Evidence-Based Complementary and Alternative Medicine

Scientific Evidence for Folkloric Antimicrobials: Ethnopharmacology-Guided Solutions

Lead Guest Editor: Dejan Stojković
Guest Editors: Gokhan Zengin and Marija Smiljkovic

Scientific Evidence for Folkloric Antimicrobials: Ethnopharmacology-Guided Solutions

Evidence-Based Complementary and Alternative Medicine

Scientific Evidence for Folkloric Antimicrobials: Ethnopharmacology-Guided Solutions

Lead Guest Editor: Dejan Stojković
Guest Editors: Gokhan Zengin and Marija Smiljkovic

Contents

Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2021, Article ID 6879278, 9 pages
https://doi.org/10.1155/2021/6879278

Research Article

Scientific Evidence of Xuebijing Injection in the Treatment of Sepsis

Sa Tian, Defang Qin, Yixuan Ye, Huawei Yang, Shuguang Chen, Tao Liu, Luming Hu, Huiming Li, Qian Niu, and Xingzhan Zhang

Department of Intensive Care Unit, Guangdong Provincial Hospital of Chinese Medicine, Zhuhai, China

Correspondence should be addressed to Xingzhan Zhang; 13928000908@163.com

Received 15 July 2021; Accepted 7 October 2021; Published 25 October 2021

Academic Editor: Dejan Stojkovic

Objectives. To systematically collate, appraise, and synthesize the current evidence on the Xuebijing injection (XBJI) for sepsis. *Methods*. Eight databases were searched for systematic reviews (SRs) or meta-analyses (MAs) on XBJI for sepsis. Assessing the Methodological Quality of Systematic Reviews-2 (AMSTAR-2), Preferred Reporting Item for Systematic Reviews and Meta-Analyses (PRISMA), and Grading of Recommendations, Assessment, Development, and Evaluation (GRADE) methods were used to assess the methodological quality, reporting quality, and evidence quality of the enrolled studies, respectively. *Results*. Out of the 13 studies that were included, all studies were rated critically low quality based on AMSTAR-2 results. Based on the results obtained from PRISMA, all studies were reported to be over 80%, while the GRADE system yielded three outcome measures rated high-quality, 16 were of moderate quality, and the rest were of low or critically low quality. *Conclusions*. The combination of XBJI and Western medicine (WM) showed significant synergy for the treatment of sepsis compared to WM alone. However, this conclusion should be treated with caution since the quality of the SRs/MAs providing the evidence was relatively low.

1. Introduction

Sepsis is a severe complication resulting from severe infection, severe trauma, burns, surgery, and shock. This complication is quite perilous, leading to septic shock and multiple organ dysfunction syndromes [1]. Sepsis is a common cause of death in the intensive care unit [2], where it accounts for one-third to one-half of hospital deaths [3], as more than six million people die from this disease worldwide each year [4]. Despite the major advances in antibiotics and supportive therapies over the last few decades, mortality from sepsis still maintains an increasing trend [5]. At present, there is still no effective treatment for sepsis. Conventional anti-infection and supportive therapies have also shown no significant improvement in the survival rate of patients with sepsis [6, 7]. Under these circumstances, complementary and alternative therapies have drawn attention.

Xuebijing injection (XBJI), a Chinese patent medicine, was developed by Professor Jinda Wang [8]. Professor Wang's work was based on the Xuefu Zhuyu decoction by Wang Qingren, a famous physician in the Qing dynasty. XBJI consists of five Chinese herbs (Radix Salviae, Carthami Flos, Chuanxiong Rhizoma, Angelicae Sinensis Radix, and Paeoniae Radix Alba) that contain approximately 30 bioactive compounds, such as hydroxysafflor yellow A, danshenol, ferulic acid, paeoniflorin, senna lactone I, and more [9]. The benefits of this medicine include detoxification and toning, elimination of bacteria and viruses, supplementation of vital energy, and improved blood circulation [9, 10]. In addition, the medicine can also inhibit the action of most inflammatory mediators and endotoxin, allowing the restoration of the immune response [11]. It was reported that XBJI can block the progression of sepsis through different mechanisms, such as antibacterial, antioxidative, and anti-endotoxin [8, 11]. Therefore, XBJI has been approved as a State Category II New Drug for the treatment of sepsis in China and has been used in clinical practice [10]. Previous studies have shown that integrated medicine can reduce mortality due to sepsis [8], but the efficacy of XBJI combined

with Western medicine (WM) still lacks scientific evidence. This study aims to systematically collate, appraise, and synthesize scientific evidence through the presentation of an overview of these SRs/MAs.

2. Methods

This study was registered in the PROSPERO registry (CRD42021264569). The methods of the Cochrane handbook and some high-quality reviews were followed [12, 13].

