, has been cultivated in various temperate regions of Europe and Asia alongside other species found in north Africa and North America [4-6, 143, 151] (Figure 2.1). Species belonging to this genus are characterized as herbaceous, either annual or perennial in nature [145].

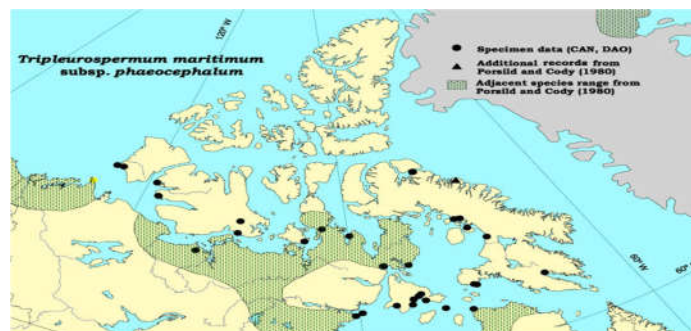
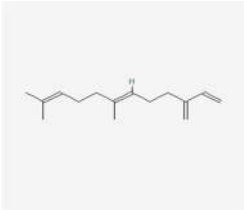
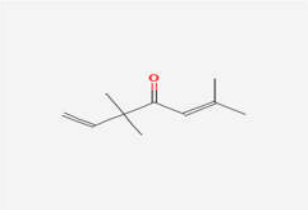
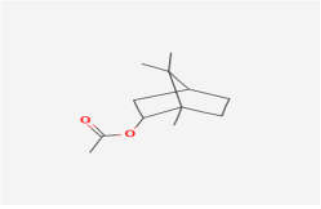
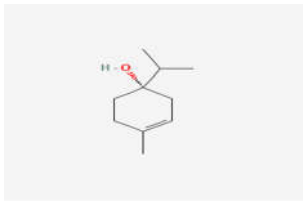
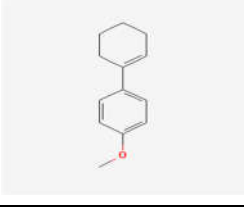
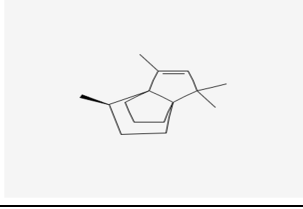
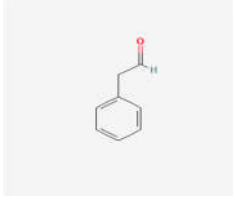
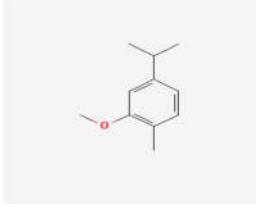
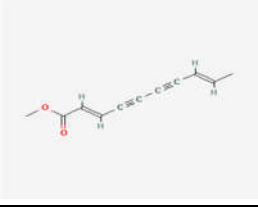
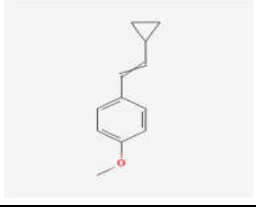
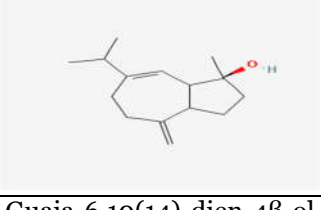
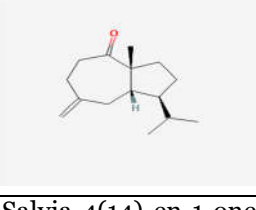
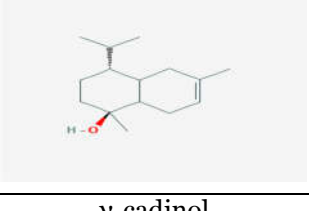
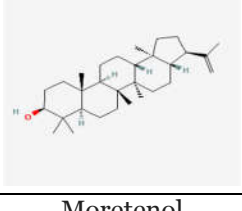


Figure 2.1. Distribution of the cytotype of *Tripleurospermum maritimum* in North America and the northern hemisphere. ● – $2n = 18$ [178]

2.3. Phytochemical Compounds of the Genus *Tripleurospermum*

The essential oil composition of *Tripleurospermum decipiens* flowers has been previously investigated, and the main reported compounds are matricaria esters [15] (Figure 2.2). Matricaria esters have also been identified as compounds of *T. inodorum* [179] and *T. disciforme* [180] (Figure 2.2). The presence of terpenoids in *Tripleurospermum decipiens* was confirmed based on a reference study [181]. Furthermore, the primary compounds found in the essential oil of the aerial parts of *Tripleurospermum disciforme* include trans matricaria ester (39.93%), cis-calamenene (22.99%), (Z)-b-farnesene (12.54%), b-maaliene (7.98%) and b-sesquiphellandrene (2.22%) [182]. Previous reports have identified the significant essential oil compounds in the flowers, leaves, and stems of *T. inodorum*. In the flowers, important compounds include artemisia ketone (14.4%), terpinene-4-ol (5.5%), 1,8-cineole (5.1%), sabinene (4.7%), and tricosane (4.6%) [16] (Figure 2). The leaf is characterized by important compounds such as caryophyllene oxide (16.0%), phytol (12.1%), spathulenol (5.9%), hexahydrofarnesyl acetone (3.8%), and salvia-4(14)-en-1-one (3.5%), which exhibit relatively high percentages. The main compounds in the stem essential oil include neryl acetate (12.8%), (E)- β -farnesene (12.5%), phytol (12.1%), guaia-6,10(14)-dien-4 β -ol (10.8%), γ -cadinol (7.8%), nonacosane (7.3%), decanoic acid (6.3%), and caryophyllene oxide (4.6%).

Phytochemical research on *T. insularum* Inceer & Hayırhıoglu-Ayaz [149] has revealed that the basic compounds of the headspace and essential oils are fatty acids and n-alkanes (38.43–59.22%), including n-octacosane, linoleic acid, and n-hexacosane [12]. Additionally, sesquiterpenes (13.45%) and β -sesquiphellandrene (9.29%) were identified as the main compounds in the essential oil of the genus *T. insularum* Inceer & Hayırhıoglu-Ayaz [149]. Moreover, β -sitosterol (14.82%) and globulol (13.45%) are notable essential compounds in the headspace [12] (see Figure 2.2).

	
cis- β -farnesene	Artemisia ketone
	
Bornyl acetate	Terpinen-4-ol
	
Anisyl, p-1-cyclohexen-1-yl-	Modephene
	
Benzene acetaldehyde	Carvacrol methyl ether
	
Matricaria ester	p-methoxy- β -cyclopropylstyrene
	
Guaia-6,10(14)-dien-4 β -ol	Salvia-4(14)-en-1-one
	
γ -cadinol	Moretenol

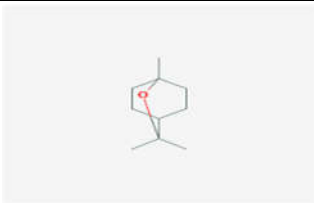
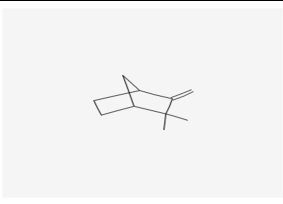
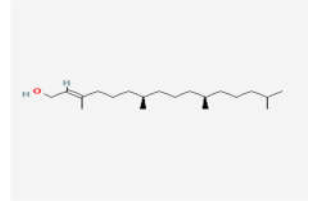



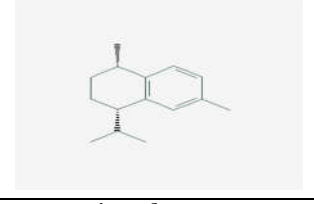
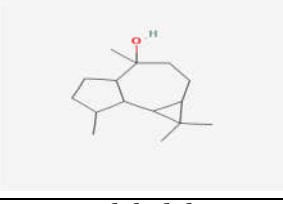
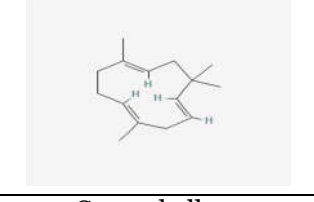
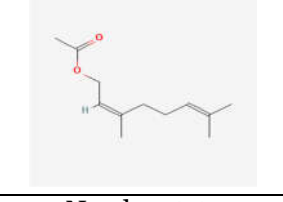
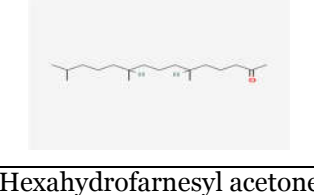
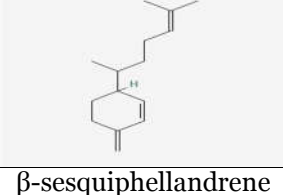
	
1 8-cineole	Camphene
	
Phytol	Camphor
	
Sabinene	p-methoxy-humulene oxide
	
Cis-calamene	Globulol
	
Caryophyllene	Neryl acetate
	
Hexahydrofarnesyl acetone	β -sesquiphellandrene

Figure 2.2. Essential oil compounds of *Tripleurospermum* spp..

Recent research revealed the presence of significant phenolic compounds in *T. inodorum*, namely apigenin, apigenin-7-O-glucoside, luteolin, luteolin-7-O-glucoside, quinic acid, and 5-O-caffeoyl quinic acid [183] (Figure 2.3).

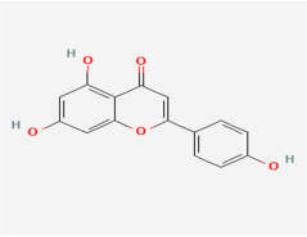
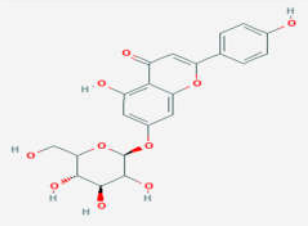
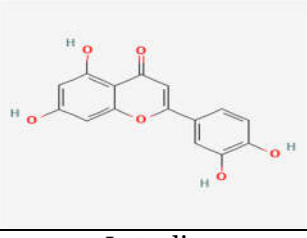
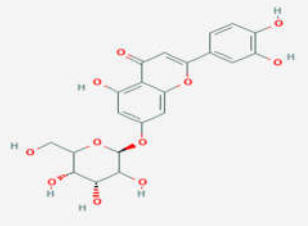
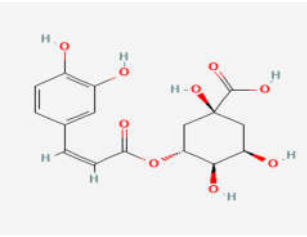
	
Apigenin	Apigenin-7-O-glucoside
	
Luteolin	Luteolin-7-o-glucoside
	
5-o-caffeoyl quinic acid	

Figure 2.3. Phenolic compounds in *Tripleurospermum* spp..

Furthermore, recent reports indicated that the genus *T. insularum* Inceer & Hayırhıoglu-Ayaz [149] contains fatty acids and n-alkanes (38.43–59.22%), such as n-octacosane, n-hexacosane, and linoleic acid (Figure 2.4).

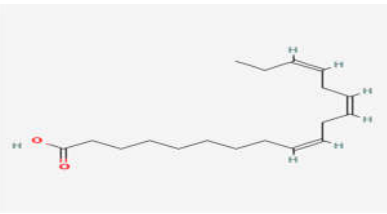

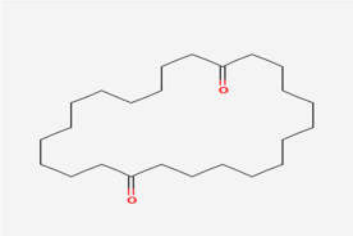
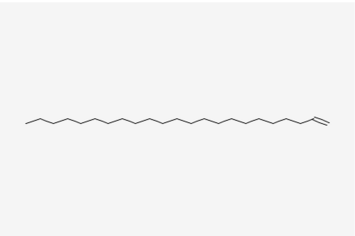
	
Linolenic acid	Hexadecanoic acid or palmitoleic acid
	
Cyclotetracosane	1-Tricosene



Figure 2.4. Fatty acids and hydrocarbon compounds of *Tripleurospermum* spp.

Plants belonging to the genus *Tripleurospermum* encompass a wide range of phytochemicals, including terpenoids, alkanes, steroids, organic acids, and aromatic compounds [17, 184]. The essential oil of these plants is characterized by the presence of p-methoxy- β -cyclopropylstyrene, (E)- β -farnesene, β -sesquiphellandrene, and cis-calamenene as major components [21].

In the case of *T. parthenium*, extensive studies have evaluated its major compounds, which include many secondary metabolites, such as camphor, chrysanthenyl acetate, comphene, and bornyl acetate [185-187] (Figure 2.1). In addition, the hot water extracts of *T. parthenium* and *T. disciforme* revealed the presence of melatonin in flowers [34]. Another study reported the detection of an acetylene derivative of dioxaspiran in the chloroform extract of *T. disciforme* [33]. The particularly volatile compounds of *T. auriculatum* from Saudi Arabia were characterized as fatty acids and their derivatives [188]. The main compounds of *T. callosum* chloroform extract have been identified as hexadecanoic and linoleic acids [21, 189] (Figure 2.4), while the predominant compound in *T. callosum* is moretenol (11.71%) (Figure 2.2). Moreover, studies have demonstrated that the most common compounds in flower oil are linoleic acid (16.18%), n-hexadecanoic acid (17.88%) (Figure 2.4), and n-nonacosane (11.04%). The major compound in the stem oil includes 1-tricosene (13.41%) (Figure 2.4), while the main compounds in the root oil are cyclotetracosane (5.88%) and n-hexadecanoic acid (6.18%) [17]. Yaşar et al. (2005) mentions that chloroform extracts of *T. callosum* flowers, stems, and roots include p-methoxy, β cyclopropylstyrene, and β -farnesene as the main compounds [17]. Additionally, the essential oil of *Tripleurospermum disciforme* is predominantly composed of sesquiterpenes β -farnesene, p-methoxy- β -cyclopropyl styrene, β -sesquiphellandrene and cis-calamenene, ρ -methoxy-humulene oxide, benzene acetaldehyde, and heptadecane [12, 21, 182, 189, 190].

A total of 21 compounds from *T. disciforme* essential oil were analyzed at different stages, including the flowering stages; and variations in the composition were observed. During the flowering stage, β -farnesene (22.46%) and β -sesquiphellandrene (17.85%) were found to be the major compounds, along with p-methoxy- β -cyclopropylstyrene (16.64%), heptadecane (10.60%) p-methoxy-humulene oxide (6.88%), and benzene acetaldehyde (9.30%) [21] (Figure 2.2). The amounts of β -farnesene, β -sesquiphellandrene, p-methoxy- β -cyclopropylstyrene, heptadecane, and benzene acetaldehyde were reported to be highest during the flowering stage and decreased

after flowering and seed maturation. A total of 38 compounds from 89.4% of the total essential oil of *T. parviflorum* were identified. The essential oil of *T. parviflorum* was shown to contain significant compounds such as β -farnesene (18.4%), β -sesquiphellandrene (10.1%), carvacrol methyl ether (7.9%), and benzene acetaldehyde (7.2%) [191]. *T. auriculatum* was analyzed for flavonoids and sterols/triterpenes (90%), tannins (78%), volatile oils (60%), and alkaloids and coumarins (50%) [192]. *T. parthenium* was reported to contain a large proportion of oxidized monoterpenes [193]. In the essential oil of *T. disciforme*, the major compounds identified were nonterpenoids, including anisole, *p*-1-cyclohexen-1-yl- (55.95%), modephene (10.00%), camphor (43.43%), and *cis*- β -farnesene (11.94%). Additionally, the amount of caryophyllene (1.66%) was found to be higher than that in *T. parthenium*. [12, 21, 182, 193]. It is worth noting that a study analyzing the chemical composition of *T. disciforme* [180] found a relatively small amount of β -esquiphellandrene (0.22%), which deviated from previous data. Another recent study [35] employed techniques such as NMR, electron impact mass spectroscopy, column chromatography, and thin layer- chromatography (see Figure 2.2) to identify three triterpenoids—taraxasterol, lupeol, and betulinic acid—in the dichloromethane extract of *T. disciforme*. Regarding *T. tenuifolium* and *T. parviflorum*, palmitic acid (C 16:0) and linoleic acid (C 18:2) were identified as the main fatty acids (see Figure 2.4) [194]. Furthermore, saturated fatty acids (SFAs) were found to be present in higher amounts than monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) in both *T. tenuifolium* and *T. Parviflorum*.

2.4. General Uses, Medicinal Uses, and Pharmacological Studies

The genus *Tripleurospermum*, belonging to the family Asteraceae, has garnered significant interest in the medical field. Ethnobotanical studies have revealed that *Tripleurospermum* species possess various medicinal properties, including sedative and anti-inflammatory effects; relief of muscle pain, fatigue, and carminative properties [195, 196], memory enhancement [197]; cholesterol regulation kidney stone management; treatment of sore throat and wounds healing; respiratory support; diabetes management; cardiac disorders; gastric pain; antiseptic properties and hair care [23-30]. Notably, comprehensive medicinal analyses have demonstrated the therapeutic potential of *T. disciforme*, which exhibits antiulcer effects [32], antibacterial properties [13, 21], antioxidant activity [12, 33], antimicrobial effects [13], and antispasmodic and antiseptic properties [195, 196].

Research on *Tanacetum parthenium*, another member of the Asteraceae family, has shown significant antibacterial activity, potentially attributed to sesquiterpene lactones, such as parthenolide and flavonoids. *T. parthenium* has been used as a neurotonic and antipyretic agent [198], as a relaxant and for muscle ache alleviation, stress management [199] and as a hair dye [200, 201]. Additionally, *T. disciforme* extract has demonstrated antimicrobial effects against *S. aureus* and *S. epidermidis* [202, 203]. The antioxidant activity of different parts of *T. disciforme* was assessed by evaluating their ability to inhibit linoleic acid peroxidation. Notably, the chloroform extract exhibited a substantial antioxidant effect, slightly lower than that of the

standard α -tocopherol [13, 204]. Moreover, a study [205] showed that extracts of *T. parviflorum* and *T. monticola* were effective in treating cough and stomachache and as antipyretic agents. *T. parviflorum* also demonstrated efficacy in the treatment of pharyngeal illnesses and vaginitis [205]. Traditional medicinal practices involve the use of the entire plant of *T. limosum* for the treatment of gastritis [133, 205, 206]. Additionally, *T. sevanense* has been utilized for hair-care purposes [207].

T. callosum flowers have been recommended to treat urinary tract disorders, kidney stones, shortness of breath, common cold, asthma, and bronchitis and as a panacea [190]. Furthermore, *T. auriculatum* was found to be effective in the treatment of neuromuscular blockade and mild hypoglycemia [192]. *T. inodorum* is known for its efficacy in alleviating gastrointestinal pain and its anti-inflammatory properties [208]. Moreover, *T. inodorum* was historically utilized in prehistoric painting techniques, and it is important to mention that apigenin, a compound found in this species, contributes to its yellow coloration [209].

2.4.1. Antioxidant Activity

The antioxidant activity of the essential oils from the newly discovered species *T. insularum*, belonging to the genus *Tripleurospermum* [149], was investigated using 2-diphenyl-1-picrylhydrazyl (DPPH) and ferric-reducing activity (FRAP) assays [12]. The essential oil's ability to scavenge stable free radicals and reduce metal intermediates may be attributed to the substantial presence of sesquiterpenes, which have known antioxidant properties [12]. The antioxidant activity of *T. insularum* is comparable to that of other *Tripleurospermum* species, such as *T. disciforme* and *T. oreades* [200], which are recognized for their abundance of caffeoyl derivatives, which are regarded as significant compounds for the antioxidant potential of the Asteraceae family [210]. The aqueous extract of *T. oreades* also showed antioxidant activity [200]. In addition, the chloroform and hydroalcoholic extracts of *T. disciforme* have been associated with antioxidant and antiulcer properties [33, 211]. The antioxidant activity of different fractions of *T. disciforme* was assessed using different methodologies, namely the linoleic peroxidation method, 2-diphenyl-1-picrylhydrazyl (DPPH) assay, Ferric reducing antioxidant power (FRAP) assay, oxygen radical absorbance capacity (ORAC), and cupric ion reducing antioxidant capacity (CUPRAC) assay [33, 212] (Table 2.1). Furthermore, the genus *Tripleurospermum*, specifically *T. disciforme*, showed significant antioxidant activity and elevated levels of total phenolic compounds and flavonoids [199].

It is important to note that the low temperatures at higher altitudes represent a crucial environmental factor contributing to the enhanced biosynthesis of several antioxidants, despite the genetic differences between different species [213, 214]. Among the essential oil compounds of *T. inodorum*, matricaria ester exhibited modest antioxidant activity [215]. Recent findings [133] demonstrated the antioxidant activity of *T. limosum*, with notable effectiveness observed in aqueous, methanol, and ethanol extracts.

Table 2.1. Antioxidant activity of the genus *Tripleurospermum*.

Taxon	Part of Plant	Extract(s) and Essential oil	Type of Study
<i>T. disciforme</i>	Petals, flowers, leaf	Water	DPPH (radical scavenging activity), FRAP (ferric-reducing activity) [12]
<i>T. oreades</i>	Aerial parts, flower	Water	FRAP assay [199], Linoleic acid peroxidation [33]
<i>T. limosum</i>	Leaf, flower, root	Water, methanol, ethanol, acetone, ethyl acetate, ethyl ether, dichloromethane, or hexane	DPPH, ABTS, hydroxyl radical assay, Superoxide radical assay, FRAP, CUPRAC assay, iron chelating assay, copper chelating assay, H ₂ O ₂ assay, β-carotene bleaching assay [133], NO assay
<i>T. oreades</i>	Aerial parts, flower	Water	DPPH-RS, linoleic acid peroxidation [33, 200]
<i>T. rosellum</i> , <i>T. corymbosum</i> , <i>T. temskyanum</i> , <i>T. caucasicum</i> , <i>T. conoclinum</i> , <i>T. callosum</i> , <i>T. Temskyanum</i> , <i>T. oreades</i> , <i>T. ziganaense</i> , <i>T. corymbosum</i> , <i>T. decipiens</i> , <i>T. disciforme</i> , <i>T. elongatum</i> , <i>T. Fissurale</i> , <i>T. heterolepis</i> , <i>T. hygrophilum</i> , <i>T. indorum</i> , <i>T. Kotschyji</i> , <i>T. melanolepis</i> , <i>T. microcephalum</i> , <i>T. monticola</i> , <i>T. oreades</i> var. <i>oreades</i> , <i>T. oreades</i> var. <i>tchihatchewii</i> , <i>T. parviflorum</i> , <i>T. Pichleri</i> , <i>T. rosellum</i> var. <i>album</i> , <i>T. rosellum</i> var. <i>album</i> , <i>T. sevanese</i> , <i>T. subnivale</i> , <i>T. tempskyanum</i> , <i>T. tenuifolium</i> , <i>T. transcaucasicum</i>	Leaf, flower, root	Methanol	ORAC, DPPH, FRAP, CUPRAC [212]
<i>T. insularum</i>	Aerial parts, flower, petal, leaf	Essential oil	DPPH (radical scavenging activity), FRAP (ferric-reducing activity) [12]
<i>T. inodorum</i>	Aerial parts	Essential oil Matricaria ester	DPPH [215]

2.4.2. Antimicrobial Activity

Previous studies have investigated the antibacterial properties of Asteraceae plant species and have found a significant inhibitory effect [198, 216]. The inhibitory effects of different essential oils have also been examined (Table 2.2). Most authors have reported a stronger inhibitory effect of essential oils against Gram-positive bacteria than against Gram-negative bacteria [193, 201, 217, 218] Interestingly, it was found that the levels of flavonoids in Gram-negative bacteria are higher than those in Gram-positive bacteria.

A noteworthy study explored and elucidated the antiyeast activity of *T. disciforme*, which is attributed to the presence of farnesol [201], a crucial compound in the essential oil of the

Tripleurospermum genus. Additionally, flavonoids were identified as antimicrobial agents, exhibiting direct antibacterial activity, synergy with antibiotics, and disease elimination in various studies [219]. The methanol extract of the genus *T. disciforme* showed antimicrobial activity against *Staphylococcus aureus* and *S. epidermidis* [13]. In addition, the essential oil of *T. disciforme* showed antimicrobial activity against *S. subtilis* and *Bacillus cereus* [21]. The phytochemical composition analysis revealed that compounds such as modephene, cis- β -farnesene, β -sesquiphellandrene, anisole, and p-1-cyclohexen-1-yl are responsible for the antibacterial activity in the essential oil of *T. disciforme*. Additionally, these essential oil compounds are present in high concentrations during the flowering period.