2.1. Strategy for Search. A systematic search was conducted utilizing PubMed, Cochrane Library, Embase, Web of Science, China National Knowledge Infrastructure, Chongqing VIP, SinoMed, and Wanfang databases from inception to June 2021. The following medical subject headings, terms, and relevant keywords were used in this search: Xuebijing, sepsis, and systematic review. The search strategies can be found in additional file 1.

2.2. Criteria of Inclusion and Exclusion. The studies that met the following criteria would be included for further evaluation. (1) Study type: participants enrolled in randomized controlled trials. (2) Subjects: patients diagnosed with sepsis according to internationally recognized diagnostic criteria. (3) Interventions: the experimental intervention was a combination of XBJI plus WM and the control intervention was WM alone. (4) Outcomes: one or more of the index of outcomes was present, such as 28-day mortality, acute physiology and chronic health evaluation (APACHE) II score, white blood cell, and procalcitonin (PCT). A study was excluded if it had the following factors: (1) it was a duplicate publication, (2) it was an expert comment or a conference report, (3) it did not undergo peer review, (4) the control group included XBJI, and (5) the lack of further data.

2.3. Literature Selection and Data Extraction. Two independent authors strictly followed the inclusion and exclusion criteria to conduct the study selection. Titles and abstracts of the literature were screened first, followed by the full text of all the initial qualified literature. The following data were extracted from each study: general information (first authors, country, and publication year), characteristics of the study (sample size and interventions), and results (outcomes and relative effect). A third author resolved any discrepancies between the two authors.

2.4. Quality Assessment. For the eligible studies that were included, two independent authors assessed the methodological quality, reporting quality, and evidence quality using the appraisal tool for systematic reviews of randomized and observational studies, Assessing the Methodological Quality of Systematic Reviews-2 (AMSTAR-2) [14], Preferred Reporting Item for Systematic Reviews and Meta-Analyses (PRISMA) [15], and Grading of Recommendations, Assessment, Development, and Evaluation (GRADE) [16], respectively. A third author resolved any discrepancies between the two authors. The items obtained from AMSTAR-2 and the checklists of PRISMA can be found in the additional file 2 and additional file 3.

3. Results

3.1. Results of the Literature Search. From the databases utilized, 132 articles were identified from the initial search. After 63 duplicate articles were removed, 69 were eliminated based on the title and abstract following the criteria. Then, the eligibility of the remaining 22 articles was evaluated by scanning the full text of each article. Finally, examining full text resulted in the exclusion of eight studies (Appendix file 4), and the remaining 14 studies [17–30] met the inclusion criteria. Flow diagram of the literature selection process is shown in Figure 1.

3.2. Basic Characteristics. The studies included were published between 2010 and 2021. Five of these reviews were published in English, while the remaining were in Chinese. The number of trials of the included reviews ranged from 11 to 49 studies, and the total number of subjects ranged from 399 to 1970. As for the intervention, all reviews compared XBJI plus WM as a treatment intervention, while the control group only utilized WM. Six reviews out of 13 applied the Jadad scale for methodological quality assessment of included trials, while the remaining seven reviews used the Cochrane criteria tool. Further details of the assessment are given in Table 1.

3.3. Quality Assessment

3.3.1. Methodological Appraisal. The methodological quality was evaluated through AMSTAR-2. Among these studies, items 2, 4, 7, 9, 11, 13, and 15 were identified as key items. The key factors affecting the methodological quality were item 2 (no review established protocol), item 4 (11 reviews did not provide the search strategy), item 7 (no review provided a list of excluded trials), item 10 (6 reviews did not report the sources of funding), and item 16 (5 reviews did not report any potential sources of conflict of interest). Further details of this assessment are given in Table 2.

3.3.2. Quality of Reporting Appraisal. The quality of reporting was evaluated using the PRISMA guidelines, which included 7 sections and 27 items. The sections of the studies, including project title, abstract, introductions, and discussion, were comprehensively reported (100%). In the Methods section, the protocol and registration numbers were not reported in any of the reviews (0%), while the searches were completely reported in three reviews (21.4%), and the additional analyses conducted in the studies were reported in 10 reviews (78.6%). In the results section, the additional analyses were reported in 8 reviews (57.1%). Furthermore, funding was only reported in 8 reviews (57.1%). Further details are given in Table 3.

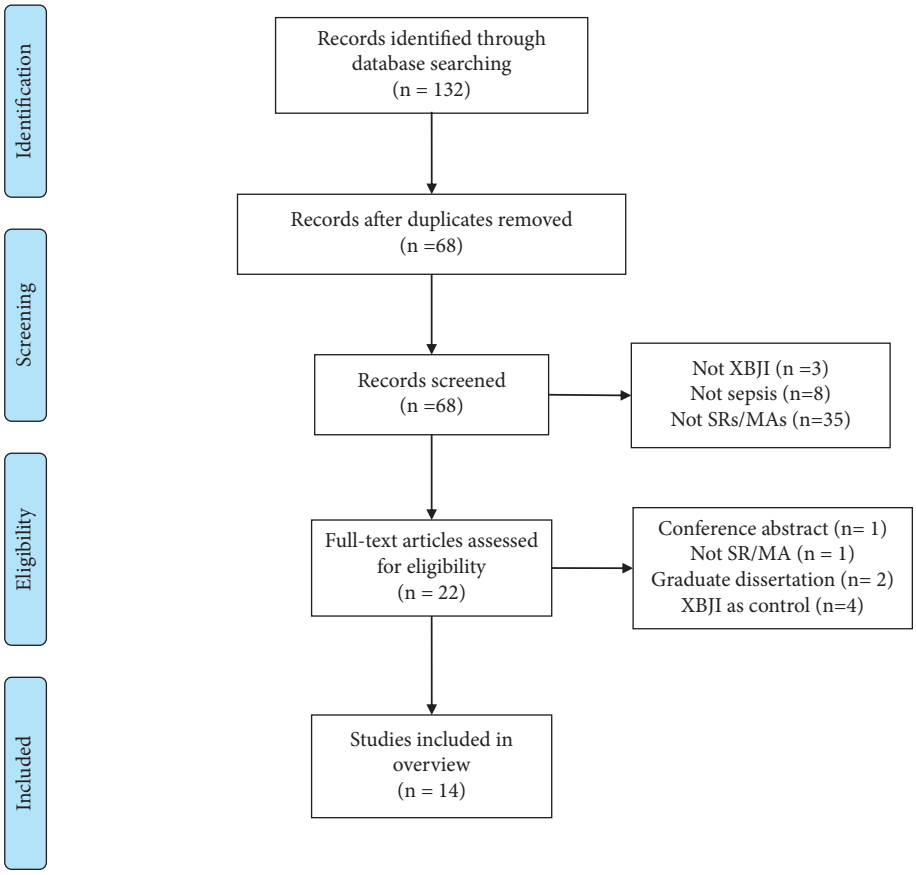


Figure 1: Flow diagram of the literature selection process.

3.3.3. GRADE Evidence Quality Classification. The 13 reviews included 43 outcome indicators that were related to the effectiveness of XBJI for sepsis. Three outcomes were identified as high quality, 16 were identified to be of moderate quality, 19 were identified to be of low quality, and the remaining 3 were identified to be with critically low quality. The risk of bias, inconsistency, imprecision, and publication bias were the main reasons for the decrease in quality. Further details are given in Table 4.

3.4. Description of Efficacy

3.4.1. Effect of the Interventions. The effects of the outcome indicators related to the effectiveness of XBJI for sepsis are given in Table 4. Twelve reviews [17, 24, 26, 27, 29, 30] reported the meta-analysis results of the 28-day mortality. The results revealed that the 28-day mortality rate of the XBJI group was lower when compared to the control group. Night reviews [17, 21, 23, 25, 27, 30] reported the outcomes for the APACHE II score revealed that XBJI combined with WM was superior to a single WM in improving the APACHE II score. Three reviews [17, 19, 20] then reported the outcomes for the duration of mechanical ventilation. These results showed that the time of mechanical ventilation of XBJI combined with the WM group was shorter than the control group, while three reviews [17, 19, 20] reported that the outcomes for the length of ICU stay showed that the XBJI plus WM group had an advantage over the WM only group in reducing the length of ICU stay. Two reviews [18, 20] reported the outcomes for body temperature changes, where their results revealed that XBJI accompanied with WM could lower body temperature better than the treatment with WM alone. Five reviews [17, 19, 21, 22, 25] then reported the serum levels of PCT for XBJI in combination with WM and the control group. The results showed that the XBJI plus WM group had a lower PCT level than the control group. Furthermore, four reviews [18, 25, 27, 28, 30] reported that the white blood cell count of the XBJI plus WM group was lower than the control group.

3.4.2. Safety of the Interventions. A total of five reviews [18, 19, 24, 25, 29] mentioned the adverse effects of XBJI for sepsis. Wherein, no adverse effects were reported in 3 reviews [18, 24, 25]. Two reviews [19, 29] identified the following side effects, including pruritus and mild diarrhea, but no significant difference was found compared with the control group.

4. Discussion

The treatment of sepsis remains unsatisfactory despite the use of combined antibiotics and therapy [18]. Therefore, it is essential to identify a more effective, innovative, and

Table 1: Basic characteristics description.