Furthermore, the antibacterial activity of essential oils is not solely dependent on their main compounds; minor compounds can also exhibit significant efficacy. Sesquiterpenes, for instance, have demonstrated inhibitory effects. Additionally, a study [220] highlighted the antibacterial activity of flavonoids, specifically kaempferol and quercetin, against *Propionibacterium acnes*. Apigenin was identified as a compound responsible for suppressing *S. typhi*, *Proteus mirabilis*, and *P. aeruginosa* [221]. Another study investigated the eradication of *S. aureus*, MRSA, and methicillin-sensitive *S. aureus* using apigenin and luteolin [222]. Researchers provided evidence supporting the therapeutic and disinfectant properties of *T. disciforme* in improving acne and resolving skin issues, particularly in teenagers [13]. Moreover, a recent report showed the strong antibacterial activity of *T. parthenium* essential oil against *Aspergillus brasiliensis* [193]. Previous findings demonstrated the strong antibacterial effects of all extracts from *T. parviflorum*, including ethanol, methanol, and ethyl acetate extracts against *S. aureus* ATCC 29213. However, none of the tested extracts showed any effect on *Candida albicans* [223]. Furthermore, another investigation reported the high inhibitory activity of *T. disciforme* essential oil against *Klebsiella pneumoniae*, *Shigella dysenteriae*, *E. coli*, and *Candida albicans* [193].

Table 2.2 Antibacterial, antifungal, and antiparasitic activities of the genus *Tripleurospermum* spp.

Part of Plant	Type of Study	Extract(s) and Essential oil	Biological Activity
Aerial part (top flower)	Cup plate diffusion method	Methanol	<i>S. aureus</i> and <i>S. epidermidis</i> [13]
Leaf	Disc diffusion assay	n-Hexane, methanol, ethanol, ethyl acetate, and water extract	<i>E. coli</i> ATCC 29998, <i>E. coli</i> ATCC 25922, <i>E. coli</i> ATCC 11230, <i>S. ATCC</i> 29213, <i>S. aureus</i> ATCC 6538P, <i>Enterobacter cloacae</i> ATCC13047, <i>Enterococcus faecalis</i> ATCC 29212, <i>Pseudomonas aeruginosa</i> ATCC 27853, and <i>Candida albicans</i> , ATCC 10239 [223]
Flower	MIC	Essential oil	<i>S. subtilis</i> and <i>Bacillus cereus</i> [21]
Flower	Agar well diffusion (AD) assay, MIC, MBC /MFC	Essential oil	<i>Aspergillus brasiliensis</i> , <i>Klebsiella pneumoniae</i> , <i>Shigella dysenteriae</i> , <i>E. coli</i> [193]

2.4.3. Anti-Inflammatory Activity

T. disciforme was shown to have significant anti-inflammatory and analgesic effects by inhibiting the release of prostaglandins and other mediators [224]. Notably, fatty acids have been recognized for their protective properties and diverse biological activities, including anti-inflammatory effects [32, 208, 217, 225].

Previous studies reported the remarkable inhibitory effect of ethyl acetate extracts from *T. tenuifolium* and *T. parviflorum* on overall in vivo anti-inflammatory activity [226]. Palmitic acid is the major and important component of *T. tenuifolium*. The substantial presence of linoleic acid and palmitic acid accounts for the significant anti-inflammatory activity observed (Table 2.3). The anti-inflammatory and analgesic activities of *T. disciforme* were assessed using carrageenan-induced edema, formalin, and the tail-flick test. Extracts of *T. disciforme* demonstrated remarkable anti-inflammatory and analgesic properties in the tested models [209]. An extract of *T. disciforme* was shown to be nontoxic at analgesic doses. Furthermore, the administration of large amounts of *T. disciforme* further confirmed its potent anti-inflammatory effects [209].

Studies on the hydroalcoholic extracts of *T. disciforme* have revealed its remarkable ability to protect against ulcer formation in rats with pyloric ligation, indicating the involvement of additional mechanism(s) beyond its acid-reducing activity. Additionally, high doses of *T. disciforme* exhibited effectiveness upon administration via injection [32]. The intensity of ulceration is typically assessed by parameters such as ulcer score or ulcer incidence [227, 228]. Essential oils containing important secondary metabolites in the flowers of *T. disciforme* were identified as contributors to the antiinflammatory activity of the genus *T. disciforme* [229]. Furthermore, a study [230] identified the major flavonoid compounds of *T. disciforme*, including apigenin, apigenin-7-glucoside, apigenin-7-glucuronide, luteolin, luteolin-7-glucoside, luteolin-7-glucuronide, quercetin, quercetin-7-glucoside, and chrysoeriol, which demonstrated effective anti-inflammatory activity [231].

Table 2.3. Anti-inflammatory activities of the genus *Tripleurospermum*.

Part of Plant	Identified Compounds	Extract	Biological Activity
Aerial part	Linoleic and palmitic acids	n-Hexane Aqueous, ethyl acetate, and methanol	Anti-inflammatory, by carrageenan- and serotonin-induced paw edema acetic-acid-induced increase in capillary permeability models [232]
Flower	Flavonoids such as apigenin, quercetin, patuletin, luteolin, and their glucosides	Water	Analgesic and anti-inflammatory, evaluated by formalin test, by inhibiting the cyclooxygenase(COX)-mediated conversion of arachidonic acid to prostanoids [209]

Flower	essential oil compounds	Hydroalcoholic and chloroform	Antiulcerogenic potential [33, 211] ; anti-inflammatory, by carrageenan induced edema, formalin, and tail-flick test [224]
Flower	Flavonoids such as flavones and flavonols, tannins, and essential oil	Hydroalcoholic	Protection against gastric ulcers in pylorus-ligated rats [32]

2.4.4. Cytotoxic Activity

The cytotoxic activity of the methanol extract of *T. disciforme* was investigated against several human cancer cell lines, including human lung adenocarcinoma (A549), human breast adenocarcinoma (MCF7), hepatocellular carcinoma (HepG2), and human colon carcinoma (HT-29) [233] (Table 2.4).

The cytotoxic activity of various extracts of *T. parviflorum*, namely methanol, n-hexane, ethanol, ethyl acetate, and water extracts, were assessed against brine shrimp (LC₅₀ < 1000) [223]. Among the tested extracts, the methanol extract showed the highest cytotoxic activity compared with that of the other extracts tested. All extracts, except the aqueous one, exhibited higher activity than the cytotoxic agent umbelliferone. However, the cytotoxicity of the *T. parviflorum* extracts was lower than that of colchicine.

Moreover, the dichloromethane extract of *T. disciforme* demonstrated antitumor activity against human gastric carcinoma (AGS) and mouse skin fibro sarcoma (WEHI-164) cell lines [35]. However, *T. disciforme* did not exhibit cytotoxic effects on PC12 cells [234]. Notably, the essential oil of the genus *T. inodorum* contains a compound called matricaria ester, known for its potent cytotoxicity against *Artemia salina*. [215]. Recent findings revealed that the methanol extract of *T. limosum* exhibited low cytotoxicity at the lowest concentration tested and no cytotoxicity at the other three concentrations [133].

Table 2.4. Cytotoxic activities of the genus *Tripleurospermum* spp..

Taxon	Activity	Extract	Biological Activity
<i>T. disciforme</i>	("-")	Methanol	(A549), (MCF7), (HepG2), and (HT-29) [233]
<i>T. parviflorum</i>	(+)	n-Hexane	Brine Shrimp Lethality Assay (BSLA) [223]
		methanol, ethanol, ethyl acetate, and water	
<i>T. disciforme</i>	(+)	Dichloromethane	(AGS) and (WEHI-164) [35]
<i>T. disciforme</i>	(-)	Methanol	PC12 cells [234]
<i>T. inodorum</i>	(+)	Essential oil (matricaria ester)	Brine Shrimp Lethality Assay (BSLA) [215]
<i>T. limosum</i>	(+)	Methanol	TM3 cells [133]

("-"), any cytotoxicity activity; (+), cytotoxicity activity

2.5. Conclusions

The primary group of phytochemicals found in the essential oils of the genus *Tripleurospermum* exhibit significant potential as sources of various compounds, such as cis- β -farnesene, 1-tricosene, phytol, guaia-6,10(14)-dien-4 β -ol, γ -cadinol, nonacosane, decanoic acid, caryophyllene oxide, linolenic acid, palmitoleic acid, heptadecane, n-hexadecanoic, n-nonacosane, apigenin, apigenin-7-glucoside, apigenin-7-glucuronide, luteolin, luteolin-7-glucoside, luteolin-7-glucuronide, quercetin, quercetin-7-glucoside, melatonin, and chrysoeriol. These compounds have demonstrated diverse therapeutic applications in multiple fields.

Traditionally, these plants have been commonly employed for the treatment of gastrointestinal disorders, inflammation, throat ailments, vaginitis, dysentery, fever, skin diseases, urinary tract disorders, kidney stones, shortness of breath, common cold, asthma, bronchitis, and muscular pain, and as a sedative, a gastro tonic, an antihemorrhagic, a carminative, a relaxant, stress-relieving agents, hair colorants, and as panaceas. This review provided an overview of the medicinal uses of *Tripleurospermum* species across different countries, thus highlighting their remarkable potential for various biological activities. Notwithstanding, further future research is required to identify the individual bioactive compounds present in *Tripleurospermum* species and elucidate their mechanisms of action.

Chapter 3

Genus *Echium* L.: Phytochemical Characterization and Bioactivity Evaluation for Drug Discovery

This chapter corresponds to a published manuscript with the following reference:

Sheydaei P, Amaral ME, Duarte AP. Genus *Echium* L.: Phytochemical Characterization and Bioactivity Evaluation for Drug Discovery. *Plants*. 2025; 14(16):2548.

<https://doi.org/10.3390/plants14162548>

Abstract

Echium L. is a genus of flowering plants from the Boraginaceae family that includes several species traditionally used in herbal medicine. *Echium* spp. have been applied for treating wounds, urinary tract infections, inflammation, respiratory ailments, cardiovascular disorders, and microbial infections. The roots and flowers are most frequently used, typically prepared as decoctions or infusions. Phytochemical studies have identified diverse bioactive compounds, including phenolics, naphthoquinones, shikonins, fatty acids, sterols, terpenoids, amino acids, and toxic pyrrolizidine alkaloids. Reported pharmacological effects include antioxidant, antimicrobial, and cytotoxic activities, primarily attributed to polyphenolic and terpenoid content. However, the presence of toxic alkaloids also raises concerns regarding safety. This review provides a comprehensive overview of the ethnomedicinal uses, phytochemical components, and pharmacological activities of *Echium* species. The bioactivities observed in genus *Echium* L. substantiate the necessity for preclinical and clinical investigations to thoroughly elucidate and validate the therapeutic potential of this genus and emphasize its relevance in the development of novel therapeutic agents.

3.1. Introduction

In recent years, there has been a substantial increase in the use of natural products for managing various diseases, primarily driven by their favorable safety profiles, economic accessibility, and widespread availability [235, 236]. Although numerous studies have explored the pharmacological potential of bioactive compounds derived from *Echium* species, a thorough evaluation of their therapeutic efficacy, safety concerns, and ethnomedicinal applications remains insufficiently addressed [41, 237-239].

The genus *Echium* L. comprises approximately 70 recognized species predominantly distributed across Europe, North Africa, and the Macaronesian archipelago, with several species extending into Western Asia. Due to their remarkable ecological adaptability, *Echium* plants have been introduced to diverse regions worldwide [240, 241]. For instance, Tunisia hosts 11 native species [242], Turkey contains 9 distinct species including *E. italicum*, *E. vulgare*, and *E. plantagineum* [243], while Egypt reports 7 native taxa [244]. In Iran, *E. amoenum* (Persian borage), a biennial or perennial species from the Boraginaceae family, is widely utilized in traditional medicine. Typically found at altitudes ranging from 60 to 2200 meters, it is consumed as an herbal infusion for managing the common cold, sore throat, gastrointestinal discomfort [41], and neurological disorders [237].

Its documented effects include sedative, analgesic [239], diuretic [238], antioxidant, and anxiolytic activities [245, 246]. Beyond Iran, other species such as *E. italicum* are traditionally used in Turkey as teas and decoctions with diuretic and sedative effects [205]. Topical applications of powdered leaves or root extracts [247], either raw or formulated with beeswax and oils [207, 248, 249].

Additionally, *E. plantagineum* L. is used as a diuretic and diaphoretic through decoction [250], whereas *E. vulgare* L. and *E. russicum* J.F. are employed in the treatment of wound healing disorders through ointments [251]. In Serbia, *E. vulgare* is known for its antitussive, aphrodisiac, demulcent, diaphoretic, diuretic, pectoral, vulnerary properties, and its use in snakebite treatment [252]. Collectively, *Echium* species exhibit an impressive spectrum of pharmacological activities including antioxidant [245, 252-255], anti-inflammatory [256], antiproliferative [257-259], antidepressant [260-262], anxiolytic [246, 263-265], antiviral [266, 267], antibacterial [268], analgesic [239], anticonvulsant [269], and lipid-regulating properties [270, 271]. These effects are largely attributed to key phenolic compounds such as shikonin and other naphthoquinones, as well as essential fatty acids like linolenic acid, which are discussed in detail in subsequent sections. However, the presence of toxic constituents—particularly pyrrolizidine alkaloids (PAs)—raises safety concerns. Notably, *E. plantagineum*, known as Salvation Jane or Paterson's Curse, has been flagged by Australian and New Zealand food safety authorities due to its high PA content. Compounds such as echimidine, echiumine, acetylechimidine, 7-O-acetyl lycopsamine, and 7-O-acetyl intermedine have led to dietary recommendations limiting the consumption of honey derived from this plant to no more than two tablespoons per day [272]. Furthermore, the concentration and composition of these toxic alkaloids vary significantly depending on the plant's geographical origin [273].

For example, while PAs such as senecionine, echimidine, and lycopsamine have been detected in *Echium*-based honey from Spain [274], no such compounds have been reported in Portuguese samples [272]. These findings underscore the pivotal role of environmental factors—such as climate, soil composition, humidity, and pest prevalence in shaping the phytochemical profiles of *Echium* species, ultimately influencing their therapeutic efficacy and toxicity [275]. Supported by both traditional knowledge and emerging scientific evidence, the *Echium* genus represents a pharmacologically valuable group of plants with significant biomedical potential. This literature review seeks to provide an integrative synthesis of the medicinal applications, phytochemical diversity, pharmacological properties, and toxicological risks associated with various *Echium* species. Additionally, by compiling ethnopharmacological data, this work aims to inform future research directions toward the evidence-based use of *Echium* spp. in modern phytotherapy. The paper concludes with a critical appraisal of current findings and suggestions for future scientific exploration

Aims and Methodology

This review provides an overview of the plant's ethnomedical applications, phytochemistry, pharmacological properties, and toxicological risks associated with various *Echium* species.

Methodology: The literature analysis necessitated those reports be disseminated in the English language, without imposing any exclusion criteria based on the publication date. Online databases like PubMed, Google Scholar, Web of Science, pubchem and Scopus were used to gather information using keywords such as *Echium* genus, ethnomedicinal uses, phytochemistry, bioactivities, and pharmacological activities.

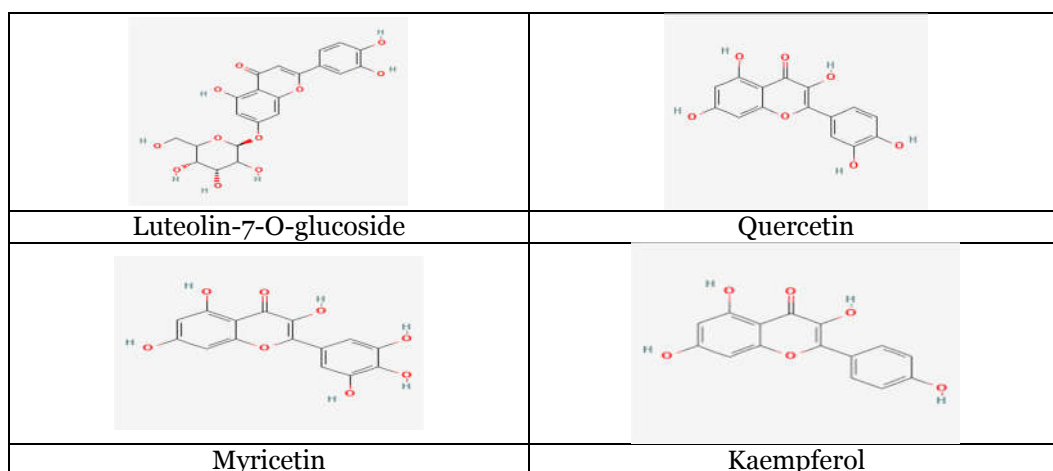
3.2. Phytochemical Characterization

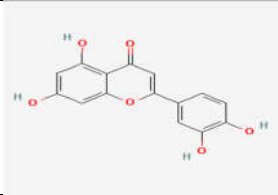
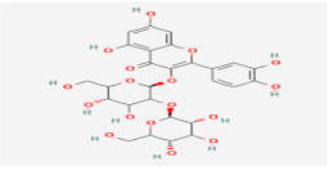
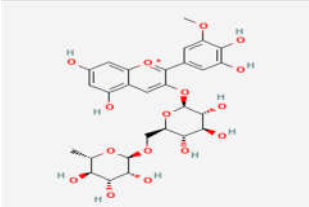
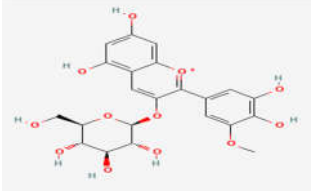
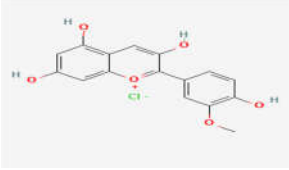
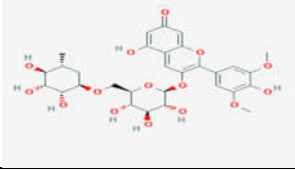
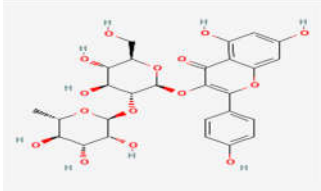

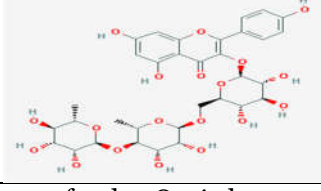
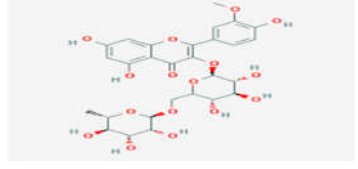
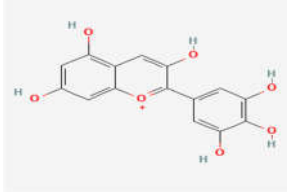
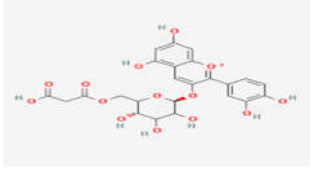
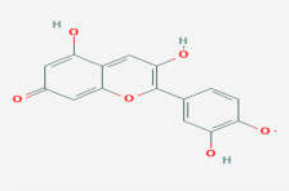

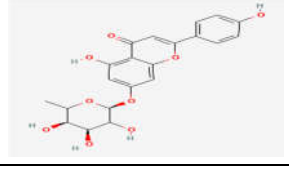
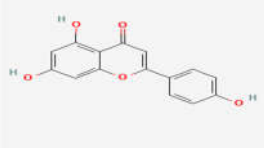
3.2.1. Polyphenolic Compound Extraction and Phenolic Profiles in *Echium* Species: Key Findings and Analytical Insights

The genus *Echium* L. is renowned for its chemically diverse flavonoid profile, which has been rigorously investigated through the application of advanced analytical methodologies. These studies have unveiled a wide array of bioactive flavonoid compounds distributed across various species within the genus, as summarized below. In *E. arenarium*, reverse-phase high-performance liquid chromatography (RP-HPLC) has identified several major flavonoids, including luteolin-7-O-glucoside, myricitrin, myricetin, and quercetin. Among these, luteolin-7-O-glucoside emerged as the most abundant and biologically active compound, underscoring its phytochemical relevance and potential therapeutic significance [276] (see Figure 3.1).

In other *Echium* species, an extensive range of flavonoid compounds has also been detected through HPLC–DAD–MS. These include kaempferol, peonidin, cyanidin, and malvidin, typically occurring as mono- or disaccharide derivatives glycosylated at the C3 position. Particularly noteworthy among these is kaempferol-3-O-neohesperidoside, a compound recognized for its notable bioactivity and pharmacological potential [277-279].

A comprehensive phytochemical investigation of *E. sericeum*, employing proton nuclear magnetic resonance (¹H-NMR) and fast atom bombardment mass spectrometry (FAB-MS), led to the identification of four predominant flavonoids: apigenin, luteolin-7-O-rutinoside, apigenin-7-O-rhamnoside, and quercetin-3-O-rhamnoside (Figure 3.1). These findings collectively highlight the critical role of sophisticated spectroscopic techniques in the structural characterization and profiling of flavonoids in plant matrices [244].



	
Luteolin	Quercetin-3-O-sophoroside
	
Petunidin-3-O-rutinoside	Petunidin-3-O-glucoside
	
Peonidin	Malvidin-3-O-rutinoside
	
Kaempferol-3-O-neheosophoroside	Kaempferol-3-O-rutinoside
	
Kaempferol-3-O-4'-rhamnosyl	Isorhamnetin-3-O-rutinoside
	
Delphinidin	Cyanidin-3-(6''-malonylglucoside)
	
Cyanidin	Rutin
	
Apigenin-7-O-rhamnoside	Apigenin

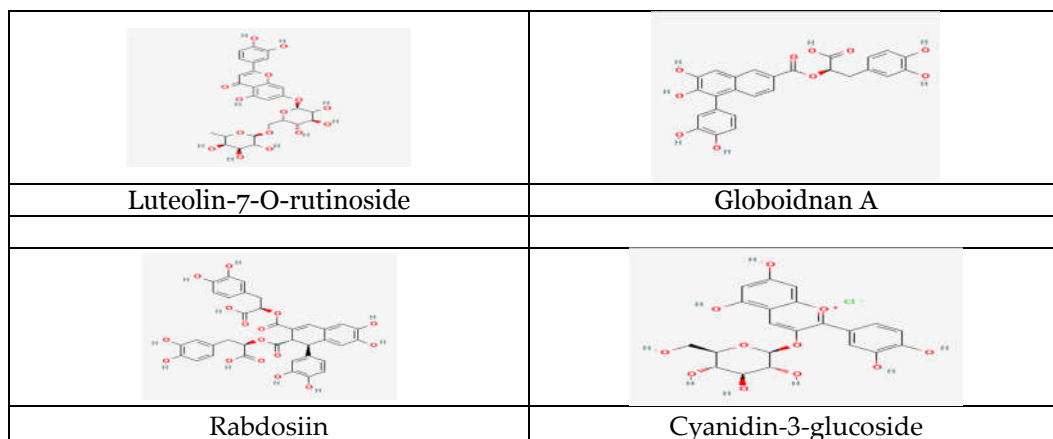


Figure 3.1. Phenolic compounds in *Echium* spp.

Research on polyphenols shows that the extraction efficiency of these bioactive compounds is greatly affected by key factors, including extraction time, temperature, solvent-to-solid ratio, and the water content of the solvent [280-284]. Among the anthocyanins evaluated, cyanidin-3-glucoside yielded the highest concentration, followed in descending order by cyanin chloride, cyanidin-3-rutinoside, and pelargonidin-3-glucoside chloride. These results were obtained using a natural deep eutectic solvent composed of choline chloride and glycerol (CHGLY), which showed superior extraction performance compared to conventional solvents such as methanol, ethanol, and water [284] (see Figure 3. 2).

Reverse-phase high-performance liquid chromatography (RP-HPLC) analysis of the ethyl acetate extract derived from *Echium arenarium* flowers revealed the presence of four major phenolic constituents: luteolin-7-O-glucoside (60.56 µg/mg of dry extract (DE)), myricitrin (27.92 µg/mg DE), myricetin (7.34 µg/mg DE), and quercetin (1.27 µg/mg DE) [276].

The essential oil derived from the hydrodistillation of the flowering aerial parts of *Echium amoenum* (Fisch. & CA Mey) identified thymol and carvacrol as the predominant monoterpenes.[285]. Furthermore, rosmarinic acid has been consistently reported as the primary phenolic compound in *E. amoenum*, *E. russicum*, and *E. vulgare* [286-289]. Notably, hot water extractions of *Echium* species yield significantly higher concentrations of rosmarinic acid compared to other extraction methods [290].