Author, year	Country	Sample size	Treatment intervention	Control intervention	Quality assessment tool	Conclusion summary
Chen et al. [17] 2018	China	17 (1247)	XBJI + WM	WM	Cochrane criteria	The XBJI and ulinastatin combination therapy appeared to be more effective for the treatment of sepsis when compared with the use of ulinastatin alone.
Li et al. [18] 2018	China	16 (1144)	XBJI + WM	WM	Cochrane criteria	This study suggested that supplementation with XBJI in addition to the conventional treatment appeared to be more effective for the treatment of sepsis as compared to the conventional treatment alone.
Xiao et al. [19] 2018	China	16 (1335)	XBJI + WM	WM	Cochrane criteria	The combination therapy appeared to be more effective for the treatment of sepsis compared to the conventional treatment alone. It was also observed that the risk of adverse events did not increase.
Zheng et al. [20] 2018	China	16 (1192)	XBJI + WM	WM	Cochrane criteria	Our results found that XBJI when combined with ulinastatin was superior to both routine therapies and the single administration of either ulinastatin or XBJI.
Xiao et al. [21] 2017	China	49 (1801)	XBJI + WM	WM	Jadad	The combination therapy appeared to be more effective for the treatment of sepsis when compared to conventional treatment alone.
Liu et al. [22] 2021	China	16 (1423)	XBJI + WM	WM	Jadad	This study suggested that supplementation with XBJI in addition to the conventional treatment appeared to be more effective for the treatment of sepsis as compared to conventional treatment alone.
Zhang et al. [23] 2021	China	15 (930)	XBJI + WM	WM	Cochrane criteria	The utilization of XBJI has a certain effect on the improvement of the inflammatory response and increased level of platelets.
Zhou et al. [24] 2016	China	8 (399)	XBJI + WM	WM	Jadad	The homogeneity of the reduced mortality rate and the available evidence was sufficient to support the use of XBJI as adjunctive therapy for sepsis.
Li et al. [25] 2016	China	11 (803)	XBJI + WM	WM	Cochrane criteria	Clinical evidence showed that the addition of XBJI to the conventional treatment could improve the clinical efficacy in the treatment of sepsis.
Xu et al. [26] 2014	China	18 (1172)	XBJI + WM	WM	Jadad	The combined use of XBJI based on conventional treatment could improve the survival rate of patients with sepsis.
Li et al. [27] 2013	China	13 (1280)	XBJI + WM	WM	Jadad	XBJI had a certain effect in improving the inflammatory response and coagulation function in patients with sepsis. These effects reduced mortality and improved the APACHE II scores.
Sun et al. [28] 2012	China	18 (1080)	XBJI + WM	WM	Jadad	The existing results showed that the application of XBJI in the treatment of sepsis could significantly reduce the white blood cell count in the plasma of patients.
Hu et al, [29] 2010	China	25 (1970)	XBJI + WM	WM	Cochrane criteria	The evidence available showed that XBJI might decrease mortality, ineffectiveness, incidence of complication, and average hospital stay. It could also reduce the APACHE II score in patients with sepsis.
Wu et al. [30] 2020	China	14 (938)	XBJI + WM	WM	Cochrane criteria	XBJI can improve the clinical symptoms, significantly reduce the mortality, and has a high clinical application value.

adjunctive medicine for clinical application [17]. XBJI has been widely used for sepsis in clinical practices in China, wherein pharmacological experiments have demonstrated that it may be a promising treatment for sepsis. As the number of SRs/MAs regarding XBJI for sepsis has increased, scientific evidence for evidence-based medicine is still weak. Hence, we conducted this study to collate, appraise, and synthesize the evidence on XBJI systematically.

Table 2: Result of methodological quality.

Reviews	AMSTAR-2																Quality
	I1	I2	I3	I4	I5	I6	I7	I8	I9	I10	I11	I12	I13	I14	I15	I16	
Chen et al. [17] 2018	Y	PY	Y	Y	Y	Y	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	CL
Li et al. [18] 2018	Y	PY	Y	Y	Y	Y	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	CL
Xiao et al. [19] 2018	Y	PY	Y	Y	Y	Y	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	CL
Zheng et al. [20] 2018	Y	PY	Y	PY	Y	Y	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	CL
Xiao et al. [21] 2017	Y	PY	Y	PY	Y	Y	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	CL
Liu et al. [22] 2021	Y	PY	Y	PY	Y	Y	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	CL
Zhang et al. [23] 2021	Y	PY	Y	PY	Y	Y	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	CL
Zhou et al. [24] 2016	Y	PY	Y	PY	Y	Y	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	CL
Li et al. [25] 2016	Y	PY	Y	PY	Y	Y	N	Y	Y	N	Y	Y	Y	Y	Y	N	CL
Xu et al. [26] 2014	Y	PY	Y	PY	Y	Y	N	Y	Y	N	Y	Y	Y	Y	Y	N	CL
Li et al. [27] 2013	Y	PY	Y	PY	Y	Y	N	Y	Y	N	Y	Y	Y	Y	Y	N	CL
Sun et al. [28] 2012	Y	PY	Y	PY	Y	Y	N	Y	Y	N	Y	Y	Y	Y	Y	N	CL
Hu et al. [29] 2010	Y	PY	Y	PY	Y	Y	N	Y	Y	N	Y	Y	Y	Y	Y	N	CL
Wu et al. [30] 2020	Y	PY	Y	PY	Y	Y	N	Y	Y	N	Y	Y	Y	Y	Y	Y	CL

Table 3: Result of reporting quality.

Items	Chen, 2018	Li, 2018	Xiao, 2018	Zheng, 2018	Xiao, 2017	Liu, 2021	Zhang, 2021	Zhou, 2016	Li, 2016	Xu, 2014	Li, 2014	Sun, 2012	Hu, 2010	Wu, 2020	Compliance (%)
# 1	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100
# 2	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100
# 3	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100
# 4	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100
# 5	N	N	N	N	N	N	N	N	N	N	N	N	N	N	0
# 6	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100
# 7	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100
# 8	Y	Y	Y	PY	PY	PY	PY	PY	PY	PY	PY	PY	PY	PY	21.4
# 9	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100
# 10	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100
# 11	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100
# 12	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100
# 13	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100
# 14	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100
# 15	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100
# 16	Y	Y	Y	Y	Y	N	Y	Y	N	Y	Y	N	Y	Y	78.6
# 17	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100
# 18	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100
# 19	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100
# 20	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100
# 21	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100
# 22	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100
# 23	Y	Y	Y	Y	Y	N	N	Y	N	Y	Y	N	N	N	57.1
# 24	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100
# 25	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100
# 26	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100
# 27	Y	Y	Y	Y	Y	Y	Y	Y	N	N	N	N	N	N	57.1

This overview summarized the scientific evidence on the effectiveness and safety of XBJI for sepsis by evaluating the methodological quality, reporting quality, and evidence quality of SRs/MAs. The current evidence indicated that subjects treated using the combination of XBJI and WM showed a significant reduction in the 28-day mortality, APACHE II score, duration of mechanical ventilation, length of ICU stays, body temperature, serum levels of PCT, and white blood cell count as compared to those treated with WM alone. However, this conclusion must be considered with caution, given the limitations of the study. According to the results of AMSTAR-2, all reviews failed to meet the key item of I2 (established protocol) and I7 (provided the list of excluded trials), which may contribute to the possibility of risk of bias and undermine the reliability of the conclusions. Then, according to the results of PRISMA, I5 (protocol and registration protocol and registration), I8 (search), I16 (additional analyses), I23 (additional analyses), and I27 (funding) were not reported adequately. This reasoning may increase the risk of bias and affect the rigor of SRs/MAs.

Table 4: Results of evidence quality.

Review	Outcomes	Certainty assessment						Relative effect (95% CI)	Quality
		Design	Limitations	Inconsistency	Indirectness	Imprecision	Publication bias		
Chen et al. [17] 2018	28 days mortality	Rct	No	No	No	No	No	RR 0.54 (0.39, 0.73)	⊕⊕⊕⊕⊕ high
	Duration of mechanical ventilation	Rct	No	No	No	No	No	SMD −1.13 (−1.30, −0.95)	⊕⊕⊕⊕⊕ high
	Length of ICU stay	Rct	No	No	No	No	No	SMD −0.84 (−1.00, −0.67)	⊕⊕⊕⊕⊕ high
	APACHE II score	Rct	No	Serious	No	No	No	SMD −1.09 (−1.49, −0.69)	⊕⊕⊕⊕○ moderate
	Serum levels of PCT	Rct	Serious	Serious	No	No	No	SMD −1.61 (−2.23, −0.98)	⊕⊕⊕○○ low
Li et al. [18] 2018	28 days mortality	Rct	Serious	No	No	No	No	RR 0.62 (0.51, 0.76)	⊕⊕⊕⊕○ moderate
	APACHE II score	Rct	Serious	Serious	No	No	No	MD = −3.51 (−4.49, −2.54)	⊕⊕⊕○○ low
	White blood count	Rct	Serious	Serious	No	Serious	Serious	MD = −8.00 (−10.18, −5.82)	⊕○○○○ very low
	Body temperature changes	Rct	Serious	No	No	No	No	MD = −0.43 (−0.55, −0.31)	⊕⊕⊕⊕○ moderate
Xiao et al. [19] 2018	Duration of mechanical ventilation	Rct	Serious	No	No	No	No	SMD −0.90 (−1.07, −0.72)	⊕⊕⊕⊕○ moderate
	Length of ICU stay	Rct	Serious	No	No	No	No	SMD −0.89 (−1.04, −0.73)	⊕⊕⊕⊕○ moderate
	28 days survival rate	Rct	Serious	No	No	No	No	RR 1.20 (1.08, 1.34)	⊕⊕⊕⊕○ moderate
	Serum levels of PCT	Rct	Serious	Serious	No	No	No	SMD −0.57 (−0.77, −0.38)	⊕⊕⊕○○ low
	APACHE II score	Rct	Serious	Serious	No	No	No	SMD −1.16 (−1.57, −0.75)	⊕⊕⊕○○ low
Zheng et al. [20] 2018	28 days mortality	Rct	Serious	No	No	No	No	RR 0.64 (0.43, 0.96)	⊕⊕⊕⊕○ moderate
	APACHE II score	Rct	Serious	Serious	No	No	No	SMD −1.21 (−1.62, −0.80)	⊕⊕⊕○○ low
	Duration of mechanical ventilation	Rct	Serious	Serious	No	No	No	SMD −1.04 (−1.40, −0.67)	⊕⊕⊕○○ low
	Length of ICU stay	Rct	Serious	No	No	No	No	SMD −0.83 (−1.03, −0.64)	⊕⊕⊕⊕○ moderate
Xiao et al. [21] 2017	28 days mortality	Rct	Serious	No	No	No	Serious	RR 0.51 (0.44, 0.59)	⊕⊕⊕○○ low
	APACHE II score	Rct	Serious	Serious	No	No	No	WMD −3.70 (−4.31, −3.09)	⊕⊕⊕○○ low
	Serum levels of PCT	Rct	Serious	Serious	No	No	No	WMD −1.26 (−1.63, −0.88)	⊕⊕⊕○○ low
	White blood count	Rct	Serious	Serious	No	No	No	WMD −1.48 (−2.03, −0.94)	⊕⊕⊕○○ low
	Body temperature changes	Rct	Serious	Serious	No	No	No	WMD −0.50 (−0.92, −0.07)	⊕⊕⊕○○ low
Liu et al. [22] 2021	28 days mortality	Rct	Serious	No	No	No	No	RR 1.20 (1.15, 1.25)	⊕⊕⊕⊕○ moderate
	White blood count	Rct	Serious	Serious	No	No	No	MD −1.95 (−3.62, −0.28)	⊕⊕⊕○○ low
	Serum levels of PCT	Rct	Serious	Serious	No	No	No	MD −1.29 (−1.97, −0.62)	⊕⊕⊕○○ low