Additional phenolic compounds identified in *E. russicum* include salvianolic acid A, rabdosiin, lithospermic acid, and eritrchin (also known as globoidnan A), enriching the phytochemical profile of the species [287] (Figure 3.2).

A novel compound (caffeoyl derivatives) has recently been identified in the leaves of *E. amoenum* using NMR spectroscopy [291]. This compound is considered an analogue of rabdosin, a phytochemical previously reported in *E. russicum* [292] and *E. plantagineum* [293].

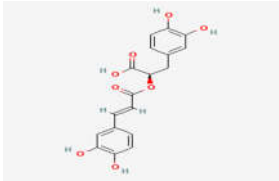
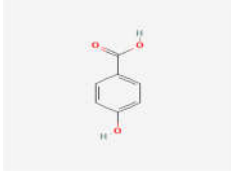
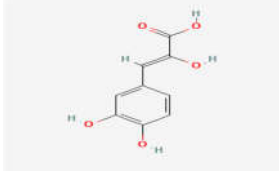
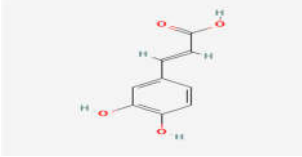
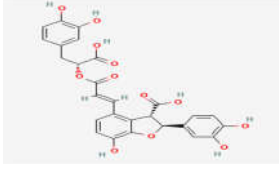
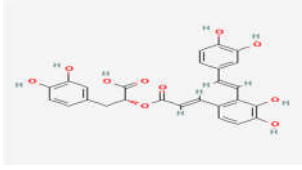
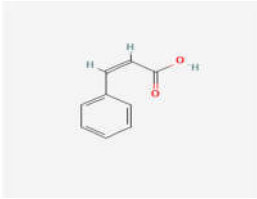
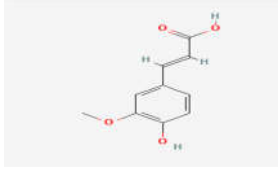
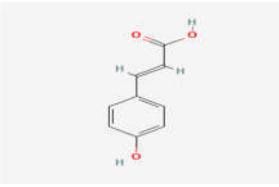
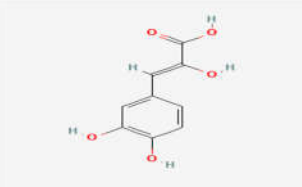
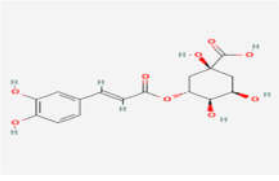
	
Rosmarinic acid	4-Hydroxybenzoic acid
	
Hydrocaffeic acid	Caffeic acid
	
Lithospermic acid	Salviolic acid A
	
cis-Cinnamic acid	Ferulic acid
	
p-Coumaric acid	Hydrocaffeic acid
	
Chlorogenic acid	

Figure 3 2. Phenolic acids in *Echium* spp..

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3.2.2. *Echium* Roots: Focus on Alkanins and Shikonins compounds

The roots of various *Echium* species are abundant in pharmacologically active naphthoquinones, notably alkanins and shikonins. These compounds are extensively documented for their wound-healing efficacy [243, 294, 295], alongside their anti-inflammatory and antimicrobial activities [296]. Among them, the ethanolic root extract of *E. italicum*—rich in naphthoquinone derivatives such as acetylshikonin, deoxyshikonin, and isovalerylshikonin was shown to significantly elevate hydroxyproline levels in experimental mice, indicating enhanced collagen synthesis and tissue regeneration [243] (Figure 3.1).

Similarly, the ether extracts of *Lithospermum erythrorhizon* and *Macrotomia euchroma* roots (both from the Boraginaceae family) have demonstrated wound-healing activity by stimulating granulation tissue formation in murine models [297]. In *Arnebia densiflora* and *Arnebia nobilis*, compounds like arnebin-1 have been implicated in wound contraction and accelerated re-epithelialization, further supporting the pivotal role of root-derived naphthoquinones in tissue repair [298, 299]. Environmental factors such as seasonality and altitude markedly influence the biosynthesis of these compounds. For instance, roots of *E. plantagineum* harvested during the summer contain significantly higher levels of shikonins compared to those collected in winter or spring [300, 301]. Additionally, roots from low-altitude areas yield three to five times more shikonins than those from higher elevations [302]. Beyond wound healing, shikonins, alkanins, and related naphthazarins exhibit a broad range of biological activities. These include strong antioxidant, antihelminthic, and purgative properties, as well as the induction of apoptosis in prostate cancer [295, 303-306] and acute myeloid leukemia cells [307]. They also display potent antibacterial effects, especially against Gram-negative bacteria [59], and have shown neuroprotective effects in Alzheimer's disease models [308]. Moreover, these compounds act as powerful allelopathic agents, inhibiting the growth and viability of competing plants, fungi, insects, and bacteria [309-311].

The remarkable therapeutic potential of naphthoquinone compounds—particularly shikonins and their derivatives underscores the importance of *Echium* and related Boraginaceae species in biomedical research. Understanding the environmental and seasonal factors that influence their biosynthesis can guide the optimization of phytopharmaceutical production and support the

development of novel, nature-based therapeutic strategies in wound management and oncology [252, 312, 313].

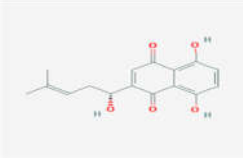
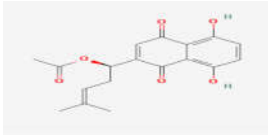
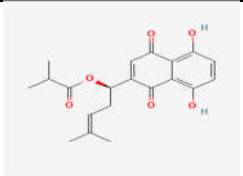
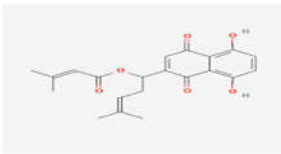
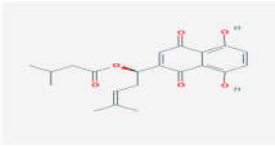
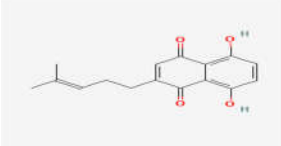
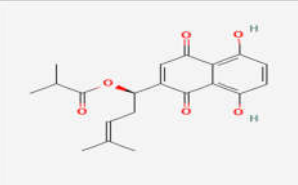
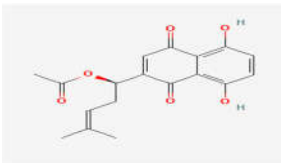
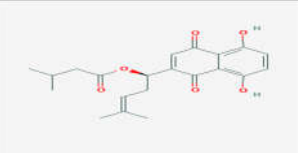
	
Shikonin	Acetylshikonin
	
Adeoxyshikonin	3,3-Dimethylacrylshikonin
	
Isovalerylshikonin	Deoxyshikonin
	
Isobutyrylshikonin	Acetylshikonin
	
Isovalerylshikonin	

Figure 3.3. Chemical structures of shikonin naphthoquinones derivatives of *Echium* spp.

Two steroids, stigmast-4-ene-3,6-dione and β -sitosterol, have been identified in the flowers of *E. vulgare*, with β -sitosterol exhibiting notable antioxidant properties [314, 315] (Figure 3.4).

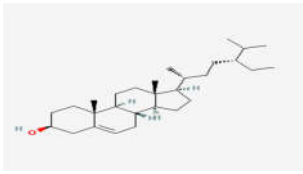
	
β -sitosterol	Stigmast-4-ene-3,6-dione

Figure 3.4. Sterons of *Echium* spp

In the essential oil obtained via hydrodistillation from the aerial parts of *E. italicum*, 22 chemical constituents have been identified, with 1-hexadecanol and pulegone a monoterpene ketone emerging as the most abundant compounds [316]. Similarly, in the essential oil of *E.*

amoenum, n-hexadecane (8.7%) and n-pentadecane (5.6%) are the predominant alkanes [317]. The seed oil of *E. amoenum* is particularly rich in polyunsaturated fatty acids (PUFAs), most notably α -linolenic acid (ALA), which constitutes approximately 40–41% of total fatty acids, followed by linoleic acid (19–20%). These PUFAs significantly outnumber the monounsaturated fatty acids (MUFAs), positioning *Echium* species as valuable botanical sources of essential fatty acids. Notably, among various *Echium* species, the highest concentration of ALA has been consistently reported in *E. amoenum* seed extracts [318]. (Figure 3.5).


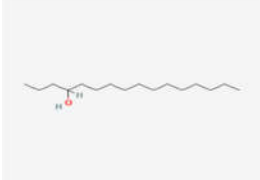



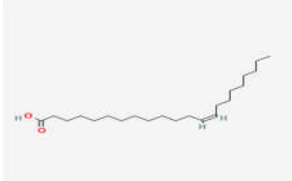
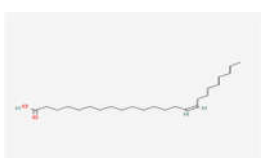
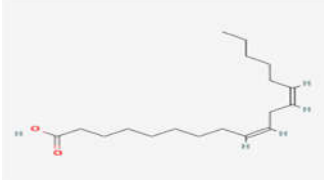
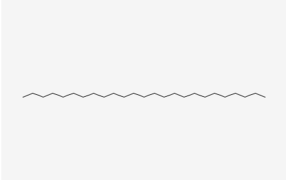
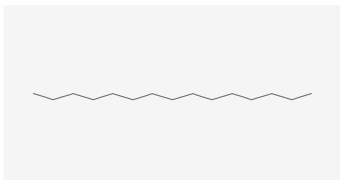
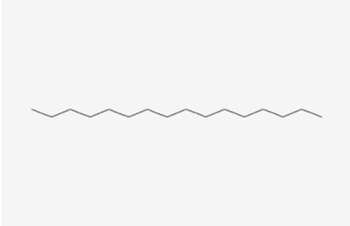
	
Palmitic acid	Hexadecanol
	
Stearic acid	Oleic acid
	
Eicosenoic acid	Erucic acid
	
Nervonic acid	Linoleic acid
	
Pentacosan	n-Pentadecane
	
n-Hexadecane	

Figure 3.5. Fatty acids and hydrocarbon compounds present in essential and seed oils of *Echium* spp.

Figure 3. 6 summarizes the major sesquiterpenes found in the essential oil of *E. amoenum*, which include cadinene (24.3%), viridiflorol (4.9%), β -muurolene (4.5%), ledene (3.8%), α -calacorene (3.0%), and γ -cadinene (2.9%) [317]. Similarly, the essential oil derived from the aerial parts of *E. humile* is characterized by the presence of bicycloelemene (15.9%), pentacosane (8.4%), p-cymen-8-ol (5.8%), β -phellandrene (4.9%), and trans-thujone (4.1%) [319].

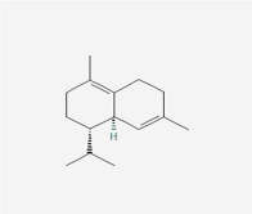
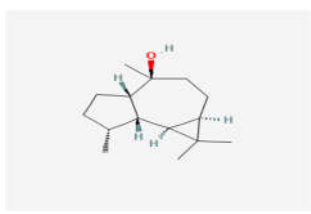
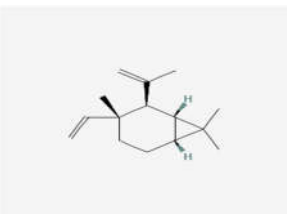

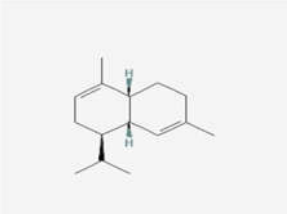
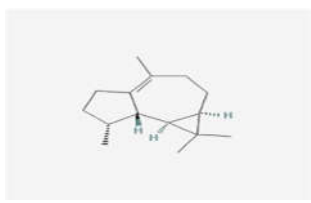
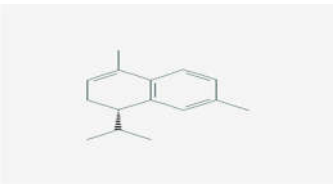
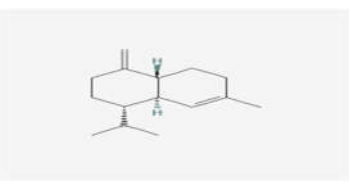

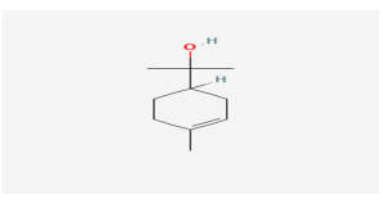
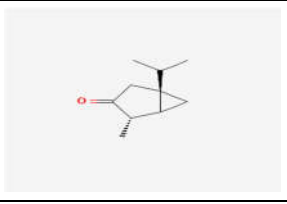
	
δ -Cadinene	Viridiflorol
	
Bicycloelemene	β -Phellandrene
	
α -Muurolene	Ledene
	
α -Calacorene	γ -Cadinene
	
Borneol	α -Terpineol
	
trans-Thujone	

Figure 3.6. Sesquiterpenes and terpenes from the essential oil of *Echium* spp.

These findings collectively highlight the diverse phytochemical landscape of *Echium* species. In particular, the presence of antioxidant steroids, bioactive monoterpenes and sesquiterpenes, and high levels of essential polyunsaturated fatty acids underscores the medicinal and nutritional relevance of this genus. The compositional variability across different species and plant parts suggests that *Echium* holds promise as a multipurpose genus for pharmaceutical [320-322].

3.2.3. Pyrrolizidine Alkaloids in *Echium* species: Toxicity, challenges and Mitigation-Strategies

Pyrrolizidine alkaloids (PAs), a class of toxic secondary metabolites, have been consistently detected across various genera within the Boraginaceae family, including *Echium*, *Symphytum*, and *Heliotropium*. Analytical techniques such as HPLC-ESI-MS and NMR spectroscopy have confirmed the presence of multiple hepatotoxic and genotoxic PA compounds—such as echimidine, echimiplatine-N-oxide, and their isomers—in methanolic extracts of several *Echium* species. In contrast, non-polar extracts, such as those obtained using hexane, showed no detectable PA content, indicating solvent-dependent variation in toxic compound recovery. These findings have raised considerable concerns about the safety of PA-containing medicinal plants and their derivatives in food and pharmaceutical products [323].

Among the approximately 600 plant species identified as containing toxic pyrrolizidine alkaloids belongs to the family Boraginaceae. This family, which includes numerous melliferous (honey-producing) plants, encompasses all species within the genera *Echium* and *Symphytum* of the subfamily Boraginoideae, as well as the genus *Heliotropium* of the subfamily Heliotropioideae contain toxic pyrrolizidine alkaloids such as echimidine, lycopsamine, and vulgarine [324]. Toxic pyrrolizidine alkaloids are capable of entering the food chain and have been identified in a wide range of food and plant-based products such as cereals, tea, honey, herbal remedies, spices, and dietary supplements, posing potential health risks to consumers [325, 326]. chronic or excessive exposure to pyrrolizidine alkaloids has been associated with severe pathological outcomes, such as pulmonary hypertension, myocardial hypertrophy, renal dysfunction, and in extreme cases, mortality [327, 328].

Due to the presence of toxic pyrrolizidine alkaloids (PAs) across all species within the Boraginaceae family, there are increasing concerns regarding the safety of herbal medicinal products derived from these plants. In response to this issue, the General Statement on the Use of Herbal Medicinal Products Containing Toxic and Unsaturated Pyrrolizidine Alkaloids has established a Tolerable Daily Intake (TDI) of 0.35 µg/day for adults [329]. This threshold represents the maximum permissible daily exposure, based on toxicological risk assessments, aimed at minimizing the potential health risks associated with chronic ingestion of these hepatotoxic and genotoxic compounds.

Furthermore, the German pharmaceutical industry has established a comprehensive and practical code of practice aimed at minimizing the presence of toxic alkaloid compounds. This protocol addresses contamination risks at all critical stages of the production chain—including

cultivation, harvesting, inspection of raw materials, pharmaceutical processing, and final product release—ensuring enhanced safety and quality control throughout [330, 331].

high-performance liquid chromatography coupled with electrospray ionization mass spectrometry (HPLC-ESI-MS) revealed the presence of pyrrolizidine alkaloids such as pyrrolizidine N-oxides, tertiary pyrrolizidine alkaloids, leptanthine-N-oxide, echimiplatine-N-oxide, leptanthine and echimiplatine in the methanolic extract of *Echium vulgare*., whereas these toxic compounds were not detected in the hexane extract [274]. Notably, analysis of the methanolic extract derived from the leaves and flowers of *E. plantagineum* with (HPLC-ESI-MS) led to the identification of 3 distinct pyrrolizidine N-oxide alkaloids such as 3'-O-acetylechiumine-N-oxide, echimiplatine-N-oxide and echiuplatine-N-oxide [332]. HPLC-ESI-MS, which is considered one of the fastest and most reliable analytical techniques for profiling pyrrolizidine alkaloids, was particularly effective in detecting these compounds in *E. plantagineum*. Furthermore, using nuclear magnetic resonance (NMR) spectroscopy, the major pyrrolizidine alkaloids were identified as echimidine and a tigloyl isomer of echimidine in two other species, *E.setosum* and *E.vulgare* [333].

Given the widespread presence and toxicological implications of pyrrolizidine alkaloids in Boraginaceae species, there is a pressing need for stricter quality control, improved regulatory frameworks, and continued development of sensitive detection methods.

3.3. General Applications, Medicinal Uses, and Pharmacological Studies of *Echium* spp.

Ethnobotanical surveys conducted in various regions (Table 3.1) confirm the traditional use of *Echium* species, particularly *E. italicum* and *E. amoenum*, for treating infections, inflammation, ulcers, skin conditions, and respiratory ailments. Additionally, in vivo experiments have demonstrated that chloroform root extracts of *E. italicum*, rich in naphthoquinones, significantly promote collagen synthesis and wound healing. Furthermore, essential oils derived from species within the Boraginaceae family are gaining recognition for their industrial relevance, especially in the pharmaceutical, cosmetic, and food sectors. Additionally, in vivo experiments have demonstrated that chloroform root extracts of *E. italicum*, rich in naphthoquinones, significantly promote collagen synthesis and wound healing. Furthermore, essential oils derived from species within the Boraginaceae family are gaining recognition for their industrial relevance, especially in the pharmaceutical, cosmetic, and food sectors.

In *E. italicum*, one of the major phenolic secondary metabolites released from its hairy roots in response to biotic and abiotic stress is shikonins, which are recognized for their antihistamine properties [103]. In fact, the shikonins extracted from the epidermal tissues of *E. italicum* have been identified in recent pharmacological studies not only for their antihistaminic properties, particularly beneficial for individuals with allergies, but also for their notable anticancer and anti-HIV activities [104–106]. Moreover, an additional recognized therapeutic effect of this compound includes the treatment of burns and eczema, neuroprotection, as well as antimicrobial, antithrombotic, and anti-inflammatory activities [104–111].

Furthermore, ethnobotanical studies conducted in the Antalya region of Turkey have identified three additional species from the Boraginaceae family, such as *E. italicum*, *E. vulgare* L., and *E. russicum* J.F. Gmellin, for their anti-ulcer, anti-inflammatory, and vulnerary activities [18].

Additionally, a traditional ethnobotanical study conducted in Bilecik Province, Turkey, identified *E. plantagineum* as a widely used medicinal plant for treating infections and skin disorders [19]. A recent study conducted on four *Echium* species, *E. italicum* L., *E. vulgare* L., *E. angustifolium* Miller., and *E. parviflorum* Moench, across different provinces of Turkey revealed that *E. italicum* contains higher levels of shikonin and its derivatives compared to the other species. These compounds represent the active ingredients in several pharmaceutical formulations [112].

Additionally, in vivo studies conducted in Turkey on *E. italicum* reported that the chloroform root extract of this species, due to its content of naphthoquinones, significantly accelerated wound healing in mice. Specifically, the treatment led to an increased level of hydroxyproline protein in the granulation tissue, indicating enhanced collagen turnover during the healing process [10]. An ethnobotanical survey conducted in Italy reported that *E. italicum* is traditionally used for the treatment of five different health conditions, making it one of the commonly used medicinal plants in the region [113] (Table 1). Similarly, an ethnobotanical study conducted in Iran, Alamut, reported that *E. amoenum*, Fisch. And May. Ahvazi 637 (IMPH), is the only species from the Boraginaceae family recognized for its traditional medicinal use [6]. The aqueous extract of *E. amoenum*, collected from Ardabil Province in Iran, has demonstrated antimicrobial activity against *S. aureus* (ATCC 8327) [37]. This finding supports the traditional use of the plant in treating fever and the common cold. Notably, *E. amoenum* has shown efficacy in treating neuropsychiatric disorders, such as anxiety and depression [30,89]. Multiple preclinical and clinical studies have highlighted the diverse therapeutic potential of its extracts, particularly the aqueous and methanolic forms. Clinical trials have demonstrated that oral administration of aqueous extracts of *E. amoenum* for 6 to 8 weeks significantly improves symptoms in patients with depression, anxiety, and obsessive-compulsive disorder. In vivo studies have further supported its neuroprotective potential, showing anticonvulsant effects when methanolic extracts are administered before picrotoxin-induced seizures and notable analgesic activity when given prior to nociceptive testing, with clinical trials supporting these claims [32,34,114,115]. They found that *E. amoenum* syrup was more effective than citalopram with fewer complications [114]. This highlights its potential as a natural, well-tolerated alternative for mood regulation.

In addition to its neuropsychiatric applications, a recent clinical trial revealed that a single oral formulation combining *E. amoenum* extract with chamomile and vitamin B6 significantly reduced the severity of premenstrual syndrome (PMS) symptoms, further supporting its use in women's health [89].

Essential oils of aromatic plants, extracted from various parts such as leaves, stems, bark, seeds, fruits, roots, and plant exudates, contain a mixture of chemical compounds that are widely used in industries such as perfumery, food production, and pharmaceuticals [116]. Among plant families, Asteraceae, Lamiaceae, and Apiaceae are the most prominent sources of these essential

oils [117]. Within the Boraginaceae family, certain species are considered industrially significant due to their production of essential oils. Notably, three species of borage identified in the Cape Verde region have been recognized as important sources of essential oils [117].

The diverse therapeutic potential and traditional applications of *Echium* species, particularly *E. italicum* and *E. amoenum*, highlight their value as candidates for further pharmacological research and pharmaceutical development. Future studies should focus on standardizing extraction methods, characterizing bioactive compounds such as shikonins and essential oils, and validating traditional claims through clinical trials to enable the safe and effective integration of these plants into modern therapeutic protocols.

Table 3.1. Medicinal uses of various *Echium* spp. and parts of plant.