Table 4: Continued.

Review	Outcomes	Certainty assessment						Relative effect (95% CI)	Quality
		Design	Limitations	Inconsistency	Indirectness	Imprecision	Publication bias		
Zhang et al. [23] 2021	28 days mortality	Rct	Serious	No	No	No	No	OR 0.52 (0.38, 0.71)	⊕⊕⊕⊕○ moderate
	APACHE II score	Rct	Serious	No	No	No	No	WMD −2.65 (−3.23, −2.08)	⊕⊕⊕⊕○ moderate
Zhou et al. [24] 2016	28 days mortality	Rct	Serious	No	No	Serious	No	RR 0.61 (0.41, 0.90)	⊕⊕⊕○○ low
Li et al. [25] 2016	Effective rate	Rct	Serious	No	No	No	No	OR 2.90 (1.89, 4.47)	⊕⊕⊕⊕○ moderate
	APACHE II score	Rct	Serious	No	No	No	No	MD −4.01 (−4.88, −3.13)	⊕⊕⊕⊕○ moderate
	White blood count	Rct	Serious	No	No	Serious	Serious	MD −4.31 (−6.73, −1.89)	⊕○○○○ very low
	Serum levels of PCT	Rct	Serious	No	No	Serious	Serious	MD −1.42 (−1.90, −0.95)	⊕○○○○ very low
Xu et al. [26] 2014	28 days survival rate	Rct	Serious	No	No	No	No	RR 1.21 (1.12, 1.29)	⊕⊕⊕⊕○ moderate
Li et al. [27] 2014	28 days mortality	Rct	Serious	No	No	No	No	OR 0.39 (0.27, 0.58)	⊕⊕⊕⊕○ moderate
	APACHE II score	Rct	Serious	Serious	No	No	No	WMD −3.43 (−4.72, −2.15)	⊕⊕⊕○○ low
	White blood count	Rct	Serious	No	No	Serious	Serious	WMD −2.94 (−3.49, −2.38	⊕○○○○ very low
Sun et al. [28] 2012	White blood count	Rct	Serious	Serious	No	No	No	WMD −1.87 (−2.92, −0.81)	⊕⊕⊕○○ low
Hu et al. [29] 2010	28 days mortality	Rct	Serious	Serious	No	No	No	RR 0.65 (0.54, 0.79)	⊕⊕⊕○○ low
Wu et al. [30] 2020	28 days mortality	Rct	Serious	No	No	No	No	RR 0.52 (0.40, 0.67)	⊕⊕⊕⊕○ moderate
	APACHE II score	Rct	Serious	Serious	No	No	No	MD −5.48 (−7.52, −3.43)	⊕⊕⊕○○ low
	White blood count	Rct	Serious	Serious	No	No	No	MD −2.26 (−3.35, −1.17)	⊕⊕⊕○○ low
	C-reactive protein	Rct	Serious	Serious	No	No	No	MD −37.43 (−56.70, −18.16)	⊕⊕⊕○○ low

ICU, intensive care unit; APACHE, acute physiology and chronic health evaluation; PCT, procalcitonin. RCT, randomized controlled trials; WMD, weighted mean difference; SMD, standard mean difference; MD, mean difference; OR, odds ratio; RR, relative risk.