Species	Medicinal Uses	Part of Plant	Extraction Techniques	Country
<i>E. italicum</i> L.	Allergic disease	hairy roots	ND	ND [334]
<i>E. italicum</i> L.	Wounds, Anti-inflammatory	Leaf, root	Poultice	Turkey[207]
<i>E. vulgare</i> , L and <i>E. russicum</i> J.F. Gmellin	Vulnerary	Root	Poultice	Turkey[207]
<i>E. vulgare</i> L.	Wound healing, Ulcer, Bruising, Pulled muscles, Ligaments and Sprains	Root	Ethanol and chloroform	Turkey [335]
<i>E. vulgare</i> L.	Blood purification, Heal wounds, Tonic, Diaphoretic, Diuretic, Astringent, Treatment of snakebite	Leaves and flowering stems	Methanol and ethanol	East Serbia [336]
<i>E. italicum</i> L.	Depurative, Diaphoretic, Diuretic, Emollient for healing respiratory infections	Aerial part	Decoction	Italy[337]
<i>E. amoenum</i> , Fisch. And May. Ahvazi 637 (IMPH)	Demulcent, Anti-inflammatory and analgesic, especially for common cold, pneumonia, anxiolytic, sedative, and other psychiatric symptoms, including obsession	Flower	brewed	Iran [41]
<i>E. amoenum</i>	Infectious diseases and for controlling fever	Flower	aqueous extraction	Iran [268]
<i>E. vulgare</i> L.	Antitussive, Aphrodisiac, Demulcent, Diaphoretic, Diuretic, Pectoral, Vulnerary, Efficacious in the treatment of snake bites, for cleaning up the blood and healing wounds.	leaves and flowering stems	ND	Serbia[252]
<i>E. italicum</i> L.	Diuretic, Sweet and sedative	aerial parts	decoction	Turkey [205]
<i>E. italicum</i> L., <i>E. vulgare</i> L. and <i>E. angustifolium</i> Miller	Wound healing	Root and Aerial parts	hydroalcoholic extract	Turkey [243]

<i>E. plantagineum</i>	diuretic and diaphoretic,	aerial part	decoction	Turkey [250]
<i>E. Arabicum</i> . R. Mill	Antiplasmodial and antitrypanosomal activity	Aerial parts	Methanolic extract	Saudi Arabia [338]
<i>E. flavum</i> Desf	Antiseptics and wound healing for ulcers and herpe	Root	mashed or fried in olive oil	Spain [339]
<i>E. vulgare</i> L.	Diuretic	Leaves	aqueous EtOH , through maceration	Italy [340]
<i>E. amoenum</i> Fisch. & C.A.Mey.	Cold and flu	Flower	ND	Iran [341]
<i>E. amoenum</i>	Reduce anxiety	Flower	aqueous EtOH	Iran [342]
<i>E. amoenum</i> Fisch. & C.A.Mey.	Improve depression	Flower	aqueous extract	Iran [261]
<i>E. amoenum</i>	Alleviate PMS symptoms		aqueous extrat	Iran [343]
<i>E. italicum</i> L.	Sedative , Burns , Rheumatism, Uterus infection	Flower, root	Infusion, Cataplasm, Decoction	Iran [43]
<i>E. amoenum</i> Fisch. & C.A.	sore throat, nerve system relaxant, digestive	Therophytes, Aerial parts	Infusion, Decoction	Iran [237]
<i>E. amoenum</i> Fisch. & C.A.	sedative, exhilarating, diuretic analgesic antioxidant, anxiolytic diaphoretic	Flowers	Infusion	Iran [41, 239, 245, 344, 345]
<i>E. hipernopicum</i>	Dietetic	Seed oil	Essential oil	Cape Verde[346]
<i>E. stenosphon</i>	Cough syrup	ND	Essential oil	Cape Verde[346]
<i>E. vulcanorum</i>	Dietetic	Seed oil	Essential oil	Cape Verde[346]
<i>E. vulgare</i> L.	as a revitalizing, anti-inflammatory, cough-relieving, asthma-relieving and phlegm-resolving agent, dry colds, biliary diseases, depression, heart and brain deficiency syndromes, palpitations, insomnia	Flower	ND	China [347]

ND¹ = Not Defined

3.4. Bioactivities of the *Echium* Genus

3.4.1. Antimicrobial Activity

Various *Echium* species particularly *E. amoenum* and *E. italicum* have garnered increasing scientific interest due to their rich content of bioactive compounds. In recent years, several in vitro studies have explored their antiviral, antibacterial, and antifungal activities, revealing promising potential for the development of novel plant-based therapeutics. The aqueous extract of *E. amoenum* flowers exhibited potent antiviral properties by inhibiting viral replication and proliferation at concentrations below 400 µg/mL [267]. Furthermore, both aqueous and ethanolic extracts demonstrated substantial antimicrobial activity against bacterial strains commonly associated with foodborne illnesses and spoilage [348]. The seed oil of *E. amoenum* revealed notable antimicrobial efficacy against *Candida albicans* and *Pseudomonas aeruginosa*, underscoring its potential as a natural therapeutic agent for managing cutaneous and mucosal infections caused by opportunistic pathogens [349]. Conversely, the aqueous extract at low concentrations did not display antibacterial effects, and its activity remained unaltered under varying pH conditions [268]. Remarkably, the methanolic flower extract showed effective antibacterial activity against *Acinetobacter baumannii*, positioning it as a potential alternative to colistin [350]. Among various extracts tested, only the ethyl acetate extract demonstrated significant inhibitory activity, particularly against Gram-positive bacteria. The ethyl acetate extract of *E. arenarium* exerted strong antibacterial effects against *Bacillus cereus* (ATCC 14579) and *S. aureus* (ATCC 2592), while aqueous and ethanol extracts were largely inactive. [276].

Phytochemical analyses identified luteolin-7-O-glucoside and myricetin as key phenolic constituents contributing to the observed antibacterial activity, particularly against *S. aureus*, with a minimum inhibitory concentration (MIC) of 62.5 µg/mL. In comparison, quercetin exhibited weaker effects, with MIC values ranging from 125 to 250 µg/mL [276].

In the case of *E. serbicum*, antibacterial assays demonstrated that all plant parts including the root, stem, flower, and leaves exhibited notable antibacterial activity against Gram-positive bacteria. While the leaf and stem extracts didn't showed sensitivity against Gram-negative strains, the root and flower extracts displayed partial sensitivity toward Gram-negative bacteria, and overall exhibited stronger effects against Gram-positive strains. These findings are consistent with previous studies suggesting that phenolic compounds and other plant secondary metabolites generally exert greater antibacterial effects on Gram-positive bacteria compared to Gram-negative ones [351].

Studies on *E. italicum* oil confirmed a dose-dependent enhancement in antibacterial activity, with increasing inhibition zones at concentrations of 250, 500, 1000, 2000, 4000, and 8000 µg/disc [316]. Similarly, recent evaluations of its extracts showed increased antimicrobial effects at higher concentrations (25, 50, 75, and 100 mg/mL) [352]. Among these, the methanol extract at 100 mg/mL produced the strongest inhibition, forming zones of 14 ± 2 mm against *S. aureus* and 14 ± 1.5 mm against *E. coli*. In contrast, the hexane extract demonstrated peak

antifungal activity against *Candida* at 50–100 mg/mL, with an inhibition zone of 15 ± 0.2 mm. Additionally, aqueous extracts of *E. amoenum* at a dose of 5 mg exhibited 50% growth inhibition against *S. aureus* [266], as shown in the Table 3.2.

Collectively, the findings from various in vitro investigations underscore the potent antimicrobial potential of *Echium* species, particularly through methanolic, ethanolic, and seed oil extracts. Notably, ethyl acetate extracts demonstrated strong inhibitory effects against Gram-positive bacteria, while key phenolic compounds such as luteolin-7-O-glucoside and myricetin were identified as primary contributors to the observed bioactivity. These results suggest that *Echium* holds substantial promise as a natural source for developing alternative antimicrobial agents, especially in the face of rising antimicrobial resistance and the urgent need for novel therapeutic strategies [351].

Table 3.2. Antimicrobial activity of *Echium* spp.

Species	Virus/bactery	Part of plant	Extract	assay	Country
<i>E. amoenum</i> L.	herpes simplex virus type I (HSV-1, KOS strain) and Hep-2	Flower	Aqueous	cytopathic effect inhibition assay (CPE)	Iran [267]
<i>E. amoenum</i> L.	<i>Staphylococcus aureus</i> (ATCC 25913), <i>Listeria monocytogenes</i> (ATCC 19117), <i>Escherichia coli</i> (ATCC 8739), <i>Yersinia enterocolitica</i> (ATCC 9610) and <i>Salmonella typhimurium</i> strain (ATCC 14028)	Flower	aqueous and ethanolic	disc diffusion method, and minimum inhibitory concentration (MIC)	Iran [348]
<i>E. amoenum</i> Fisch. & C.A.Mey.	methicillin-resistant <i>Staphylococcus aureus</i> (MRSA), <i>Staphylococcus epidermidis</i> , <i>Pseudomonas aeruginosa</i> , <i>Candida albicans</i> , and <i>Aspergillus niger</i>	seed	Chloroform and methanol	MIC	Iran[349]
	<i>Staphylococcus aureus</i> 8327	flower	aqueous	agar-well diffusion method and MIC	Iran [268]
<i>E. amoenum</i> Fisch. & C.A.Mey	<i>Acinetobacter baumannii</i>	flower	methanolic	disc diffusion method	Iran [350]
<i>E. arenarium</i> (Guss)	<i>Bacillus cereus</i> ATCC 14579, <i>Listeria monocytogenes</i> ATCC 19115, <i>Staphylococcus aureus</i> ATCC 25923, methicillin-resistant <i>Staphylococcus aureus</i> (MRSA), <i>Enterococcus faecalis</i> ATCC 29212, <i>Escherichia coli</i> ATCC 35214, <i>Pseudomonas aeruginosa</i> ATCC 27853 and <i>Klebsiella pneumonia</i> CIP 104727	Aerial part	Water, cyclohexane, dichloromethane and ethyl acetate	disk diffusion method and Minimal inhibitory concentration (MIC)	Tunisia [276]
<i>E. italicum</i> L.	<i>Bacillus subtilis</i> , <i>Staphylococcus aureus</i> , <i>Escherichia coli</i> , <i>Salmonella typhi</i> , <i>Pseudomonas aeruginosa</i> , <i>Aspergillus niger</i> and <i>Candida albicans</i>	flowering aerial parts	hydrodistillation	disk diffusion method and minimal inhibitory concentration (MIC)	Iran[316]

<i>E. italicum</i> L.	<i>Bacillus megaterium</i> , <i>Escherichia coli</i> , <i>S. aureus</i> , <i>C. albicans</i> , <i>K. pneumoniae</i>	Flower, stems and leaves	Methanol and hexane	Agar well diffusion method	Turkey[352]
<i>E. serbicum</i> L.	<i>Bacillus mycoides</i> ATCC 6462, <i>Bacillus subtilis</i> ATCC 6633, <i>Enterococcus faecalis</i> ATCC 29212, <i>Micrococcus luteus</i> ATCC 1024, <i>Micrococcus lysodeikticus</i> ATCC 4698, <i>Staphylococcus aureus</i> ATCC 25923, <i>Staphylococcus epidermidis</i> ATCC 14990, <i>Escherichia coli</i> ATCC 25922, <i>Klebsiella pneumoniae</i> ATCC 13883, <i>Pseudomonas aeruginosa</i> ATCC 19429	flowers, leaves, stems and roots	Methanol	MIC	[351]
<i>E. italicum</i> L.	<i>Bacillus subtilis</i> , <i>Staphylococcus aureus</i> , <i>Escherichia coli</i> , <i>Salmonella typhi</i> , <i>Pseudomonas aeruginosa</i> , <i>Aspergillus niger</i> and <i>Candida albicans</i>	Arial part	Essential oil	disc diffusion method	Iran [316]
<i>E. amoenum</i> Fisch. & C.A.Mey.	<i>Staphylococcus aureus</i>	Flower	Aqueous	Agar well diffusion method	Iran [266]

MIC¹ = Minimal Inhibitory Concentration.

3.4.2. Antioxidant Activity

Antioxidant compounds derived from medicinal plants play a critical role in mitigating oxidative stress induced by free radicals and have attracted considerable attention in pharmaceutical and nutritional research. Among these, various species of the *Echium* genus particularly *Echium amoenum* have been extensively studied due to their high content of phenolic, flavonoid, and anthocyanin compounds. This section summarizes the findings of previous studies on the antioxidant properties of *Echium* species, with a focus on the influence of solvent type, extraction techniques, drying conditions, and ecological factors on the retention and enhancement of antioxidant activity.

In *E. amoenum*, anthocyanins such as cyanidin-3-glucoside [235] have shown a significant protective effect on human endothelial cells exposed to oxidative stress, especially at higher concentrations (100–1000 μ M) [254].

Aqueous extracts displayed stronger antioxidant activity than acetone extracts, emphasizing the influence of solvent type and polarity on compound efficacy [353]. Antioxidant activity varies based on the type and concentration of the solvent used [354].

Noteworthy, recent reports indicated that the case of *E. amoenum*, a species commonly reported in Iran, drying conditions particularly temperature are critical for maintaining total phenolic content (TPC), total flavonoid content (TFC), anthocyanins, and overall antioxidant capacity. A drying temperature of 60 °C and an air velocity of 0.86 m/s are optimal for preserving phenolics, phenylpropanoids, flavonoids and anthocyanins compounds [355].

In *E. vulgare*, the efficiency of phytochemical extraction particularly polysaccharides with demonstrated antioxidant and anti-*Listeria* activity has been shown to depend on several parameters, including extraction time (73.8 min), microwave power (769.2 W), extraction temperature (42.3 °C), and the water-to-raw-material ratio (61.4 mL/g) [255].

Notably, drying conditions such as 60 °C temperature and 0.86 m/s air velocity are optimal for preserving phenolics, flavonoids, and anthocyanins.

In a study investigating the influence of geographical location on the antioxidant activity of *E. italicum*, plant samples were collected from three provinces in Turkey: Gaziantep, Hakkari, and Mersin. The results indicate that samples collected from the Mersin region exhibit significantly higher antioxidant activity, as well as elevated levels of Total Oxidant Status (TOS) and the Oxidative Stress Index (OSI), in comparison to samples obtained from the other regions. These findings highlight the significant impact of environmental and geographical factors on the antioxidant capacity of *E. italicum* [356]. Ethanol and acetone proved to be the most effective solvents for extracting antioxidant compounds [357]. Furthermore, ultrasound-assisted extraction (UAE) conducted under optimized conditions specifically at 41.84 °C for 45 minutes, with a liquid-to-solid ratio of 10:0.78 (mL/g), resulted in the highest recovery efficiency for shikonins and phenolic antioxidants [358].

Numerous studies have established a direct correlation between antioxidant activity and the presence of phenolic compounds [359]. Nevertheless, some research indicates that such a relationship may not exist [360]. This disparity may arise because not all plant phenolic compounds exhibit antioxidant properties. The antioxidant activity is influenced by the position of hydroxyl groups on aromatic rings and the oxidation state of unpaired electrons within the phenolic structure [361]. This disparity may arise because not all plant phenolic compounds exhibit antioxidant properties. The antioxidant activity is influenced by the position of hydroxyl groups on aromatic rings and the oxidation state of

unpaired electrons within the phenolic structure [361]. For example, among *E. arenarium* extracts, ethyl acetate extracts exhibited the strongest antioxidant activity despite lower overall phenolic content, suggesting compound-specific effects [276] (Table 3).

The antioxidant activity of *E. amoenum* flower decoction is attributed to phenolic acids like rosmarinic acid and various flavonoids [245]. Rosmarinic acid, a significant compound in the Boraginaceae family, exhibits stronger antioxidant activity than vitamin E. It plays a crucial role in scavenging free radicals [362] inhibiting low-density lipoprotein oxidation [362] suppressing oil oxidation [363], suppressing arachidonate metabolism [364] preventing hemolysis [365], and exhibiting hyaluronidase and h-hexosaminidase activities [366]. Due to the presence of flavonoids, and phenolic compounds, glycerol (CHGLY)-based natural deep eutectic solvent (NADES) demonstrate superior antioxidant activity compared to methanol, ethanol, and water in extracts from *Echium amoenum* flowers species [284]. Among the three types *E. amoenum* flower extracts, the ultrasonic extract, alkaline fraction, and polyphenol extract exhibited the highest antioxidant activities, respectively [367].

The phenolic compounds in plants can stabilize metal ions that catalyze hydrogen peroxide decomposition, thereby preventing oxidative reactions [368, 369]. In *Echium* spp. flower extracts, phenolic compounds such as apigenin, luteolin-7-O-rutinoside, apigenin-7-O-rhamnoside, and quercetin-3-O-rhamnoside in ethyl acetate and butanol extracts have been identified as key contributors to strong antioxidant activity [244].

Important ecological factors like temperature, rainfall, UV light, light intensity, altitude, longitude, and latitude significantly influence the concentration of phenolic compounds (Figure 3.7).

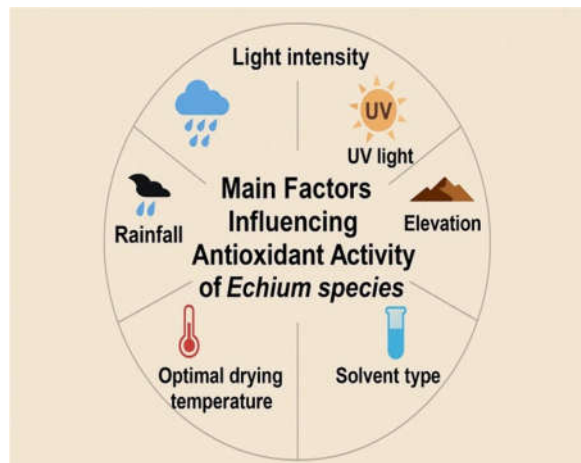


Figure 3.7. Main factors enhancing the antioxidant activity of *Echium* spp.

Plants growing at higher altitudes have been reported to contain more phenolic and flavonoid compounds than those at lower altitudes.[370-372]. For instance, extracts from *Echium* spp. seeds and stems in the Bushehr and Ramsar regions exhibited higher phenolic content and stronger antioxidant activity compared to borage seeds from Alamut and Boumehen [373].

Drying conditions also affect the preservation of bioactive compounds in *Echium amoenum* flowers. High temperatures combined with moderate airspeed were found to retain bioactive compounds and enhance antioxidant activity [355]. Similarly, studies on lemon myrtle leaves indicate that freeze-drying preserves the highest phytochemical and antioxidant properties among six drying methods.

However, due to its high energy consumption, microwave drying is preferred in the industry for its energy efficiency while preserving antioxidant activity and phytochemical content [374].

The optimal methods for preserving phytochemical compounds in borage plants are decoction, infusion, and hydromethanolic extraction [290]. The species of *Echium rubrum* and *Echium vulgare* exhibited the highest hydroxyl radical scavenging activity and iron chelating ability. Among them *Echium vulgare* was reported to have a higher flavonoid content [252]. When comparing the antioxidant activity of methanol and hexane extracts of *E. italicum*, the hexane extract demonstrated an IC₅₀ value of 63.3 µg/ml, while the methanol extract exhibited a significantly lower IC₅₀ value of 20.7 µg/ml (66). This indicates that the methanol extract contains a higher concentration of compounds capable of neutralizing free radicals, which accounts for its superior antioxidant activity compared to the hexane extract [352].

Among the extracts analyzed from *E. humile* Desf, the ethyl acetate and methanol extracts exhibited the highest polyphenol content, as determined by HPLC-MS analysis. Among the identified phenolic compounds, *p*-coumaric acid was the most abundant, followed by cirsiol [375].

Among the extracts of *E. vulgare* (chloroform, ethyl acetate, ethanol, acetone, and petroleum), the ethanolic extract exhibited the highest antioxidant activity with an IC₅₀ value of 49.48 ± 1.33 µg/ml, followed by the acetone extract (50.50 ± 1.10 µg/ml) and the chloroform extract (51.34 ± 1.06 µg/ml). In the case of *Echium italicum*, the acetone extract demonstrated the most pronounced inhibition of lipid peroxidation, with an IC₅₀ value of 42.54 ± 1.13 µg/ml, followed by the chloroform extract (43.29 ± 1.20 µg/ml) and the ethanol extract (44.56 ± 1.29 µg/ml) [376] (Table 3.3).

Among the *Echium* species, *E. amoenum* as illustrated in Figure 3.8, is the most extensively studied for its antioxidant properties.



Figure 3.8. *E. amoenum* species (<https://www.inaturalist.org/photos/288847187>, accessed on 14 July 2025).

Notably, the roots of *Echium* species have consistently exhibited the highest antioxidant activity, which can largely be attributed to their elevated levels of phenolic compounds [158].

Table 3.3. Antioxidant activity of *Echium* spp.

Scientific name	Part of plant	Extract	Type of study	Reference
<i>E. amoenum</i> Fisch. & C.A. Mey	Petals	dichloromethane	Ferric Reducing Antioxidant Power Assay (FRAP)	[254]
<i>E. amoenum</i>	Petals	ethanol, methanol, acetone, ethanol 80% and water extracts	1,1 Diphenyl 2-Picryl Hydrazyl (DPPH)	[353]
<i>E. arenarium</i> (Guss)	Arial part	ethyl acetate extracts cyclohexane, dichloromethane and water	1DPPH; β -carotene bleaching assay	[276]
<i>E. amaenum</i> Fisch & C.A. Mey	flowers	decoction	blood total antioxidant capacity (TAC), lipid peroxidation (LPO) and total thiol (SH) molecules	[245]
<i>E. amoenum</i>	flower	Ethanol, water , methanol and a choline chloride and glycerol (CHGLY)	DPPH and FRAP	[284]
<i>E. amoenum</i> Fisch & C.A. Mey	petals	Ultrasonic extract, Polyphenol fraction, and Alkaloid fraction	DPPH	[367]
<i>E. pycnanthum</i> Pomel	roots	hydromethanolic	DPPH, b-carotene bleaching test, 1,1-diphenyl-2-picrylhydrazyl, 2,2-azino-bis-3-ethyl benzthiazoline-6-sulfonic acid (ABTS), Chelating effect on ferrous ions, Iron reducing power	[369]
<i>E. sericeum</i> (Vahl)	aerial parts	chloroform, ethyl acetate and n-butanol the ethanolic extract	DPPH	[244]
<i>E. amoenum</i> , <i>E. italicum</i>	Leaf, steam and seed	aqueous EtOH	DPPH and FRAP	[373]
<i>E. amoenum</i>	petal	Methanol and 70% acetone	DPPH	[355]
<i>E. italicum</i>	Root	n-hexane	DPPH	[358]
<i>E. amoenum</i>	flower	Decoction, infusion, methanolic and hydroalcoholic extract	DPPH, FRAP, ABST	[290]

<i>E. vulgare L. and, E. Rubrum L.</i>	Aerial parts	Methanol and ethanol	total antioxidant capacity, DPPH free-radical scavenging, the inhibitory activity toward lipid peroxidation, Fe ³⁺ -reducing power, Fe ²⁺ -chelating ability and hydroxyl radical scavenging activity	[252]
<i>E. italicum L.</i>	Flowers, stems and leaves	Methanol and hexane	DPPH	[352]
<i>E. italicum L.</i>	Arial parts	chloroform, ethyl-acetate, ethanol, acetone and petroleum ether extracts	DPPH, total phenolic content, flavonoid content, inhibitory activity against lipid peroxidation, hydroxyl radical scavenging activity	[357]
<i>E. serbicum L.</i>	flowers, leaves, stems and roots	Methanol	DPPH, ABTS, Reducing power activity, Inhibition of lipid peroxidation	[351]
<i>E. humile Desf</i>	Aerial part	hexane, dichloromethane, ethyl acetate, methanol and aqueous	DPPH, ABTS FRAP and TAC	[375]
<i>E. vulgare L. and E. italicum L.</i>	flower	chloroform, ethyl-acetate, ethanol, acetone, petroleum	Determination of inhibition lipid peroxidation by ammonium thiocyanate	[376]

3.4.3. Cytotoxicity Activity

A key determinant of the pharmacological efficacy of plant extracts is the polarity of the solvent used during extraction, which influences the spectrum of isolated compounds.

This study evaluates the cytotoxic potential of different solvent extracts—including methanol, hexane, chloroform, acetone, and ethyl acetate obtained from *Echium* species, tested against various cancer cell lines and normal cell lines.

The methanolic extract of *E. italicum* demonstrated marked cytotoxic activity against the human breast cancer cell line MCF-7, with an IC₅₀ value of 202.2 µg/mL. In contrast, the hexane extract exhibited substantially lower potency, yielding an IC₅₀ of 853 µg/mL. These results suggest that the methanolic extract contains a higher concentration of bioactive anticancer constituents compared to the hexane fraction [352].

Among the tested extracts of *E. vulgare* L. and *E. italicum* L., those prepared using chloroform and acetone showed the most pronounced antiproliferative effects, particularly against the murine tumor fibroblast cell line (L2OB) [376].

At concentrations ranging from 1 to 100 µg/mL, the hexane extract derived from *E. italicum* seeds exhibited no significant cytotoxicity against either HepG2 (human hepatoma) or MCF-7 (human breast cancer) cell lines. However, at a concentration of 200 µg/mL, the hexane extract displayed a markedly enhanced cytotoxic effect against HepG2 cells, surpassing the activity of the methanolic extract. Interestingly, no inhibitory effect was observed on MCF-7 cells at any tested concentration of the hexane extract [377]. This implies that, in the case of HepG2 cells, the hexane fraction harbors compounds with greater cytotoxic potency than those present in the methanolic extract.

Similarly, hexane, dichloromethane, and ethyl acetate extracts from the flowering aerial parts of *E. amoenum* showed no cytotoxic effects on J774.1A murine macrophages at concentrations between 1 and 100 µg/mL. Nevertheless, a concentration-dependent response was observed, as the highest cytotoxicity was recorded at 200 µg/mL in J774.1A cells [378] (Table 3.4).

Furthermore, between the hexane and methanolic extracts, the hexane extract obtained from the aerial parts of *E. caucasicum* exhibited notable cytotoxic activity against (B16F10) malignant melanoma cancer cells [379]. Therefore, evaluating the hexane extract in *Echium* species such will be important in future studies, as it is likely to contain potent anticancer compounds.

In contrast, in *E. creticum*, among the aqueous, ethyl acetate, and methanolic extracts tested, only the methanolic extract demonstrated significant cytotoxic effects against breast cancer cells (MCF-7) [380]. Interestingly, in another study on the same species, *E. creticum*, both ethanolic and aqueous leaf extracts exhibited significant cytotoxicity against HeLa cancer cells. This activity was attributed to the presence of various phytochemical constituents, including alkaloids, tannins, coumarins, saponins, flavonoids, and polyphenols [381].

It has also been reported that methanolic extracts derived from the roots, flowers, and leaves of *E. serbicum* exhibit selective cytotoxicity against various cancer cell lines, HCT-116, SW-480, MDAMB-231, and normal MRC-5 cancer cells. This selective activity is primarily attributed to the presence of major bioactive constituents such as rosmarinic acid and chlorogenic acid. [351].

A study conducted on honey extracts derived from *E. plantagineum*, (Figure 3.9) collected in different regions of Portugal revealed, through HPLC analysis, the absence of toxic pyrrolizidine alkaloids. Instead, the extracts were rich in phenolic compounds such as hydroxybenzoic acid, hydroxycinnamic

acid, p-coumaric acid, caffeic acid, and quercetin. Cytotoxicity assays demonstrated that while the extracts had no adverse effects on normal MRC-5 fibroblast cells, they significantly reduced the viability of AGS gastric cancer cells [272].



Figure 3.9. *E. plantagineum* in Portugal (https://jb.utad.pt/especie/Echium_plantagineum#imagem-14486, accessed on July 14, 2025)

These findings suggest that the phenolic compounds present in Portuguese *Echium* species exert selective anticancer effects, targeting tumor cells while sparing normal ones. This observation aligns with previous studies on other *E. serbicum* L species, where phenolics were shown to decrease the viability of breast and colon cancer cells without harming normal cells [351].

The data presented underscore the critical role of solvent polarity in shaping the cytotoxic profile of *Echium* extracts. Collectively, these findings emphasize the necessity of strategic solvent selection to optimize the recovery of targeted anticancer compounds and to guide the development of effective plant-based therapeutics.

Table 3.4: Cytotoxicity activities of *Echium* spp

Taxon	Extract	Biological activity	assay	Part of plant	Ref
	Methanol and hexane	HepG2, MCF-7	MTT	seed	[377]
<i>E. vulgare</i> L and <i>E. italicum</i> L	chloroform, ethyl-acetate, ethanol, acetone, petroleum	Hep2, RD, L2OB	MTT	flower	[376]
	hexane, dichloromethane and ethyl acetate	J774.1A macrophage cell line	MTT	flower	[378]
<i>E. serbicum</i> L	Methanol	HCT-116, SW-480, MDAMB-231 and MRC-5	MTT	flowers, leaves, stems and roots	[351]
<i>E. plantagineum</i> L.	Methanol	AGS and MRC-5	MTT	pollen	[272]
<i>E. arenarium</i> Guss	Hydromethanolic	U266	MTT	root and aerial parts	[382]
<i>E. angustifolium</i> Mill	n-hexane (HCT116 and HEPG2	SRB	Aerial part	[383]
<i>E. caucasicum</i>	n-hexane and Methanol	B16F10, HFFF2	MTT	Aerial parts	[379]
<i>E. creticum</i>	Aqueous, ethyl acetate extracts, Methanol	MCF-7	MTT	leaves and stem	[380]
<i>E. italicum</i> L.	Methanol and hexane	HepG2 and MCF 7	MTT	flowers, stems and leaves	[352]

3.5. Conclusions

This review emphasizes the phytopharmaceutical significance of *Echium* species, which are characterized by a rich diversity of bioactive secondary metabolites, including flavonoids, phenolic acids, anthocyanins, naphthoquinone derivatives, terpenoids, and essential polyunsaturated fatty acids. Notable compounds such as luteolin-7-O-glucoside, kaempferol-3-O-neohesperidoside, and rosmarinic acid exhibit a broad spectrum of pharmacological effects, including antioxidant, anti-inflammatory, neuroprotective, and antimicrobial activities. The roots of several *Echium* species are particularly enriched with alkanins and shikonins naphthoquinone compounds with well-documented wound-healing, collagen-stimulating, and anticancer properties. In addition, volatile constituents such as thymol, carvacrol, and pulegone contribute further to the antimicrobial and antifungal potential of these plants. The seed oil of *E. amoenum*, rich in alpha-linolenic acid (ALA), highlights the genus's relevance in pharmaceutical, nutraceutical, and cosmeceutical applications.

The use of advanced analytical platforms, especially spectroscopic techniques, has significantly enhanced our ability to identify and characterize these metabolites across diverse plant matrices. However, the presence of pyrrolizidine alkaloids (PAs) toxic compounds associated with hepatotoxicity—remains a critical safety concern. Therefore, future research should prioritize the development of optimized extraction protocols aimed at minimizing PA content while preserving the therapeutic efficacy of the extracts. Investigations into alternative plant parts and innovative processing strategies may also help balance efficacy with consumer safety.

Altogether, the phytochemical complexity of *Echium* species underscores their substantial biomedical potential and justifies further research to isolate, characterize, and understand the mechanisms of action of their active constituents. These efforts are essential for the rational development of safe and effective phytopharmaceuticals derived from this valuable genus. The observed antimicrobial, antioxidant, and cytotoxic activities of *Echium* extracts underscore their promise in the development of novel therapeutic agents. These findings support the need for further pharmacological and clinical research to fully explore and validate the medicinal potential of this versatile genus.

Chapter 4

Material and Methods

4.1 Ethnobotanical studies

4.1.1 Field surveys/interviews

This survey was conducted over a six-month period during the first semester of 2023. A semi-structured, face-to-face questionnaire was administered to 100 local individuals in the herbal markets of Guilan, Qazvin, and Alborz provinces. The questionnaire primarily focused on ethnobotanical claims and traditional beliefs held by local communities in these three regions. It also gathered demographic and personal information, including respondents' age, gender, relationship with the locality, education level, knowledge level, most used plants, plant identification, utilized plant parts, and methods of preparation. Subsequently, scientific identification and authentication of the cited plants were carried out using Flora Iranica [384], Flora of Iran [385], and various herbal literature sources [79, 386]. Additionally, the accuracy of the scientific names of the medicinal plants was verified through www.theplantlist.org.

4.1.2 Quantitative analysis of ethnobotanical results

All statistical was firstly prepared using Microsoft Excel (Windows, Washington, DC, USA). To evaluate the consistency of traditional knowledge regarding plant use across different ailment categories,

The Informant Consensus Factor (ICF) was employed Informant Consensus Factor

The informant consensus factor (ICF) was obtained using the Equation (1):

$$ICF = (Nur - Nt)/(Nur - 1) \quad (\text{Equation 1})$$

This metric is computed using the total number of use-reports (Nur) for a specific illness category and the number of distinct plant species (Nt) utilized in that category. The ICF, which ranges from 0 to 1, reflects the degree of agreement among informants. A higher ICF value suggests a concentrated reliance on a few specific species, possibly indicating effective knowledge transmission or a shared cultural understanding. In contrast, lower values imply inconsistency in plant selection, potentially due to sporadic knowledge sharing or arbitrary choices by informants [387, 388].

Fidelity Level (FL) was also assessed to identify the most culturally significant plant species associated with specific health conditions, especially in cases where multiple species are used for the same ailment. The FL was calculated using the following formula:

$$FL (\%) = (Np / N) \times 100 \quad (\text{Equation 2})$$

Here, Np represents the number of participants who cited a plant for a particular disease, and N denotes the total number of participants who reported that plant for any ailment. Higher FL percentages indicate a stronger preference and consensus on using a given species for a specific health issue [387, 388].

Additionally, the Relative Frequency of Citation (RFC) index does not differentiate between specific uses or categories of use for a plant, It is simply a general record of how often a plant is cited in relation to any reported use by an individual. This means that it reflects the frequency with which a plant is mentioned in ethnobotanical studies, but it does not provide detailed information about the specific purpose or category (e.g., medicinal, culinary) for which the plant is used.

The RFC is expressed as:

$$\text{RFC} = \text{FC} / \text{N} \quad (\text{Equation 3})$$

Where FC is the number of informants who mentioned a particular plant, and N is the total number of informants. The index varies between 0 (if a species is not mentioned at all) and 1 (if every participant mentioned the plant). Unlike other indices, RFC does not account for the category of use or the number of different uses reported [387, 388].

Lastly, the Use Value (UV) index was calculated to gauge the overall significance of each plant species in the local traditional knowledge system. This was determined using:

$$\text{UV} = \sum \text{U}_i / \text{N} \quad (\text{Equation 4})$$

Where U_i represents the number of different uses cited by each informant for a species, and N is the total number of informants. A higher UV indicates a broader or more frequent application of a plant in ethnomedicinal practices [387, 388].

4.2 Extracts and fractionation of plant materials

4.2.1 Plant materials

The dried flowering tops of *Tripleurospermum disciforme* were purchased in Guilan, Iran local market. A voucher specimen was deposited in the herbarium of Guilan University of Medical Sciences, Iran.

4.2.2 Fractionation of methanolic crude extracts

About 1500 g of the top flowers of *T. disciforme* was extracted via the percolation method by methanol at room temperature for 24, 48, and 72 h. The extract was concentrated using a rotary evaporation system. The methanolic crude extract of *T. disciforme* was re-extracted with increasing polarity solvents, n-hexane, chloroform, ethyl acetate, and methanol, respectively, using an ultrasound bath for 20 min, three times, with frequent shaking at 40°C. The extracts fractions were concentrated using the same rotary evaporation system (vacuum (mbar) for a solvent boiling point of 40°C according to the equipment list), weighed, and stored in 4 °C, until phytochemical characterization and analysis of the biological activities.

4.3 Phytochemical characterization and Phenolic Profile

An untargeted metabolomic approach was employed to characterize the phytochemical composition of the samples. Analyses were conducted by ultra-high performance liquid chromatography (UHPLC) coupled to a trapped ion mobility time-of-flight mass spectrometer (timsTOF-MS; Bruker Daltonics, Germany), equipped with a VIP-HESI electrospray ionization source. Samples were previously dissolved in minimal volumes of hexane, ethyl acetate, or methanol, and 5 µL of each solution was

injected into a ZORBAX Eclipse XDB-C18 RRHD column (2.1 × 100 mm, 1.8 μm; Agilent Technologies, USA). Chromatographic separation was achieved using a binary mobile phase consisting of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B), under a flow rate of 0.4 mL/min. The gradient programme lasted 30 min, including a re-equilibration phase.

Mass spectrometric detection was performed in both positive and negative ionization modes. The capillary voltage was set at ±4500 V and the end plate was offset at ±500 V. Nitrogen was used as the nebulising gas (8 bar), drying gas (8 L/min at 240 °C), and sheath gas (4 L/min at 450 °C). Spectra were acquired over an *m/z* range of 20–1300 in both MS and MS/MS mode using Parallel Accumulation–Serial Fragmentation (PASEF). Ion mobility separation was recorded over a $1/K_0$ range of 0.45–1.45 V·s/cm² with a ramp time of 100 ms.

Data processing was carried out using *Data Analysis* (v6.1) and *MetaboScope* (v7.0.1) software from Bruker. Spectral features (“buckets”) were extracted based on *m/z*, retention time, and collisional cross section (CCS), applying an intensity threshold of 10,000 counts. Compound annotation was performed using three strategies: (i) spectral library matching (SL), (ii) an in-house analyte list (AL) of phenolic compounds (name and molecular formula only), and (iii) SmartFormula (SF) calculations restricted to CHNOPS atom sets.

Combining these approaches, a total of 43 tentative identifications were obtained through SL + AL + SF. Additional matches were retrieved using AL + SF (237 features), SL + SF (64 features), and SF alone (1605 features).

4.4 Assessment of biological activities

4.4.1 Antioxidant activity

The antioxidant activity was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. A calibration curve was constructed with methanol solutions of DPPH (85–4.25 mg/L). Absorbance was measured at 517 nm, and the capacity of free radical scavenging was calculated accordingly to Luís et al. [389]. The IC₅₀ was calculated graphically using the extract concentration versus the corresponding radical scavenging capacity. The antioxidant activity was expressed through the antioxidant activity index (AAI), calculated as follows: AAI = (final concentration of DPPH in the control)/(IC₅₀). The AAI allowed for the classification of the antioxidant activity of the samples as poor (AAI ≤ 0.5), moderate (0.5 < AAI ≤ 1.0), strong (1.0 < AAI < 2.0), or very strong (AAI ≥ 2.0) [389, 390].

4.4.2 Antimicrobial activity

Microorganisms and media

Hexane, chloroform, ethyl acetate, and methanol extracts fractions were tested against three Gram-positive (*Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, and *Bacillus cereus* ATCC 11778) and five Gram-negative (*Pseudomonas aeruginosa* ATCC 27853, *Salmonella* Typhimurium ATCC 13311, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Acinetobacter baumannii* LMG 1025) bacteria. All the strains were cultured on Brain Heart Infusion Agar (BHIA) and incubated at 37 °C for 24 h before any antimicrobial assay.

Determination of Disc Diffusion Assay

The antimicrobial activity of *Tripleurospermum disciforme* (C.A.Mey.) Sch.Bip was evaluated by disc diffusion assay, following the M2-A8 method as described by the Clinical Laboratory and Standards Institute (CLSI) for bacteria. Bacteria inocula were prepared by the direct suspension of colonies in saline solution (NaCl 0.85% (w/v)) and adjusted to 0.5 McFarland (about 1 to 2×10^8 (CFU/mL)). Discs with a diameter of 6 mm were each impregnated with 20 μ L of each extract dissolved in dimethylsulfoxide (DMSO; Scharlab, Spain) at a concentration of 10 mg/mL. For the negative control, DMSO was used instead. Then, the Müeller–Hinton Agar (MHA) (Liofilchem, Italy) plates were inoculated with the cellular suspension, and the discs previously prepared were placed over the agar. The plates were incubated at 37 °C for 24 h. Following incubation, all the plates were visually checked for inhibition zones, and the diameters were measured in mm. Each experiment was performed three independent times.

Determination of Minimum Inhibitory Concentration (MIC)

The MIC of the extracts fractions was evaluated by the microdilution method as described by [391]. Serial dilutions of each of extract (from 5000 μ g/mL to 39 μ g/mL) were prepared in a 96-well plate (50 μ L per well) in Müeller–Hinton Broth (MHB) (Liofilchem, Italy). The cellular suspension was prepared as described above, diluted in MHB, and added to wells to obtain a final cellular concentration of 5×10^5 CFU/mL in each well. A positive control was used containing tetracycline. Wells without the tested plant extracts were used as a growth control, and wells without bacteria were used as a negative growth control. The plate was incubated for 24 h at 37 °C; after that, 30 μ L of resazurin (0.1%) was added in each well of the microtiter plate and incubated at 37 °C for 2 h. No color change (blue) indicated inhibition of the tested bacterium. The MIC was considered the lowest concentration of the extract that inhibited bacterial growth. Each assay was performed at least three independent times.

4.4.3 In Vitro Studies Using NHDF and MCF-7 Cells

Cell culture

Human breast adenocarcinoma cells (MCF-7; ATCC® HTB-22™) and normal human dermal fibroblasts (NHDF; ATCC® PCS-201-012™) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Unless otherwise specified, all chemicals (analytical grade), reagents, culture media, and supplements were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cells were routinely maintained in 75 cm² culture flasks at 37 °C in a humidified atmosphere containing 5% CO₂. NHDF cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), L-glutamine (0.02 M), HEPES (0.01 M), sodium pyruvate (0.001 M), and 1% antibiotic/antimycotic (10,000 U/mL penicillin, 10 mg/mL streptomycin, and 25 mg/mL amphotericin B). The culture medium was renewed every 48 h until cells reached approximately 90–95% confluence. MCF-7 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, high glucose) supplemented with 10% FBS and 1% antibiotic/antimycotic. At confluence, adherent cells were detached using trypsin-EDTA solution (0.125 g/L trypsin and 0.02 g/L EDTA). Prior to each experiment, cell viability was

assessed by trypan blue exclusion using a Neubauer haemocytometer, and suspensions were adjusted to the required density in the appropriate complete culture medium.

Preparation of the Solutions of the Compounds Under Study

Hexane, chloroform, methanol, and ethyl acetate extracts fractions of *T. disciforme* (C.A.Mey.) Sch.Bip. extract were dissolved at a final concentration of 10 mg/mL in their corresponding solvents.

The mixtures were sonicated for approximately 3 h at 40 °C, filtered, and the resulting supernatant was stored at 4 °C.

Cytotoxicity Assay and Protein Quantification

NHDF and MCF-7 cells were seeded at a density of 2×10^4 cells/mL in a 96-well plate (200 µL/well). Two plates were prepared for each cell type. After incubation for 48 h at 37 °C in a humidified atmosphere containing 5% CO₂, cell adherence was verified and culture medium was replaced with treatment solutions.

Cells were exposed for 48 h to *T. disciforme* (C.A.Mey.) Sch.Bip. extracts fractions (hexane, chloroform, methanol, or ethyl acetate) diluted in complete culture medium at final concentrations of 0.05, 0.5, and 1 mg/mL. Negative controls included untreated cells, while solvent controls received 20 µL of the corresponding solvent, equivalent to the highest volume present in the treatment wells. All experiments were conducted in triplicate and independently repeated at least twice. In one plate per cell type, antiproliferative activity was assessed using the MTT assay. Following 48 h exposure, the medium was aspirated and cells were washed with 100 µL phosphate-buffered saline (PBS).

MTT solution (5 mg/mL in PBS) was added to each well and incubated for 4 h at 37 °C. After removal of the MTT solution, formazan crystals were solubilised in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA). Absorbance was measured at 570 nm using an xMark™ microplate spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA).

In parallel plates, total protein content was quantified using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA, cat. no. 23227), according to the manufacturer's instructions. Briefly, after treatment, cells were washed with PBS, and plates were wrapped in aluminum foil and frozen at -20 °C for at least 24 h. The working reagent was prepared by mixing 50 parts of Reagent A with 1 part of Reagent B. Plates were thawed, and 20 µL of PBS was added to each well, followed by 200 µL of working reagent. Plates were mixed on a shaker for 30 s and incubated at 37 °C for 30 min. After cooling to room temperature, absorbance was recorded at 562 nm, and protein concentration was determined against a standard curve.

4.5 Statistical Analysis

All statistical analyses were performed using GraphPad Prism version 10.6.0 (GraphPad Software, La Jolla, CA, USA). Comparisons among multiple groups were conducted using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. For pairwise

comparisons, Student's t-test was applied. A p-value < 0.05 was considered statistically significant.

For each experimental condition, the mean absorbance values obtained from the MTT assay and protein quantification were calculated, and the ratio of MTT/protein was determined. This normalization allowed for the evaluation of metabolic activity relative to total protein content, thereby distinguishing whether changes in MTT reduction reflected variations in cell number (biomass) or alterations in cellular metabolic activity.

Chapter 5

Bioactive Compounds and Traditional Uses of *Tripleurospermum disciforme* (C. A. Mey.) Sch. Bip.: A Comprehensive Study on Its Therapeutic Potential

This chapter corresponds to a published manuscript with the following reference:

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Abstract

Tripleurospermum disciforme (C.A.Mey.) Sch. Bip. is known as the “Plain Chamomile” of the Asteraceae family, the most prominent plant family and one that has been studied extensively due to its numerous genera and species. In traditional herbal medicine, *T. disciforme* has been used to treat digestive, neurological, and skin disorders. This study aimed to document ethnobotanical knowledge and assess the pharmacological potential of medicinal plants, specifically *T. disciforme*, across the provinces of Guilan, Alborz, and Qazvin in Iran, through ethnobotanical surveys. These surveys identified the most utilized plant families as Lamiaceae, Asteraceae, and Theaceae, with *T. disciforme* cited by 42% of participants, emphasizing its significance in local traditional medicine. Given its high relative frequency of citation and reported medicinal applications, *T. disciforme* extracts fractions were subjected to UHPLC–timsTOF–MS analysis for further phytochemical profiling and a series of biological assays. Several phenolic compounds such as neochlorogenic acid, caffeic acid, and p-hydroxyphenylethanol acetate were recurrently detected across extracts. The ethyl acetate extract fraction demonstrated potent antioxidant activity in the DPPH assay ($IC_{50} = 12.496 \mu\text{g/mL}$) and exhibited antimicrobial activity against *Bacillus cereus* ($MIC = 312 \mu\text{g/mL}$). Additionally, the hexane extract fraction revealed notable cytotoxic effects against MCF-7 human breast cancer cells. To the best of our knowledge, to date, this is the first investigation of *T. disciforme* to integrate ethnobotanical and ethnopharmacological approaches to medicinal plant research in these regions of Iran.

Keywords: ethnobotany; *Tripleurospermum disciforme*; UHPLC–timsTOF–MS, antimicrobial activity; antioxidant activity; MTT/Protein Assay

5.1. Introduction

Iran, with its expansive geography and diverse climate zones, is recognized as one of the most botanically rich countries in the world. Hosting more than 8000 identified plant species, including 1727 endemics, Iran ranks among the top ten countries globally in terms of floristic diversity [2, 392]. This exceptional plant wealth provides fertile ground for the study of medicinal flora, fostering the intersection of traditional herbal wisdom and modern scientific exploration. One notable example is *Tripleurospermum disciforme* (C.A.Mey.) Sch.Bip., also known as plain chamomile, which belongs to the Anthemideae tribe within the Asteraceae family, one of the largest plant families globally, locally known as “babuneh Dashti”. This herbaceous species, which may be perennial or biennial, typically reaches heights of 10 to 70 cm [3]. It is taxonomically recognized by the homotypic synonyms *Chrysanthemum disciforme* C.A.Mey., *Chamaemelum disciforme* (C.A.Mey.) Vis., and *Matricaria disciformis* (C.A.Mey) DC. [393]. Ancient Persian medico-pharmaceutical manuscripts reference various botanical names for “babuneh”, such as *Anthemis nobilis* L., *Anthemis wiedemanniana* Fisch. & Mey., *Matricaria chamomilla* L., and *Tripleurospermum disciforme* (C.A.Mey) Schultz Bip. [394]. Today, the chamomile-like plant sold in Iranian herbal markets under the name “babuneh” is most commonly *T. disciforme*, which exhibits botanical features distinct from the more internationally known *Matricaria chamomilla* [386].

The genus *Tripleurospermum* includes around 40 species, primarily distributed across temperate regions of Europe and Asia, with additional representation in North Africa and North America [4-6]. Turkey is considered the primary biodiversity centre for this genus [6, 7], while Russia harbours seventeen species [8], and six species are reported from Iran [9]. Ethnobotanical studies of *Tripleurospermum* species have demonstrated that they possess anti-inflammatory properties, alleviate gastric pain, serve as antiseptics, benefit hair care, and enhance memory [236]. Phytochemical studies of *Tripleurospermum* species have revealed a wide range of bioactive compounds, including terpenoids, alkanes, steroids, organic acids, and aromatic constituents [17, 236].

In addition, triterpenoids such as taraxasteol, lupeol, and betulinic acid have been identified in *T. disciforme* (C.A.Mey.) Sch.Bip., with lupeol and betulinic acid reported to inhibit tubulin polymerization in gastric cancer cells (AGS) [395]. These compounds have also shown notable cytotoxic activity against diverse cancer cell lines, including breast, cervix, colon, prostate, lung, ovary, skin, hematopoietic, and lymphoid cancers [396-398], further highlighting the therapeutic relevance of *T. disciforme* (C.A.Mey.) Sch.Bip..

The therapeutic efficacy of *T. disciforme* is primarily attributed to its rich phenolic profile, which imparts anti-ulcer [32], antibacterial [13, 21], antioxidant [33], antispasmodic, and antiseptic activities [195]. Despite its widespread presence across Iran, the rapid pace of technological and societal transformation poses a serious threat to the preservation of traditional herbal knowledge. As such, comprehensive documentation is imperative to safeguarding this cultural and pharmacological legacy [40]. Ethnobotany serves as a vital bridge between ancestral knowledge and contemporary science, focusing on the documentation, analysis, and interpretation of plant use within specific cultural and ecological contexts [5].

This study represents the most extensive investigation of *T. disciforme* to date and constitutes the first ethnobotanical and ethnopharmacological assessment of medicinal plants in the provinces of Guilan, Qazvin, and Alborz. By integrating indigenous knowledge with modern quantitative ethnobotanical methodologies, this research pioneers a systematic evaluation of regional medicinal flora through a set of analytical indicators, offering a foundational contribution to both the local and global herbal medicine literature. Additionally, the aerial parts of *T. disciforme* (C.A.Mey.) Sch. Bip. were extracted using various solvents to isolate secondary metabolites. The resulting extracts were subsequently analyzed by ultra-high-performance liquid chromatography coupled with trapped ion mobility spectrometry and tandem mass spectrometry (UHPLC–timsTOF–MS) to assess their antioxidant, antimicrobial, and cytotoxic activities.