Based on the results of GRADE, only three outcome indicators provided high-quality evidence, 16 provided moderate-quality evidence, and the remaining 24 provided low or critical low-quality evidence. These results indicate that the conclusions of the reviews may differ from the true results and therefore cannot be used as an evidence-based basis. Furthermore, it is worth noting that almost all the included SRs/MAs indicated that XBJI plus WM seems to have significant clinical efficacy in the therapy of patients with sepsis. However, most authors did not wish to draw definitive conclusions due to low methodological quality or the small size of the enrolled studies.

The pathogenesis of sepsis includes inflammation, immune dysregulation, and coagulopathy, with uncontrolled inflammation being the most critical for patients [31]. According to traditional Chinese medicine, the basic pathogenesis of sepsis involves the accumulation of toxins in the interior and extremities, leading to siltation, stagnation, and weakened body resistance [10]. XBJI was then created according to this theory as a possible treatment for sepsis [32]. XBJI was composed of five herbs containing approximately 30 bioactive compounds, including hydroxysafflor yellow A, danshenol, ferulic acid, paeoniflorin, and senna lactone I, among others [17]. Therefore, XBJI has the effects of "multiingredient, multitarget, and multipathway," including detoxifying and toning, elimination of bacteria and viruses, supplementing vital energy, and invigorating blood circulation [19]. Modern pharmacological studies have uncovered the potential therapeutic mechanisms of XBJI for sepsis. It was reported that XBJI could regulate the immune status of the body by inhibiting the release of inflammatory mediators, reducing the total accumulation of endotoxins,

bacterial toxin detoxification, and reducing the total amount of oxygen free radicals in the circulatory system. These effects help regulate the overall microcirculatory status of the body, protect and restore vascular endothelial function, and increase the total blood perfusion of the organs [19]. Moreover, XBJI also reduces the release of mast cells, which reduces the synthetic activity of fibroblasts. These effects lead to the avoidance of inflammatory exudation and increase the permeability of blood vessels [11]. Furthermore, XBJI helps the body absorb necrotic material and hematoma, promoting rehabilitation [33]. Thus, the use of XBJI is considered a promising approach for the treatment of sepsis.

To our knowledge, this is the first study to systematically collate, appraise, and synthesize the scientific evidence on XBJI for sepsis. However, we found that most of the included reviews were of poor quality, which could result in these studies having low credibility. Furthermore, the AMSTAR-2 tool, PRISMA checklist, and the GRADE system are highly subjective as different reviewers have their independent judgment. The subjectivity of the authors may then lead to varying results as subjective factors or errors cannot be eliminated.

5. Conclusion

The combination of XBJI and WM showed significant synergy for the treatment of sepsis compared to the use of WM alone. It provided a new and prospective therapeutic method for sepsis. However, this conclusion should be treated with caution as the quality of SRs/MAs providing evidence was generally low.

Abbreviations

XBJI:	Xuebijing injection
WM:	Western medicines
SR:	Systematic review
MA:	Meta-analysis
AMSTAR-2:	Assessing the Methodological Quality of Systematic Reviews-2
GRADE:	Grading of Recommendations, Assessment, Development, and Evaluation
PRISMA:	Preferred Reporting Item for Systematic Reviews and Meta-Analyses
ICU:	Intensive care unit
APACHE:	Acute physiology and chronic health evaluation
PCT:	Procalcitonin
RCT:	Randomized controlled trials
WMD:	Weighted mean difference
SMD:	Standard mean difference
MD:	Mean difference
OR:	Odds ratio
RR:	Relative risk.

Data Availability

The data generated or analyzed during this study are included within this article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Sa Tian designed and drafted the study. Yixuan Ye, Defang Qin, Huawei Yang, and Shuguang Chen performed the literature search, literature selection, and data extraction. Tao Liu, Luming Hu, Huiming Li, and Qin Niu performed the quality assessment. All authors approved the final version of the manuscript.

References

[1] M. Singer, C. S. Deutschman, C. W. Seymour et al., "The third international consensus definitions for sepsis and septic shock (Sepsis-3)," *Journal of the American Medical Association*, vol. 315, no. 8, pp. 801–810, 2016.

[2] K. M. Kaukonen, M. Bailey, S. Suzuki, D. Pilcher, and R. Bellomo, "Mortality related to severe sepsis and septic shock among critically ill patients in Australia and New Zealand, 2000–2012," *JAMA*, vol. 311, no. 13, pp. 1308–1316, 2014.