In this context, we hypothesize that *Tripleurospermum disciforme*, traditionally used as a medicinal plant, may contain bioactive metabolites with antioxidant, antimicrobial, and cytotoxic potential. To address this, ethnobotanical documentation was combined with phytochemical and biological analyses of specimens collected from Rasht, aiming to scientifically validate traditional knowledge and provide a basis for future pharmacological studies.

5.2. Results and Discussion

5.2.1. Ethnobotanical Surveys

In this ethnobotanical study, a total of 100 residents from the provinces of Guilan, Qazvin, and Alborz were interviewed. Participants were recruited through a convenience sampling strategy, based on voluntary participation at local herbal markets. The results indicated that individuals aged between 30 and 49 years represented the primary users of medicinal plants, accounting for 58% of respondents, whereas those aged over 70 showed the lowest rate of use, comprising only 1%. These findings are in line with ethnobotanical research conducted in Angola, where individuals within the same age range were identified as the most frequent users of medicinal plants [399].

Furthermore, this study revealed that 84% of medicinal plant users were natives of the Guilan, Alborz, and Qazvin provinces. The data also support previous findings indicating that women were the predominant consumers of medicinal plants compared to men [399]. Women constituted 65% of users, a trend that reflects their traditional roles in the preparation and administration of herbal remedies, knowledge that is often transmitted through generations, predominantly from mother to daughter. These results are consistent with ethnobotanical studies conducted in countries such as Algeria [388], Morocco [400], Angola [399], and Turkey [29], which have similarly reported that women possess greater awareness and knowledge regarding the traditional use of medicinal plants for treating various ailments.

Ethnobotanical studies reveal that a significant portion of people acquire medicinal plants by purchasing them at markets or specialized stores (Figure 5.1A). This trend is driven by several factors, including convenience, the elimination of the need for wild plant identification and collection, and the assurance of quality and authenticity in commercially available products. Moreover, the decline in traditional knowledge and limited expertise in identifying medicinal plants has led many individuals to favour pre-prepared forms of these plants over collecting raw plant materials, reflecting a cultural transition towards processed and commercialized products.

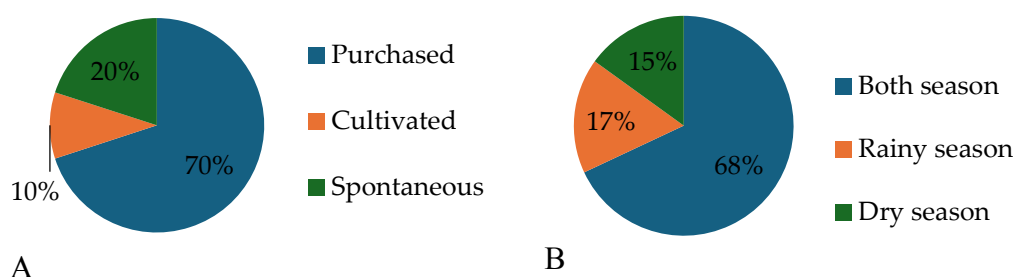


Figure 5.1. Use of medicinal plants according to forms of obtention (A) and season of collection (B).

Ethnobotanical research further indicates that the use of medicinal plants reaches its peak during transitional seasons, particularly in both the rainy season and the dry season (Figure 5.1B). Our

findings align with ethnobotanical studies from Angola, which also reported the highest usage of medicinal plants during these seasons [399].

In the provinces of Guilan, Qazvin, and Alborz, the reliance on medicinal plants was predominantly influenced by communal knowledge and shared experiences, with 65% of users indicating that their use was based on the experiences of others (Figure 5.2A). A similar pattern was observed in Angola, where the traditional knowledge of medicinal plants was largely verified and transmitted through collective experience [399].

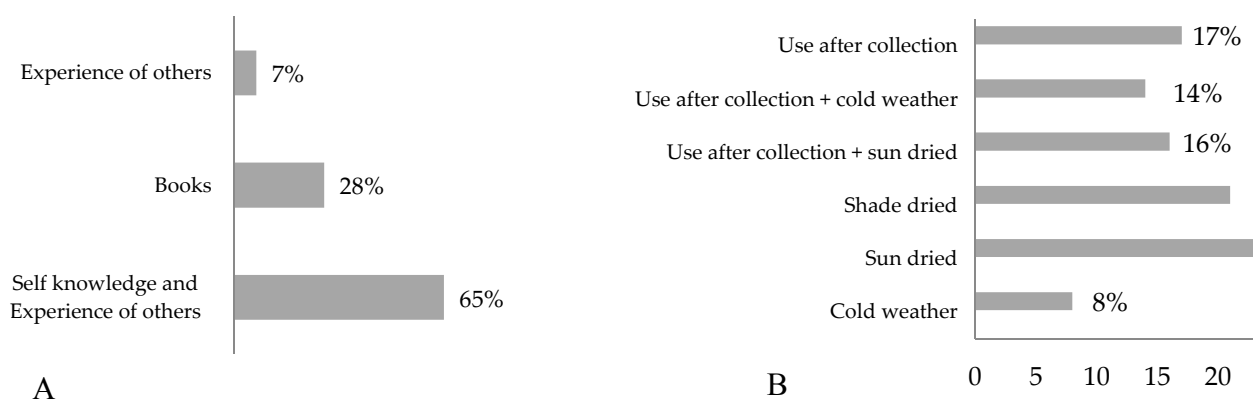


Figure 5.2. Use of medicinal plants according to information source (A) and plant conservation mode (B).

As for plant conservation practices, 21% of participants reported using shade-drying methods to preserve medicinal plants (Figure 5.2B), reflecting a common and effective approach in maintaining the quality and efficacy of herbal remedies. Sun-drying was another common practice, employed by 24% of individuals, as it facilitates medium- to long-term preservation. Meanwhile, 17% of the respondents used medicinal plants immediately after their collection, foregoing any preservation process.

The present survey demonstrated that the most frequently cited plants belong to the Lamiaceae family (Figure 5.3A), which accounted for 63 mentions. This was followed by plants from the Asteraceae (45) and Theaceae (37) families. The Lamiaceae family is one of the most significant plant families in Iran, comprising 46 genera and 406 species, of which 165 are endemic. This family is known for its wide range of biological and medicinal applications [401, 402].

According to our findings, the two families Lamiaceae and Asteraceae represent the most widely used medicinal plants. Similar results have been reported in other regions of Iran, including the forests of Arasbaran [38], where these families are dominant, in Sirjan, Kerman Province, where Lamiaceae is identified as the largest medicinal plant family [83], and in the Saravan region of Baluchistan, where Lamiaceae also plays a prominent role in traditional medicine [88]. Additionally, in Bojnourd, North Khorasan, Lamiaceae and Asteraceae were reported as the most commonly used medicinal plant families [218].

Based on the therapeutic indications of the medicinal plants, most were employed to treat disorders of the digestive system (26%), followed by those related to nervous system disorders (20%) (see Figure 5.3B). Additionally, the surveys frequently mentioned ailments associated with the skin, infections, and issues related to the cardiovascular, urinary, and respiratory systems, and for wound healing.

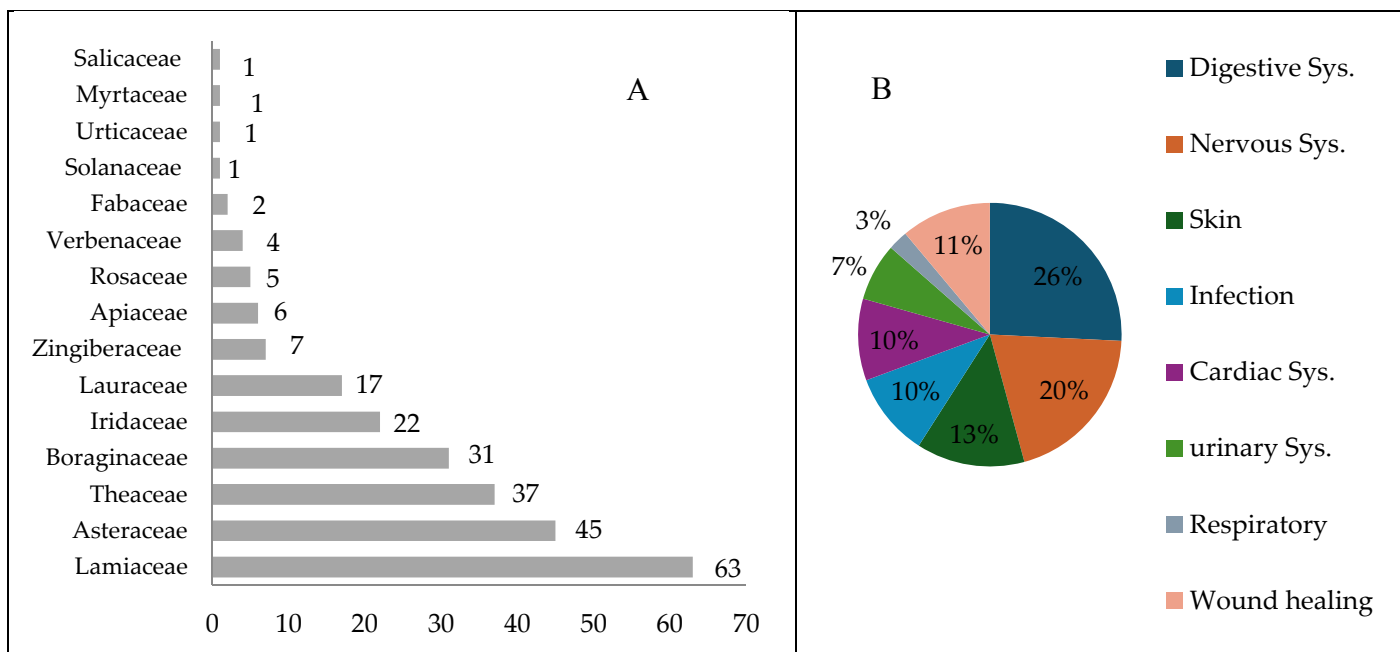


Figure 5.3. Most cited taxa (A) and therapeutic indication (B).

Moreover, a separate study conducted by [78] revealed that in Guilan Province, particularly in the regions of Amlash and Rudsar, the most commonly utilized medicinal plants belonged to the Lamiaceae family. These plants were primarily used to treat digestive disorders, accounting for 20% of reported cases. This trend aligns with findings from ethnobotanical investigations in other parts of Iran, such as Kohgiluyeh and Boyer-Ahmad Province [85], where the Asteraceae and Lamiaceae families are predominant, and digestive ailments represented the most frequently addressed health concerns. Similarly, research conducted by [42], in the village of Juban, also located in Guilan Province, confirmed that the main reason for medicinal plant use among locals was the treatment of digestive disorders. This pattern is not unique to Iran. In Turkey as well, the majority of medicinal plant applications were reported for gastrointestinal disorders [23, 28].

Regarding preparation methods, infusion was the most preferred technique among users, employed in 45% of cases, followed by decoction (19%), use in natural form (15%), and maceration (13%). Comparable trends have been observed in other regions of Iran, where infusion and decoction are also the predominant methods of preparation [82].

These methods are often preferred due to their simplicity and the use of water as a solvent, as highlighted in previous studies [387, 388]. Their ease of preparation makes infusion and decoction particularly popular techniques for extracting active compounds from medicinal plants. As for the parts of medicinal plants most frequently used, leaves alone (28%) and mixtures of leaves and flowers (17%) were the most cited.

Furthermore, the highest proportion of medicinal plant use has been attributed to leaves, accounting for 39% in the Arasbaran forests of northern Iran [38], the Zangelanlo district in Northeast Iran [24], and Lorestan Province [90], and 31% in the Saravan region of Baluchistan, Iran [88]. In addition, the most common methods of preparation reported by local communities were infusions and decoctions, while poultices were the least commonly used. These patterns are consistent with our findings [38]. Similarly, ethnobotanical studies conducted in North Khorasan have also identified infusions and decoctions as the primary methods of medicinal plant use, in agreement with our results [218].

It is important to note that, although this approach provided direct access to key informants and facilitated the collection of relevant data, the sample size (n = 100) was relatively limited and no stratification by age, gender, or specific communities was performed. Consequently, the results should be interpreted with caution, as the data may not fully represent the wider population and may be subject to selection bias, particularly favouring individuals more actively involved in local herbal trade. Nevertheless, despite these limitations, the survey offers valuable exploratory evidence on regional practices and provides a foundation for future studies employing stratified or larger-scale sampling strategies.

The Informant Consensus Factor (ICF) values ranged from 0 to 1, indicating the level of agreement among informants on plant use for specific ailments. Low values suggest diverse or uncertain knowledge, while high values reflect strong consensus and likely indicate culturally important and trusted remedies. The recorded traditional uses were grouped by therapeutic indications (Table 5.1).

Table 5.1. Informant consensus factor (ICF).

Therapeutic Indications	Number of Used Reports (N_{ur})	Number of Taxa (N_t)	ICF Value
Cardiac system	37	11	0.67
Urinary system	26	10	0.58
Nervous system	74	11	0.82
Respiratory disorder	9	3	0.63
Skin disorders	49	11	0.74
Digestive system	95	11	0.85
Wound healing	41	8	0.77

The highest ICF value (0.85) was verified for the digestive system, followed by the nervous system (0.82) and wound healing (0.77). Although all ICF values were greater than 0.5, the lowest ICF was obtained for the urinary system. The strong preference of the local population for specific medicinal plants, based on their therapeutic applications, is supported by high fidelity level (FL) values (Table 5.2).

Table 5.2. Fidelity level (FL) values for the most cited medicinal plants.

Therapeutic Indications	Plant Species	FL (%)
Digestive system	<i>Tripleurospermum disciforme</i>	62%
	<i>Camellia sinensis</i>	76%
	<i>Echium amoenum</i>	25%
	<i>Mentha</i> spp.	100%
	<i>Cinnamomum zeylanicum</i>	85%
Skin disorders	<i>Tripleurospermum disciforme</i>	37%
	<i>Thymus kotschyanus</i>	30%
Wound healing	<i>Thymus kotschyanus</i>	50%
Nervous system	<i>Tripleurospermum disciforme</i>	38%
	<i>Echium amoenum</i>	68%
	<i>Stachys lavandulifolia</i>	77%
Urinary system	<i>Echium amoenum</i>	31%
Cardiac disorder	<i>Crocus haussknechtii</i>	73%
Respiratory disorder	<i>Zingiber officinale</i>	77%

FL= 100% (high fidelity level): Everyone who mentioned the plant used it for the same disease. This suggests strong reliability and specialized use. Lower FL values: The plant is used for multiple diseases, meaning it may not be strongly associated with just one condition.

For instance, *Mentha* spp. (*Mentha pulegium* L., *Mentha arvensis* L., *Mentha aquatica* L., *Mentha piperita* L.) exhibited the highest FL value (100%), indicating its exclusive use for treating digestive ailments. This underscores a significant cultural reliance on *Mentha* spp. for gastrointestinal disorders. Traditionally, *Mentha piperita* has been employed to address biliary conditions, indigestion, enteritis, flatulence, gastritis, intestinal colic, and spasms affecting the bile ducts, gallbladder, and digestive system [403]. *Cinnamomum zeylanicum* (cinnamon) follows with an FL value of 85%, highlighting its notable role in digestive health. Numerous studies worldwide have documented cinnamon's medicinal efficacy in addressing digestive diseases [404]. Other species, such as *Camellia sinensis* (76%) and *Tripleurospermum disciforme* (62%), also hold significant relevance but exhibit slightly more diversified uses. For instance, *Tripleurospermum* species are applied in treating digestive, skin, and respiratory disorders [236]. Traditional medicine also values *Camellia sinensis* L. (tea) for its effectiveness in managing digestive issues, including its use in alleviating bloody diarrhea [405].

Tripleurospermum disciforme and *Thymus vulgaris* exhibit FL values of 37% and 30%, respectively, in the treatment of dermatological conditions, suggesting their broader medicinal roles beyond skin-

related disorders. Additional ethnobotanical evidence from Lorestan Province, Iran, confirms that *Thymus daenensis*, *Thymus eriocalyx*, *Mentha spicata*, and *Mentha longifolia* are traditionally employed as gastronomics and for managing skin disorders [90]. Notably, *Tripleurospermum disciforme* (C.A.Mey.) Schultz has traditionally been used for the treatment of prostate disorders, kidney stones, stomach aches, and hypoglycemia. Similarly, *Thymus lancifolius* Celak has been applied in managing stomach aches, hypothermia, diarrhea, flatulence, and gastric ulcers. Moreover, the therapeutic potential of *Mentha spicata* L. and *Mentha longifolia* (L.) Hudson has been validated for their effectiveness in alleviating stomach aches, vomiting, food poisoning, fever, diarrhea, infections, and even snake bites [27].

Of particular interest is *Thymus kotschyanus*, which demonstrates a higher FL value of 50% specifically for wound healing, emphasizing its specialized role in tissue regeneration. This therapeutic potential is attributed to its high concentrations of thymol and carvacrol, phenolic compounds renowned for their antibacterial and anti-ulcer properties [406-408].

In the domain of gastrointestinal health, *Echium amoenum* presents a relatively modest FL value of 25%, reflecting its more targeted, albeit limited, use for digestive complaints. This aligns with findings from Alamout, Qazvin, where its application for stomach-related issues is well documented [41]. Likewise, *Stachys lavandulifolia* is traditionally utilized for ailments such as headaches, hypothermia, hyperlipidemia, hypoglycemia, gastric ulcers, and infections [27, 41, 409]. Furthermore, an ethnobotanical study in North Khorasan identified *Echium amoenum* Fisch. & C.A.Mey., *Tripleurospermum disciforme* (C.A.Mey.), and *Stachys turcomanica* Trautv. as the most frequently cited sedative agents [218].

Crocus sativus L. (saffron) stands out with a notable FL value of 73% for cardiac disorders. The therapeutic efficacy of its petal extract is largely due to bioactive flavonoids such as kaempferol and crocin, which confer significant antioxidant properties that combat lipid peroxidation, thereby contributing to the prevention and management of atherosclerosis and cardiovascular diseases [410-412]. Additionally, *Crocus sativus* L. has demonstrated considerable effectiveness in the treatment of depressive disorders [413, 414].

Finally, *Zingiber officinale* (ginger) emerges as a leading traditional remedy for respiratory ailments, boasting the highest FL value of 77%. It is extensively used for managing conditions such as coughs, colds, influenza, sore throats, and throat infections, underscoring its prominence in ethnomedicinal practices [415, 416].

Table 5.3 highlights the importance and utilization of various medicinal plant species by presenting the number of participants who cited each species, along with key metrics such as Relative Frequency of Citation (RFC) and Use Value (UV). These indices, commonly used in ethnobotanical studies, help to measure the cultural significance and practical applications of plant species in traditional medicine.

Table 5.3. List of medicinal plants cited and their relative frequency citation (RFC) and use value (UV).

Plant Species	Number of Participants That Cited the Species	RFC	UV
<i>Tripleurospermum disciforme</i>	42	0.42	1.72
<i>Thymus kotschyanus</i>	40	0.4	1.75
<i>Echium amoenum</i>	32	0.32	1.31
<i>Mentha</i> spp.	30	0.3	1
<i>Crocus sativus</i> (Saffron)	15	0.15	1
<i>Camellia sinensis</i>	14	0.14	1
<i>Cinnamomum zeylanicum</i>	14	0.14	1
<i>Lavandula angustifolia</i>	10	0.1	1
<i>Stachys lavandulifolia</i>	9	0.09	1
<i>Malva sylvestris</i> L.	8	0.08	1
<i>Rosmarinus officinalis</i>	7	0.07	1
<i>Zingiber officinale</i>	7	0.07	1
<i>Ziziphora clinopodoides</i>	7	0.07	1
<i>Salvia sclarea</i> L.	6	0.06	1
<i>Lauruscerasus officinale</i> L.	5	0.05	1
<i>Curcuma longa</i>	4	0.04	1
<i>Aloysia citrodora</i>	4	0.04	1
<i>Cichorium intybus</i>	2	0.02	1
<i>Eryngium planum</i>	2	0.02	1
<i>Salix aegyptiaca</i>	1	0.01	1
<i>Achillea millefolium</i> L.	1	0.01	1
<i>Cuscuta epithymum</i>	1	0.01	1
<i>Ziziphus vulgaris</i>	1	0.01	1
<i>Citrus aurantium</i>	1	0.01	1
<i>Nigella arvensis</i>	1	0.01	1
<i>Elettaria cardamomum</i>	1	0.01	1
<i>Adiantum capillus-veneris</i> L.	1	0.01	1
<i>Foeniculum vulgare</i> Mill.	1	0.01	1
<i>Juniperus communis</i>	1	0.01	1
<i>Urtica urens</i>	1	0.01	1
<i>Cuminum cyminum</i>	1	0.01	1
<i>Syzygium aromaticum</i>	1	0.01	1
<i>Cassia angustifolia</i>	1	0.01	1
<i>Glycyrrhiza glabra</i> L	1	0.01	1
<i>Portulaca oleracea</i> L	1	0.01	1

The most frequently cited species in the dataset were *Tripleurospermum disciforme* and *Thymus kotschyanus*, referred by 42 and 40 participants, respectively, with high RFC values. Their UVs, 0.42 and 0.4, respectively, suggest that they are not only culturally significant, but also frequently utilized for medicinal purposes. These findings indicate a high level of familiarity and reliance on these plants in the community. Other species such as *Echium amoenum* (32 participants, RFC 0.32, UV 1.31) and *Mentha* spp. (*Mentha pulegium* L. *Mentha arvensis* L. *Mentha aquatica* L. *Mentha piperita* L) (30 participants, RFC 0.3, UV 1) are also notable. They reflect a moderate level of both recognition and practical use in traditional applications. These plants may play a role in treating common ailments, thus maintaining a prominent place in the ethnobotanical landscape.

Our findings align with previous ethnobotanical investigations conducted in Bojnord, North Khorasan Province, Iran, where *Tripleurospermum disciforme* (C.A.Mey.) Sch.Bip. and *Echium amoenum* Fisch. & C.A.Mey were also cited with notably high RFC, reflecting their prevalent use in local

traditional medicine. Specifically, *Tripleurospermum disciforme* (C.A.Mey.) Sch.Bip. was associated with a substantial number of use reports [218]. Similarly, in the Central Zagros region of Lorestan Province, species such as *Mentha longifolia*, *Mentha spicata*, *Mentha piperita*, *Thymus daenensis*, and *Thymus eriocalyx* exhibited high RFC values, suggesting widespread therapeutic application. The FL for *Tripleurospermum disciforme* (C.A.Mey.) Sch.Bip. (BabunehDashti) has been reported at 58 [90], underscoring its therapeutic relevance. Furthermore, in Hamedan Province, Iran, *T. disciforme* (C.A.Mey.) Sch.Bip. has been traditionally utilized for the treatment of kidney stones. Additional species, including *Thymus lancifolius* Celak and *Achillea biebersteinii*, have been employed in ethnoveterinary medicine, particularly for managing digestive disorders [27]. *Thymus lancifolius* Celak has also been verified for its effectiveness in treating hypothermia, stomach aches, and diarrhea, as well as for its carminative and anti-ulcer properties [27].

Zingiber officinale Roscoe is traditionally recognized for its role in treating vomiting, indigestion, colds, and musculoskeletal pain [417]. It also possesses well-documented anti-inflammatory and antioxidant properties [418], in addition to demonstrated glucose and lipid lowering effects, contributing to its potential role in obesity management [419]. Its ethnomedical significance extends beyond Iran; for instance, it is used in Ghana and Uganda for treating tuberculosis [420, 421], and in India it is employed as an antiemetic and for its anticancer properties [422].

The UV metric, which indicates the relative importance of a plant based on the frequency of its reported use by informants, further supports these findings. The highest UVs were recorded for *Tripleurospermum disciforme* (1.72), *Thymus kotschyanus* (1.75), and *Echium amoenum* (1.31), aligning well with their RFC scores. Interestingly, several species with lower citation frequencies, such as *Crocus sativus* (saffron), *Camellia sinensis*, *Rosmarinus* spp., *Cinnamomum zeylanicum*, *Lavandula angustifolia*, *Stachys lavandulifolia*, *Malva sylvestris*, and *Zingiber officinale*, still exhibited a UV of 1. This suggests that while their use may be more specialized or context-specific, they nonetheless hold significant medicinal value.

Previous studies have validated the therapeutic potential of *Lavandula angustifolium* (lavender), citing its effectiveness as a sedative and in the treatment of nervousness, insomnia, circulatory disorders, dyspeptic symptoms, and even aggression reduction [413, 423, 424]. Of the 36 plant species identified in the present study, many have also been reported across various regions of Iran. These include *Lavandula angustifolia* Mill., *Camellia sinensis* L., *Salix aegyptiaca* L. *Salix aegyptiaca* Var. *Lonigifron*, *Stachys lavandulifolia* Vahl., *Citrus aurantium* L., *Cinnamomum zeylanicum* Nees., *Rosmarinus officinalis*, *Mentha pulegium* L., *Mentha longifolia* (L.) Hudson, *Mentha piperita* L., *Mentha aquatica* L., *Mentha spicata*, *Crocus sativus* L., *Echium amoenum* Fisch. & C.A.Mey., *Tripleurospermum disciforme* (C.A.Mey.) Sch.Bip, *Tripleurospermum disciforme* L., *Rosmarinus officinalis* L., *Rosa x damascena* Mill., *Thymus vulgaris* L., *Thymus daenensis*, *Thymus eriocalyx*, *Thymus lancifolius* Celak., *Adiantum capillus-veneris* L., *Salvia sclarea* L., *Malva sylvestris* L., *Achillea santolinoides* subsp. *Wilhelmsii* (K.Koch) Greuter., *Achillea millefolium* L., *Achillea biebersteinii* Afan., *Achillea wilhelmsii* C. Koch., *Achillea vermicularis* Trin, *Urtica urens* L., *Ziziphora clinopodioides* Lam., *Salvia officinalis* (sag), and *Camellia sinensis* [27, 41, 78, 79, 90, 218, 413, 425].

5.2.2. *Tripleurospermum disciforme*

Among the medicinal plants documented in the ethnobotanical survey, *Tripleurospermum disciforme* emerged as the most frequently cited species, recording the highest relative frequency of citation (RFC) and use value (UV). These indices reflect both widespread community reliance and strong consensus regarding its therapeutic effectiveness in regions of Iran, particularly in the management of digestive, respiratory, skin, and nervous system disorders, thereby justifying its selection for further scientific exploration. The subsequent chemical and biological analyses provide initial experimental support for its traditional uses, particularly in gastrointestinal health, and demonstrate how ethnobotanical knowledge can effectively guide pharmacological investigations.

5.2.2.1. Phytochemical Characterization

The untargeted metabolomic profiling of *Tripleurospermum disciforme* (C.A.Mey.) Sch.Bip extracts revealed a diverse array of secondary metabolites. A total of 43 features were annotated based on a combination of spectral library matching (SL), an in-house analyte list of phenolic compounds (AL), and SmartFormula (SF) calculations (Table 5.4). These annotations were supported by high-resolution mass spectrometric data, including accurate *m/z* values, retention times, collisional cross section (CCS) values, and MS/MS fragmentation patterns. Notably, several phenolic compounds such as neochlorogenic acid, caffeic acid, and p-HPEA-AC (p-hydroxyphenylethanol acetate) were recurrently detected across extracts.

Table 5.4. Compounds detected in *Tripleurospermum disciforme* (C.A.Mey.) Sch.Bip. extracts by UHPLC–timsTOF–MS analysis.

Compound Name	Formula	Measured <i>m/z</i>	Retention Time (min)	CCS (Å ²)	Detected in These Solvents
2',4',6'-Trihydroxyacetophenone	C ₈ H ₈ O ₄	169.04974	6.54	120.8	Ethyl acetate, Hexane, Methanol
2-Acetylbenzoic acid	C ₉ H ₈ O ₃	163.04001	8.46	125.8	Ethyl acetate, Hexane, Methanol
3,4-Dicaffeoylquinic acid	C ₂₅ H ₂₄ O ₁₂	515.11879	9.57	211.5	Ethyl acetate
3-Feruloylquinic acid	C ₁₇ H ₂₀ O ₉	367.10357	8.85	188.7	Ethyl acetate, Hexane, Methanol
4-(3,4-Dimethoxyphenyl)-3-buten-1-ol	C ₁₂ H ₁₆ O ₃	209.11765	8.33	130.8	Ethyl acetate, Methanol
4-(4-Hydroxyphenyl)-2-butanone	C ₁₀ H ₁₂ O ₂	165.09078	11.66	121.0	Ethyl acetate, Hexane, Methanol
4-Hydroxycoumarin	C ₉ H ₆ O ₃	163.03905	6.2	116.2	Ethyl acetate, Hexane, Methanol
5-Hydroxymethyl-7-methoxybenzofuran	C ₁₀ H ₁₀ O ₃	179.07166	12.19	119.9	Ethyl acetate, Hexane, Methanol
6-Gingerol	C ₁₇ H ₂₆ O ₄	295.19226	14.38	155.9	Ethyl acetate, Hexane, Methanol
6-Hydroxyluteolin	C ₁₅ H ₁₀ O ₇	303.05216	9.26	153.2	Ethyl acetate, Hexane, Methanol

6-Hydroxyluteolin 7-O-rhamnoside	C ₂₁ H ₂₀ O ₁₁	447.09297	8.6	198.6	Ethyl acetate, Hexane, Methanol
7,3',4'-Trihydroxyflavone	C ₁₅ H ₁₀ O ₅	269.04531	11.38	153.4	Ethyl acetate, Hexane, Methanol
9-Dehydroxyeurotinone	C ₁₅ H ₁₂ O ₅	271.06046	11.57	154.4	Ethyl acetate, Hexane, Methanol
Agrostophyllidin	C ₁₇ H ₁₆ O ₄	283.0973	13.03	171.6	Ethyl acetate, Hexane, Methanol
Altechromone A	C ₁₁ H ₁₀ O ₃	191.07067	10.22	123.7	Ethyl acetate, Hexane, Methanol
Caffeic acid	C ₉ H ₈ O ₄	179.03505	5.92	127.8	Ethyl acetate, Hexane, Methanol
Chrysin	C ₁₅ H ₁₀ O ₄	253.05016	12.84	152.2	Ethyl acetate, Hexane, Methanol
Deoxyarbutin	C ₁₁ H ₁₄ O ₃	195.10202	10.04	126.7	Ethyl acetate, Hexane, Methanol
Eriodictyol	C ₁₅ H ₁₂ O ₆	287.05448	10.72	161.3	Methanol
Kaempferol	C ₁₅ H ₁₀ O ₆	287.0567	11.17	148.6	Ethyl acetate, Methanol
Myricetin 3-O-rhamnoside	C ₂₁ H ₂₀ O ₁₂	463.08758	9.96	200.0	Ethyl acetate, Hexane, Methanol
N-Phenylacetyl aminoacetic acid	C ₁₀ H ₁₁ NO ₃	194.08138	7.13	124.0	Ethyl acetate, Methanol
Neochlorogenic acid	C ₁₆ H ₁₈ O ₉	353.08764	0.83	180.7	Ethyl acetate, Hexane, Methanol
Olivetol	C ₁₁ H ₁₆ O ₂	181.1228	12.28	115.8	Hexane, Methanol
Scutellarein	C ₁₅ H ₁₀ O ₆	287.05658	10.79	128.5	Hexane, Methanol
p-HPEA-AC	C ₁₀ H ₁₂ O ₃	181.08631	4.25	108.7	Ethyl acetate

Differences in the phytochemical profiles were evident depending on the solvent used, with methanol and ethyl acetate fractions showing greater extractive efficiency compared to hexane fraction particularly for polar and mid-polar metabolites. These findings highlight the importance of solvent selection in non-targeted metabolomic studies and reflect the chemical diversity of the extracts.

The annotation strategy, integrating accurate mass, isotopic pattern, MS/MS fragmentation, and ion mobility data, aligns with current standards in untargeted metabolomics and offers a high degree of confidence for structural assignment. Although further validation through analytical standards or orthogonal techniques could complement the results, the adopted workflow provides a comprehensive and reliable overview of the metabolite composition of *Tripleurospermum disciforme* (C.A.Mey.) Sch.Bip. extracts.

A detailed list of the annotated compounds, along with their chromatographic, spectral, and ion mobility properties, is available in Supplementary Table S1. In the table, some compounds still appear more than once (e.g., 4-(4-hydroxyphenyl)-2-butanone, scutellarein, neochlorogenic acid), which reflects differences in retention times across extracts and/or the presence of isomeric forms. CCS

values were used to support this interpretation, since CCS differences of $\leq 2\%$ are generally attributed to experimental variability, while differences above 3% combined with distinct retention times strongly suggest the occurrence of isomers.

Figures 5.4 and 5.5 show the base peak chromatograms (BPCs) obtained in positive (A–C) and negative (D–F) ionization modes for the hexane (A), ethyl acetate (B), and methanol (C) extracts.

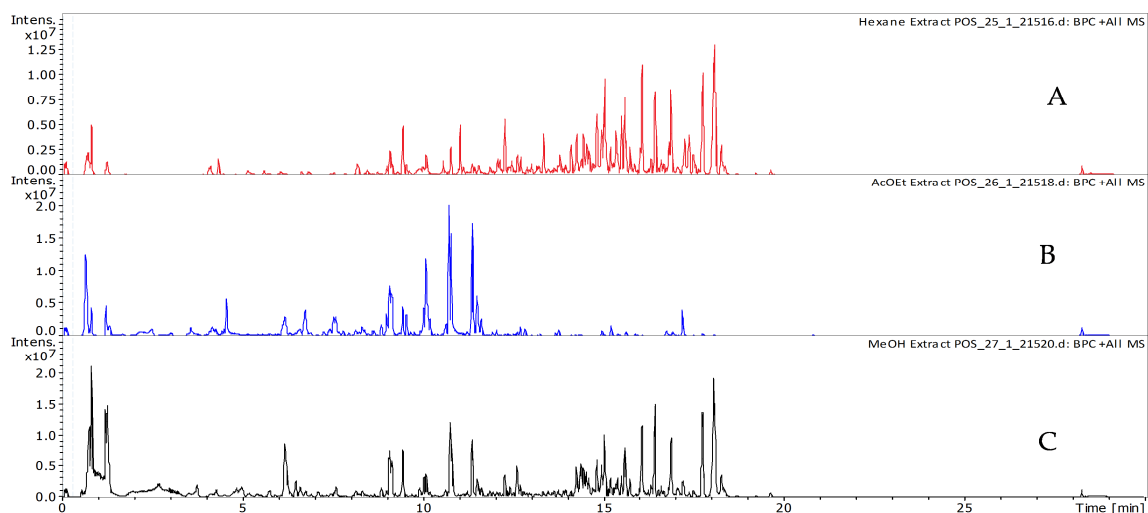


Figure 5.4. Base peak chromatograms (BPCs) of the extracts in positive ionization mode: (A) hexane fraction (B) ethyl acetate fraction, and (C) methanol fraction.

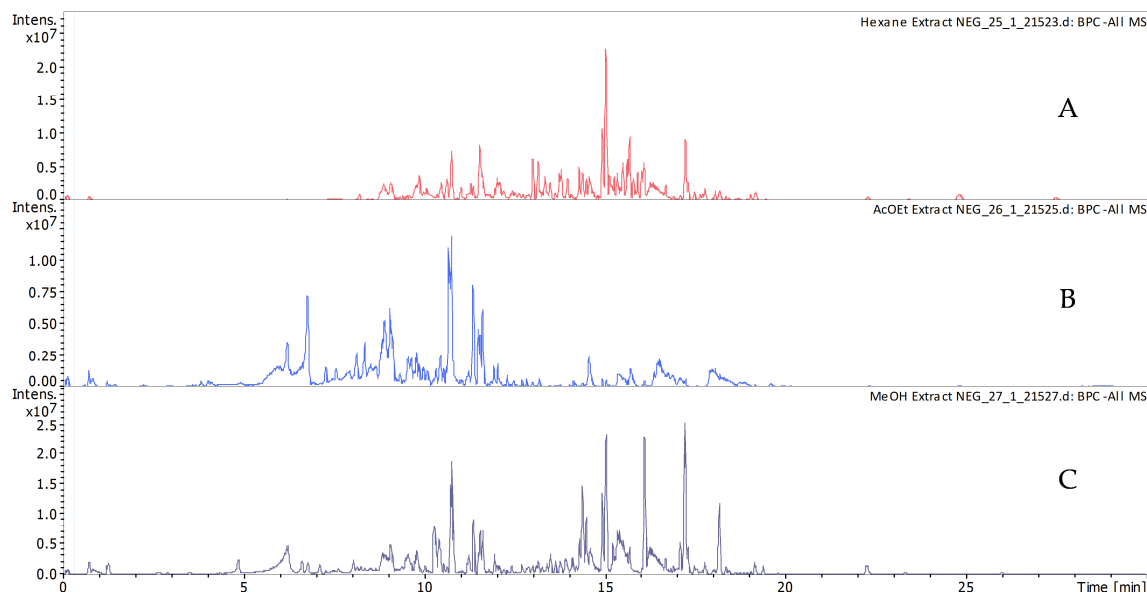


Figure 5.5. Base peak chromatograms (BPCs) of the extracts in negative ionization mode: (A) hexane fraction, (B) ethyl acetate fraction, and (C) methanol fraction.

5.2.2.2. Antioxidant Activity

Among the evaluated extracts of *Tripleurospermum disciforme* (C.A.Mey.) Sch.Bip., the ethyl acetate extract fraction exhibited the most potent antioxidant activity, as evidenced by its IC_{50} value of $12.496 \pm 4.153 \mu\text{g/mL}$ in the DPPH free radical scavenging assay, while the methanol and chloroform extracts fractions (which came from successive extractions) displayed moderate activity compared to the gallic

acid standard. Notably, all extracts fractions, except for the hexane (AAI < 0.5), demonstrated antioxidant activity ranging from moderate to strong (Table 5.5).

Table 5.5. Antioxidant properties of hexane, chloroform, ethyl acetate, and methanol extracts fractions of *Tripleurospermum disciforme* (C.A.Mey.) Sch.Bip.

Samples	* DPPH Free Radical Scavenging Assay		
	IC ₅₀ (µg/mL)	** AAI	Antioxidant Activity
n-Hexane	290.020 ± 80.639	0.183 ± 0.013	No activity
Ethyl acetate	12.496 ± 4.153	4.211 ± 0.339	Very strong
Chloroform	71.984 ± 20.112	0.720 ± 0.050	Moderate
Methanol	64.774 ± 24.764	0.834 ± 0.068	Moderate
Gallic acid	3.923 ± 1.259	13.001 ± 0.672	Very strong

* DPPH—2,2-diphenyl-1-picrylhydrazyl; ** AAI—antioxidant activity index.

In contrast, recent studies [133] reported that the methanol extract fraction of *Tripleurospermum limosum* (Maxim.) Pobed (which was a first extraction) exhibited strong antioxidant activity (IC₅₀ = 0.0304 mg/mL) for DPPH assay, but also across various assays, including ABTS, hydroxyl, and superoxide radical scavenging tests. It is important to highlight that numerous studies have emphasized the critical role of solvent selection in extracting phenolic compounds. Water and aqueous methanol are particularly effective solvents for isolating phenolic compounds from plants in the genus *Tripleurospermum*, including *Tripleurospermum insularum* Inceer & Hayırlıoglu-Ayaz [12] and *Tripleurospermum disciforme* (wild chamomile) [199], as well as for extracting flavonoids from *Tripleurospermum disciforme* [13].

The antioxidant activity observed in *Tripleurospermum disciforme* aligns with findings for the methanolic extracts of *Tripleurospermum insularum* [12] and *Tripleurospermum limosum* [133], both of which exhibit notable DPPH scavenging capabilities. Additionally, research on the Asteraceae family has identified the presence of significant caffeoyl derivatives in *Tripleurospermum disciforme* [33], *Tripleurospermum oreades* (Boiss.) Rech.f. [73], and *Tripleurospermum insularum* Inceer & Hayırlıoglu-Ayaz [12], which are recognized for their robust antioxidant properties [426].

Here, considering the very strong antioxidant activity of ethyl acetate extract fraction, it can be suggested that chlorogenic acid may play a role in this activity, due to its absence in other extracts. Chlorogenic acid (5-O-caffeoylquinic acid) is a phenolic compound belonging to the hydroxycinnamic acid family. It is known for its wide range of beneficial health effects, including managing metabolic syndrome, antidiabetic and anti-inflammatory activity, lipid-lowering and blood pressure-regulating properties, and predominantly significant antioxidant activity [427-431]. When evaluating antioxidant activity and correlating it with plant composition, multiple factors can influence the results, including seasonal and geographic variation, which directly affect phytochemical profiles.

Notably, cooler temperatures prevailing at higher elevations act as a key ecological driver, stimulating the production of antioxidant compounds regardless of interspecific genetic variation [212, 236].

Consistently, numerous studies have shown that plants adapted to high-altitude environments tend to accumulate greater amounts of phenolic constituents compared to those growing at lower elevations [370, 371, 432].

5.2.2.3. Antimicrobial Activity

In the disc diffusion assay (Supplementary Table S2), limited antimicrobial activity was observed for the tested fractions. The most notable effect was found in the ethyl acetate extract *fraction*, which exhibited inhibitory activity against *Staphylococcus aureus* and *Bacillus cereus*, with inhibition zones of 11.3 mm and 12 mm, respectively. Additionally, the chloroform extract *fraction* showed inhibitory activity against *B. cereus*, with an inhibition zone of 10 mm. In contrast, the methanol and hexane fractions displayed low to no antimicrobial activity against the tested bacterial strains.

Regarding MIC determination (Table 5.6), the strongest activity was observed against *B. cereus*, with MIC values ranging from 2500 to 312 µg/mL for *T. disciforme* extracts. *S. aureus* also exhibited some susceptibility, though at higher MIC values of 5000 and 2500 µg /mL. These findings provide initial evidence for the antimicrobial potential of *T. disciforme* and lay the groundwork for future studies aimed at identifying specific bioactive compounds and exploring a broader spectrum of microbial targets, thereby linking ethnobotanical knowledge with pharmacological evaluation.

Table 5.6. Minimum inhibitory concentration (µg/mL) of hexane, chloroform, ethyl acetate, and methanol extracts fractions of *Tripleurospermum disciforme* (C.A.Mey.) Sch.Bip.

Strains	MIC (µg/mL)				
	Hexane	Ethyl Acetate	Chloroform	Methanol	Tetracycline
<i>Staphylococcus aureus</i> ATCC 25923	1250	5000	5000	5000	2
<i>Escherichia coli</i> ATCC 25922	>5000	>5000	>5000	>5000	4
<i>Klebsiella pneumoniae</i> ATCC 13883	>5000	>5000	>5000	>5000	8
<i>Acinetobacter</i> <i>baumannii</i> LMG 1025	5000	5000	5000	>5000	2
<i>Bacillus cereus</i> ATCC 11778	2500	312	625	625	0.25
<i>Pseudomonas</i> <i>aeruginosa</i> ATCC 27853	>5000	>5000	>5000	>5000	16
<i>Salmonella</i> <i>Typhimurium</i> ATCC 13311	>5000	>5000	>5000	>5000	8
<i>Enterococcus faecalis</i> ATCC 29212	>5000	5000	>5000	>5000	16

As found in this study, previous studies showed that *T. disciforme* collected from Tehran, Guilan, and Fars provinces has demonstrated low antibacterial activity in methanol extracts fraction, with inhibition zones of 12 mm and 14 mm against *Staphylococcus epidermidis* and *S. aureus*, respectively, at high concentrations of extracts (64 mg/mL) [13]. However, Tofighi et al. (2015) reported no antibacterial activity of *T. disciforme* methanolic extracts against *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, *Aspergillus niger*, *Bacillus cereus*, and *Bacillus subtilis* [13].

Derivatives of 4-hydroxycoumarin have been shown to possess activity against *Bacillus cereus* and *Bacillus subtilis* [433-435].

Similarly, chlorogenic acid (5-O-caffeoylquinic acid) exhibits activity against the *Bacillus* genus [436, 437], with the study by Wu et al. (2020) [89] demonstrating that the chlorogenic acid caused a notable reduction in intracellular adenosine triphosphate (ATP) levels, possibly by affecting the material and energy metabolism or interfering with cellular signal transduction pathways. Additionally, it exerted a bacteriostatic effect by disturbing the intracellular metabolic balance of the tricarboxylic acid (TCA) cycle and glycolytic pathway, ultimately resulting in metabolic dysfunction and the death of *B. subtilis* [438].

Therefore, the presence of these compounds in the ethyl acetate extract fraction may support the antimicrobial activity of this extract. In turn, several studies have highlighted the strong antimicrobial properties of essential oils derived from various plants, including *T. disciforme* (C.A.Mey.) Sch.Bip, which achieved a MIC of 250 µg/mL against *S. aureus* or of 125 µg/mL against *B. subtilis*, *Klebsiella pneumoniae*, and *E. coli* and 62.5 µg/mL against *P. aeruginosa* [193]. Based on the evidence obtained from this study, we conclude that *T. disciforme* extracts exhibit weaker antibacterial properties compared to its essential oil, indicating that the antibacterial potential of *T. disciforme* resides primarily in volatile compounds rather than its crude fractions.

It is well-established that the chemical composition of plants is influenced by various factors such as species, solvent type, extraction method, and ecological conditions, all of which subsequently affect their antimicrobial efficacy [439].

However, further studies are required to explore its full therapeutic potential and to identify the specific bioactive compounds responsible for its antimicrobial properties.

5.2.2.4. Cellular Viability

The effect of *T. disciforme* (C.A.Mey.) Sch.Bip. extracts on cell viability was assessed in two distinct human cell lines: NHDF (normal human dermal fibroblasts) and MCF-7 (breast adenocarcinoma). Extracts fractions were obtained using four different solvents, chloroform, methanol, ethyl acetate, and hexane, applied at increasing concentrations (0.05%, 0.5%, and 1% v/v). Cell viability was quantified using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and normalized to total protein content, allowing for the correction of differences in cell density or growth rates (Figure 5.6).

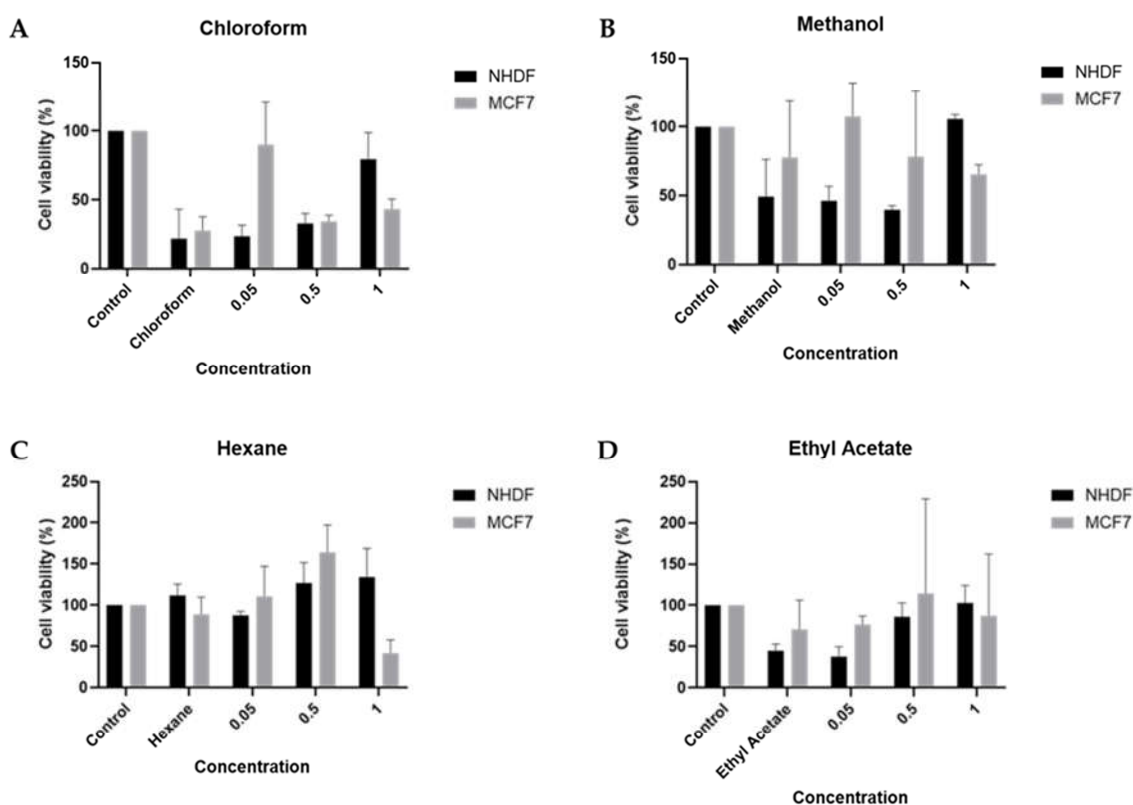


Figure 5.6. Effect of *Tripleurospermum disciforme* (C.A.Mey.) Sch.Bip. extracts on the viability of NHDF and MCF-7 after 48 h exposure. Extracts fractions were obtained using four solvents, (A) chloroform, (B) methanol, (C) hexane, and (D) ethyl acetate, and tested at three concentrations (0.05, 0.5, and 1 mg/mL). For internal conditions, Control refers to cells with medium, followed by the solvent controls (20 μ L of the corresponding solvent, equivalent to the highest volume present in the treatment wells).

In NHDF cells, the chloroform and methanol extracts fractions displayed similar patterns: both the solvent controls and the lowest extract concentrations (0.05 and 0.5 mg/mL) significantly reduced cell viability when compared to the untreated control. Specifically, chloroform alone reduced viability ($p = 0.0003$ vs. control), as did the 0.05 mg/mL ($p = 0.0004$) and 0.5 mg/mL ($p = 0.0010$) concentrations. However, at 1 mg/mL, a significant increase in viability was observed compared to the chloroform-only control ($p = 0.0031$) and to lower concentrations ($p = 0.0132$ vs. 0.5 mg/mL), although not significantly different from the untreated control ($p = 0.42$). A comparable trend was seen with the methanol extract: methanol alone reduced viability ($p = 0.0056$), as did 0.05 mg/mL ($p = 0.0037$) and 0.5 mg/mL ($p = 0.0016$), while the highest concentration significantly improved viability ($p = 0.0026$ vs. methanol control).

Regarding ethyl acetate, the concentrations 0.5 and 1 significantly increased viability when compared to solvent-only control, at $p = 0.028$ and $p = 0.0031$, respectively. In contrast, the hexane extract did not produce any statistically significant changes in NHDF viability across all tested concentrations, despite an apparent upward trend in cell viability at higher doses. In MCF-7 cells, a markedly different response profile was observed.

The chloroform fraction induced a strong cytotoxic effect: chloroform alone significantly reduced viability ($p = 0.0011$ vs. control), and both 0.5 mg/mL ($p = 0.0024$) and 1 mg/mL ($p = 0.0067$) concentrations maintained this reduction. Interestingly, the 0.05 mg/mL extract fraction concentration increased viability compared to chloroform-only ($p = 0.0034$), but remained below control, suggesting a biphasic response.

In contrast, the methanol and ethyl acetate extract's fractions produced no statistically significant effects on MCF-7 viability at any concentration. Although fluctuations were noted, particularly at 0.5 mg/mL ethyl acetate, where a slight increase in viability was observed, the high variability and lack of significance indicate minimal impact on tumour cell metabolism or survival.

The hexane extract fraction, however, induced this effect in MCF-7 cells. At 0.5 mg/mL, viability was significantly increased ($p = 0.027$ vs. hexane-only). In contrast, the 1 mg/mL concentration caused a significant reduction in viability ($p = 0.0010$ when compared to 0.5 mg/mL), suggesting dose-dependent cytotoxicity after initial stimulation.

Collectively, these findings indicate that *T. disciforme* (C.A.Mey.) Sch.Bip. contains biologically active compounds capable of modulating cell viability in a solvent-concentration dependent manner, with clear differences in sensitivity between normal and tumoral cells. While NHDF cells exhibited cytoprotective responses at higher extract concentrations (particularly with chloroform and methanol), MCF-7 cells were more susceptible to cytotoxic effects, especially with the chloroform and hexane extracts. In most of the concentrations, for all solvents, there is no tumour cell proliferation, suggesting a favourable profile for further investigation.

These results highlight the potential of *T. disciforme* (C.A.Mey.) Sch.Bip. as a source of bioactive molecules with selective effects on human cells. The extract concentrations that enhanced viability in NHDF cells did not promote proliferation in tumour cells, and in some cases induced cytotoxicity. Further studies are needed to clarify these differential effects and to explore their potential applications in cytoprotection or anticancer therapy

5.3. Materials and Methods

5.3.1. Ethnobotanical Surveys

5.3.1.1. Guilan, Qazvin, and Alborz Provinces Geo-Ethnographical Profile

Guilan Province is in the north and northwest of Iran, covering an area of 14,711 Km². It lies between latitudes 36°33' to 38°27' N and longitudes 48°32' to 50°36' E. The province is administratively divided into 16 counties, 52 cities, 43 districts, 109 rural areas, and 2583 villages, with a total population of 2,530,686. Geographically, Guilan Province is positioned south of the Caspian Sea, boasting approximately 300 km of coastline, and north of the Alborz Mountains, where the average elevation reaches 3000 m. Due to its unique topographical and climatic conditions, Guilan is recognized as one of the wettest provinces in Iran [440].

The northern provinces of the country Guilan, Mazandaran, and Golestan, as well as the Talesh region in Azerbaijan, are home to 3234 vascular plant species, encompassing 856 genera and 148 families. Of these, 475 species of medicinal plants have been identified in the forests of Guilan Province [100]. The Hyrcanian forests, which closely resemble the forests of Central Europe, are situated in the northern provinces of Iran Guilan, Mazandaran, and Golestan. Stretching 800 km along the western Caspian

Sea and the northern Alborz Mountain Range, these forests cover an area of 1.84 Mha and are 110 km wide [108].

The population of Qazvin Province was approximately 1.3 million, with 600,000 residing in Qazvin City. Despite comprising only 1% of Iran's total land area, it is in the northern half of the country, between latitudes 35°37' and 36°45' N and longitudes 48°45' and 50°50' E. Although it ranks as the 26th largest province in Iran, Qazvin boasts a remarkably diverse climate due to its position between the arid central plateau and the northeastern mountainous regions. The province also exhibits significant altitudinal variation, with Siyalan Mountain reaching 4175 m as its highest point and the banks of Manjil Dam Lake at 300 m as its lowest. This elevation disparity greatly influences both precipitation and temperature. Annual rainfall varies from 210 mm in the eastern regions to 550 mm in the northwest, while temperature fluctuations are considerable, with extreme highs recorded in the central plains and severe lows in the northwestern highlands of Avaj. Average temperatures gradually decrease from the lowlands toward the foothills and mountainous areas [441]. Alborz Province, covering 519,391.5 ha, constitutes approximately 0.3% of Iran's total land area. Geographically, it lies between 50° and 51.30° east longitude and 30.35° and 30.36° north latitude, positioned along the southern slopes of the Alborz mountain range. The province exhibits diverse topographical characteristics. Its northern areas ascend to altitudes of 4104 m, forming a continuous mountain range stretching from east to west. Meanwhile, the central region consists primarily of plains, sitting at an average altitude of approximately 1500 m. Overall, most of the province lies at elevations ranging from 1500 to 2000 m [107].

5.3.1.2. Field Interview

This survey was conducted over a six-month period during the first semester of 2023. A semi-structured, face-to-face questionnaire was administered to 100 local individuals in the herbal markets of the Guilan, Qazvin, and Alborz provinces. The questionnaire primarily focused on ethnobotanical claims and traditional beliefs held by local communities in these three regions. It also gathered demographic and personal information, including respondents' age, gender, relationship with the locality, education level, knowledge level, most used plants, plant identification, utilized plant parts, and methods of preparation. Subsequently, scientific identification and authentication of the cited plants were carried out using *Flora Iranica* [384], *Flora of Iran* [385], and various herbal literature sources [79, 386]. Additionally, the accuracy of the scientific names of the medicinal plants was verified through www.theplantlist.org.

5.3.1.3. Quantitative Analysis of the Ethnobotanical Results

All statistical results were firstly prepared using Microsoft Excel (Windows, Washington, DC, USA). Informant Consensus Factor (ICF) was utilized to assess the consistency of traditional knowledge regarding plant usage across various ailment categories, using Equation (1)

$$ICF = (N_{ur} - N_t) / (N_{ur} - 1) \quad (1)$$

Where N_{ur} is the total number of use-reports for a specific illness category, and N_t is the number of taxa utilized in that category.

The ICF, which ranges from 0 to 1, reflects the degree of agreement among informants. A higher ICF value suggests a concentrated reliance on a few specific species, possibly indicating effective knowledge transmission or a shared cultural understanding.

In contrast, lower values imply inconsistency in plant selection, potentially due to sporadic knowledge sharing or arbitrary choices by informants [387, 388].

Fidelity level (FL) index was assessed to identify the most culturally significant plant species associated with specific health conditions, especially in cases where multiple species are used for the same ailment. The FL was calculated using Equation (2)

$$FL = (N_p/N \times 100) \quad (2)$$

Where N_p is the number of participants who cited a plant for a particular disease, and N is the total number of participants who reported that plant for any ailment.

Higher FL percentages indicate a stronger preference and consensus on using a given plant species for a specific health issue, by the participants of the study area [387, 388].

Relative Frequency of Citation (RFC) index does not differentiate between specific uses or categories of use for a plant; it is simply a general record of how often a plant is cited in relation to any reported use by an individual.

This means that it reflects the frequency with which a plant is mentioned in ethnobotanical studies, but it does not provide detailed information about the specific purpose or category (e.g., medicinal, culinary) for which the plant is used. The RFC index is expressed by Equation (3)

$$RFC = FC/N \quad (3)$$

Where FC is the number of informants who mentioned a particular plant frequency of citation, and N is the total number of informants in the survey.

The index varies between 0 (when no one considers a plant to be useful) and 1 (if all the participants mention a plant as useful). Unlike other indices, RFC index does not account for the category of use or the number of different uses reported [387, 388].

Use value (UV) index was calculated using Equation (4) to gauge the overall significance of each plant species in the local traditional knowledge system, i.e.,

$$UV = \sum U_i/N \quad (4)$$

Where U_i represents the number of different uses cited by each informant for a species, and N is the total number of informants in the survey.

A higher UV indicates a broader or more frequent application of a plant in ethnomedicinal practices [387, 388].

5.3.2. Collection and Identification of Plant Materials

The dried flowering tops of *Tripleurospermum disciforme* were sourced from a local market in Guilan, Iran. A voucher specimen was subsequently deposited in the herbarium of Guilan University of Medical Sciences.

5.3.3. Preparation and fractionation of plant materials

About 1500 g of the top flowers of *T. disciforme* (C.A.Mey.) Sch.Bip. was extracted via the percolation method by methanol at room temperature for 24, 48, and 72 h. The extract was concentrated using a rotary evaporation system. The crude extract of *T. disciforme* was re-extracted with increasing polarity solvents, n-hexane, chloroform, ethyl acetate, and methanol, respectively, using an ultrasound bath for 20 min, three times, with frequent shaking at 40 °C. The extracts fractions were concentrated using the same rotary evaporation system, weighed, and stored in 4 °C, until phytochemical characterization and analysis of the biological activities.

5.3.4. Phytochemical Characterization and Phenolic Profile

An untargeted metabolomic approach was employed to characterize the phytochemical composition of the samples. Analyses were conducted by ultra-high performance liquid chromatography (UHPLC) coupled to a trapped ion mobility time-of-flight mass spectrometer (timsTOF-MS; Bruker Daltonics, Germany), equipped with a VIP-HESI electrospray ionization source. Samples were previously dissolved in minimal volumes of hexane, ethyl acetate, or methanol, and 5 µL of each solution was injected into a ZORBAX Eclipse XDB-C18 RRHD column (2.1 × 100 mm, 1.8 µm; Agilent Technologies, USA). Chromatographic separation was achieved using a binary mobile phase consisting of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B), under a flow rate of 0.4 mL/min. The gradient programme lasted 30 min, including a re-equilibration phase.

Mass spectrometric detection was performed in both positive and negative ionization modes. The capillary voltage was set at ±4500 V and the end plate was offset at ±500 V. Nitrogen was used as the nebulising gas (8 bar), drying gas (8 L/min at 240 °C), and sheath gas (4 L/min at 450 °C). Spectra were acquired over an *m/z* range of 20–1300 in both MS and MS/MS mode using Parallel Accumulation–Serial Fragmentation (PASEF). Ion mobility separation was recorded over a $1/K_0$ range of 0.45–1.45 V·s/cm² with a ramp time of 100 ms.

Data processing was carried out using *Data Analysis* (v6.1) and *MetaboScape* (v7.0.1) software from Bruker. Spectral features (“buckets”) were extracted based on *m/z*, retention time, and collisional cross section (CCS), applying an intensity threshold of 10,000 counts. Compound annotation was performed using three strategies: (i) spectral library matching (SL), (ii) an in-house analyte list (AL) of phenolic compounds (name and molecular formula only), and (iii) SmartFormula (SF) calculations restricted to CHNOPS atom sets.

Combining these approaches, a total of 43 tentative identifications were obtained through SL + AL + SF. Additional matches were retrieved using AL + SF (237 features), SL + SF (64 features), and SF alone (1605 features).

5.3.5. Biological Activities Evaluation

5.3.5.1. Antioxidant Activity

The antioxidant activity was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. A calibration curve was constructed with methanol solutions of DPPH (85–4.25 mg/L). Absorbance was measured at 517 nm, and the capacity of free radical scavenging was

calculated accordingly to Luís et al. [389]. The IC_{50} was calculated graphically using the extract concentration versus the corresponding radical scavenging capacity. The antioxidant activity was expressed through the antioxidant activity index (AAI), calculated as follows: $AAI = (\text{final concentration of DPPH in the control}) / (IC_{50})$. The AAI allowed for the classification of the antioxidant activity of the samples as poor ($AAI \leq 0.5$), moderate ($0.5 < AAI \leq 1.0$), strong ($1.0 < AAI < 2.0$), or very strong ($AAI \geq 2.0$) [389, 390].

5.3.5.2. Antimicrobial Activity

Microorganisms and Media

Hexane, chloroform, ethyl acetate, and methanol extracts fractions were tested against three Gram-positive (*Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, and *Bacillus cereus* ATCC 11778) and five Gram-negative (*Pseudomonas aeruginosa* ATCC 27853, *Salmonella* Typhimurium ATCC 13311, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Acinetobacter baumannii* LMG 1025) bacteria. All the strains were cultured on Brain Heart Infusion Agar (BHIA) and incubated at 37 °C for 24 h before any antimicrobial assay.

Determination of Disc Diffusion Assay

The antimicrobial activity of *Tripleurospermum disciforme* (C.A.Mey.) Sch.Bip was evaluated by disc diffusion assay, following the M2-A8 method as described by the Clinical Laboratory and Standards Institute (CLSI) for bacteria. Bacteria inocula were prepared by the direct suspension of colonies in saline solution (NaCl 0.85% (*w/v*)) and adjusted to 0.5 McFarland (about 1 to 2×10^8 (CFU/mL)). Discs with a diameter of 6 mm were each impregnated with 20 μ L of each extract fraction dissolved in dimethylsulfoxide (DMSO; Scharlab, Spain) at a concentration of 10 mg/mL. For the negative control, DMSO was used instead. Then, the Müeller–Hinton Agar (MHA) (Liofilchem, Italy) plates were inoculated with the cellular suspension, and the discs previously prepared were placed over the agar. The plates were incubated at 37 °C for 24 h. Following incubation, all the plates were visually checked for inhibition zones, and the diameters were measured in mm. Each experiment was performed three independent times.

Determination of Minimum Inhibitory Concentration (MIC)

The MIC of the extract's fractions was evaluated by the microdilution method as described by [391]. Serial dilutions of each of fraction (from 5000 μ g/mL to 39 μ g/mL) were prepared in a 96-well plate (50 μ L per well) in Müeller–Hinton Broth (MHB) (Liofilchem, Italy). The cellular suspension was prepared as described above, diluted in MHB, and added to wells to obtain a final cellular concentration of 5×10^5 CFU/mL in each well. A positive control was used containing tetracycline. Wells without the tested plant fractions were used as a growth control, and wells without bacteria were used as a negative growth control. The plate was incubated for 24 h at 37 °C; after that, 30 μ L of resazurin (0.1%) was added in each well of the microtiter plate and incubated at 37 °C for 2 h. No color change (blue) indicated inhibition of the tested bacterium. The MIC was considered the lowest

concentration of the extract fraction that inhibited bacterial growth. Each assay was performed at least three independent times.

5.3.6. In Vitro Studies Using NHDF and MCF-7 Cells

5.3.6.1. Cell Culture

Human breast adenocarcinoma cells (MCF-7; ATCC® HTB-22™) and normal human dermal fibroblasts (NHDF; ATCC® PCS-201-012™) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Unless otherwise specified, all chemicals (analytical grade), reagents, culture media, and supplements were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cells were routinely maintained in 75 cm² culture flasks at 37 °C in a humidified atmosphere containing 5% CO₂. NHDF cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), L-glutamine (0.02 M), HEPES (0.01 M), sodium pyruvate (0.001 M), and 1% antibiotic/antimycotic (10,000 U/mL penicillin, 10 mg/mL streptomycin, and 25 mg/mL amphotericin B). The culture medium was renewed every 48 h until cells reached approximately 90–95% confluence. MCF-7 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, high glucose) supplemented with 10% FBS and 1% antibiotic/antimycotic.

At confluence, adherent cells were detached using trypsin-EDTA solution (0.125 g/L trypsin and 0.02 g/L EDTA). Prior to each experiment, cell viability was assessed by trypan blue exclusion using a Neubauer haemocytometer, and suspensions were adjusted to the required density in the appropriate complete culture medium.

5.3.6.2. Preparation of the Solutions of the Compounds Under Study

Hexane, chloroform, methanol, and ethyl acetate extracts fractions of *T. disciforme* (C.A.Mey.) Sch.Bip. were dissolved at a final concentration of 10 mg/mL in their corresponding solvents. The mixtures were sonicated for approximately 3 h at 40 °C, filtered, and the resulting supernatant was stored at 4 °C.

5.3.6.3. Cytotoxicity Assay and Protein Quantification

NHDF and MCF-7 cells were seeded at a density of 2×10^4 cells/mL in a 96-well plate (200 µL/well). Two plates were prepared for each cell type. After incubation for 48 h at 37 °C in a humidified atmosphere containing 5% CO₂, cell adherence was verified and culture medium was replaced with treatment solutions.

Cells were exposed for 48 h to *T. disciforme* (C.A.Mey.) Sch.Bip. extracts fractions (hexane, chloroform, methanol, or ethyl acetate) diluted in complete culture medium at final concentrations of 0.05, 0.5, and 1 mg/mL. Negative controls included untreated cells, while solvent controls received 20 µL of the corresponding solvent, equivalent to the highest volume present in the treatment wells. All experiments were conducted in triplicate and independently repeated at least twice.

In one plate per cell type, antiproliferative activity was assessed using the MTT assay. Following 48 h exposure, the medium was aspirated and cells were washed with 100 µL phosphate-buffered saline (PBS). MTT solution (5 mg/mL in PBS) was added to each well and incubated for 4 h at 37 °C. After removal of the MTT solution, formazan crystals were solubilised in dimethyl sulfoxide (DMSO; Sigma-

Aldrich, St. Louis, MO, USA). Absorbance was measured at 570 nm using an xMark™ microplate spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA).

In parallel plates, total protein content was quantified using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA, cat. no. 23227), according to the manufacturer's instructions. Briefly, after treatment, cells were washed with PBS, and plates were wrapped in aluminum foil and frozen at $-20\text{ }^{\circ}\text{C}$ for at least 24 h. The working reagent was prepared by mixing 50 parts of Reagent A with 1 part of Reagent B. Plates were thawed, and 20 μL of PBS was added to each well, followed by 200 μL of working reagent. Plates were mixed on a shaker for 30 s and incubated at $37\text{ }^{\circ}\text{C}$ for 30 min. After cooling to room temperature, absorbance was recorded at 562 nm, and protein concentration was determined against a standard curve.

5.3.6.4. Statistical Analysis

All statistical analyses were performed using GraphPad Prism version 10.6.0 (GraphPad Software, La Jolla, CA, USA). Comparisons among multiple groups were conducted using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. For pairwise comparisons, Student's *t*-test was applied. A *p*-value < 0.05 was considered statistically significant.

For each experimental condition, the mean absorbance values obtained from the MTT assay and protein quantification were calculated, and the ratio of MTT/protein was determined. This normalization allowed for the evaluation of metabolic activity relative to total protein content, thereby distinguishing whether changes in MTT reduction reflected variations in cell number (biomass) or alterations in cellular metabolic activity.

5.4. Conclusions

This multidisciplinary investigation integrates ethnobotanical knowledge, phytochemical profiling, and biological evaluation to elucidate the therapeutic potential of *T. disciforme*. Ethnobotanical surveys conducted across three Iranian provinces highlight the relevance of this species in traditional medicine, particularly for gastrointestinal disorders. Untargeted metabolomic profiling revealed a chemically diverse extract composition, enriched in phenolic compounds and other bioactive secondary metabolites, with solvent polarity playing a decisive role in extraction efficiency and metabolite diversity. Supporting these traditional claims, antimicrobial assays demonstrated moderate effects of *T. disciforme* extracts, notably against *B. cereus*. While these findings substantiate the ethnomedical relevance of *T. disciforme* and advocate for its continued pharmacological exploration, we acknowledge certain limitations. The biological assessment addressed only a limited range of activities and therefore does not capture the full therapeutic potential of the species. This study focused on crude extracts, without further isolation of individual active compounds, which restricts the ability to attribute the observed effects to specific compounds. Studies focusing on cellular viability are of particular interest, as it seems that healthy and tumour cells behave differently when exposed to the same treatment. Nonetheless, collectively, our results provide a first step in bridging ethnobotanical knowledge and laboratory-based pharmacological evidence for *T. disciforme*. Future studies should include fractionation, bioassay-guided isolation, quantification of key compounds, and additional biological assays (e.g., anti-inflammatory, and safety assessments) to fully validate and expand the therapeutic potential of this medicinal plant.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/doi/s1>: Table S1. Annotated phytochemical compounds identified in the extract's fraction of *Tripleurospermum disciforme* using untargeted UHPLC–timsTOF–MS analysis. Compound annotations were performed based on spectral library matching (SL), an in-house analyte list (AL) of phenolic compounds, and SmartFormula (SF) calculations. The table includes the compound name, molecular formula, accurate m/z, retention time (RT), collision cross section (CCS), and the solvents in which each compound was detected (methanol, ethyl acetate, and hexane); Table S2: Diameter of inhibition zone (mm) of methanol, hexane, ethyl acetate, and chloroform extract's fractions of *Tripleurospermum disciforme*.

Chapter 6

Concluding Remarks and Future Perspectives

Concluding Remarks and Future Perspectives

This research highlights the remarkable phytopharmaceutical potential of *Echium* species and *Tripleurospermum disciforme* through a multidisciplinary approach integrating ethnobotanical knowledge, phytochemical profiling, and biological evaluation.

The review of *Echium* species underscores their rich reservoir of bioactive secondary metabolites including flavonoids, phenolic acids, anthocyanins, naphthoquinone derivatives, terpenoids, and essential polyunsaturated fatty acids which collectively contribute to their broad spectrum of antioxidant, anti-inflammatory, neuroprotective, and antimicrobial properties. The presence of naphthoquinone compounds such as alkanins and shikonins in the roots, alongside volatile constituents like thymol and carvacrol, further reinforces their therapeutic value and positions this genus as a promising source for pharmaceutical, nutraceutical, and cosmeceutical applications.

In parallel, the ethnobotanical and pharmacological investigation of *T. disciforme* revealed its cultural importance in Iranian traditional medicine, particularly for gastrointestinal disorders, and demonstrated its moderate antimicrobial activity, notably against *Bacillus cereus*. Metabolomic profiling highlighted a chemically diverse composition enriched in phenolic compounds, with solvent polarity playing a decisive role in metabolite extraction efficiency. These findings substantiate traditional claims and provide foundational evidence for its potential pharmacological relevance.

Despite these promising results, several knowledge gaps remain. For *Echium* species, the hepatotoxicity risk posed by pyrrolizidine alkaloids (PAs) requires the development of optimized extraction protocols that minimize PA content while preserving therapeutic efficacy. For *T. disciforme*, the reliance on crude extracts and a limited range of biological assays restricts the ability to identify specific active compounds and fully characterize the therapeutic potential.

Future research should therefore focus on the fractionation and bioassay-guided isolation of key active constituents to establish structure–activity relationships. In parallel, it will be essential to carry out quantitative analyses of the major compounds, with a view to standardizing extracts for pharmacological use. It is also important to broaden the scope of biological evaluation, including anti-inflammatory and safety assessments, as well as studies on differential cellular responses between healthy and tumour cells. Furthermore, the optimization of extraction and processing methods is crucial to enhance efficacy and minimize toxicity, particularly regarding pyrrolizidine alkaloids (PAs) in *Echium*. Finally, pharmacological and clinical investigations are needed to validate efficacy and ensure safety for potential therapeutic applications.

Taken together, this work provides a solid scientific basis for future pharmacognostic research and supports the rational development of safe and effective phytopharmaceuticals derived from these two medicinally valuable plant taxa.

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