[3] V. Liu, G. J. Escobar, J. D. Greene et al., "Hospital deaths in patients with sepsis from 2 independent cohorts," *Journal of the American Medical Association*, vol. 312, no. 1, pp. 90–92, 2014.

[4] C. Fleischmann, A. Scherag, N. K. J. Adhikari et al., "Assessment of global incidence and mortality of hospital-treated sepsis. Current estimates and limitations," *American Journal of Respiratory and Critical Care Medicine*, vol. 193, no. 3, pp. 259–272, 2016.

[5] V. Y. Dombrovskiy, A. A. Martin, J. Sunderram, and H. L. Paz, "Rapid increase in hospitalization and mortality rates for severe sepsis in the United States: a trend analysis from 1993 to 2003*," *Critical Care Medicine*, vol. 35, no. 5, pp. 1244–1250, 2007.

[6] R. P. Dellinger, M. Levy Mitchell, R. Andrew et al., "Surviving Sepsis Campaign: international guidelines for management of severe sepsis and septic shock, 2012," *Intensive Care Medicine*, vol. 39, no. 2, pp. 165–228, 2013.

[7] X. Y. Sun, X. F. Ding, H. Y. Liang et al., "Efficacy of mesenchymal stem cell therapy for sepsis: a meta-analysis of preclinical studies," *Stem Cell Research & Therapy*, vol. 11, no. 1, p. 214, 2020.

[8] C. Li, P. Wang, M. Li et al., "The current evidence for the treatment of sepsis with Xuebijing injection: bioactive constituents, findings of clinical studies and potential mechanisms," *Journal of Ethnopharmacology*, vol. 265, p. 113301, 2021.

[9] S. T. Ma, P. X. Liu, W. Long, J. Yu, and Y. Xu, "Effects of the multi-target capability of Xuebijing and its inflammatory pharmacodynamic material basis," *Acta Physico-Chimica Sinica*, vol. 15, no. 10, pp. 2080–2086, 2009.

[10] Q. Yin and C. S. Li, "Treatment effects of Xuebijing injection in severe septic patients with disseminated intravascular coagulation," *Evidence Based Complementary and Alternative Medicine*, vol. 2014, Article ID 949254, 2014.

[11] Y. Y. Feng, Y. Y. Xie, Y. P. Wang et al., "Molecular mechanism of Xuebijing injection in treatment of sepsis according to "drug-target-pathway" network," *Acta Physico-Chimica Sinica*, vol. 52, no. 4, pp. 556–562, 2017.

[12] J. K. Huang, X. H. Qin, M. Shen, and Y. Huang, "An overview of systematic reviews and meta-analyses on acupuncture for post-stroke aphasia," *European Journal of Integrative Medicine*, vol. 37, 2020.

[13] J. Huang, Y. Wang, S. Huang et al., "A critical overview of systematic reviews of shenfu injection for heart failure," *Cardiovascular Therapeutics*, vol. 2021, no. 16, 9 pages, 2021.

[14] B. J. Shea, B. C. Reeves, G. Wells et al., "AMSTAR 2: a critical appraisal tool for systematic reviews that include randomized or non-randomised studies of healthcare interventions, or both," *BMJ*, vol. 358, no. j4008, p. 1, 2017.

[15] A. Liberati, D. G. Altman, J. Tetzlaff et al., "The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate healthcare interventions: explanation and elaboration," *BMJ*, vol. 339, no. jul21 1, p. b2700, 2009.

[16] G. H. Guyatt, A. D. Oxman, G. E. Vist et al., "GRADE: an emerging consensus on rating quality of evidence and strength of recommendations," *BMJ*, vol. 336, no. 7650, pp. 924–926, 2008.

[17] G. Chen, Y. Gao, Y. Jiang et al., "Efficacy and safety of Xuebijing injection combined with ulinastatin as adjunctive therapy on sepsis: a systematic review and meta-analysis," *Frontiers in Pharmacology*, vol. 9, p. 743, 2018.

[18] C. Li, P. Wang, L. Zhang et al., "Efficacy and safety of Xuebijing injection (a Chinese patent) for sepsis: a meta-analysis of randomized controlled trials," *Journal of Ethnopharmacology*, vol. 224, pp. 512–521, 2018.

[19] S.-H. Xiao, L. Luo, X.-H. Liu, Y.-M. Zhou, H.-M. Liu, and Z.-F. Huang, "Curative efficacy and safety of traditional Chinese medicine Xuebijing injections combined with ulinastatin for treating sepsis in the Chinese population," *Medicine (Baltimore)*, vol. 97, no. 23, p. e10971, 2018.

[20] J. Zheng, X. Xiang, B. Xiao et al., "Xuebijing combined with ulinastation benefits patients with sepsis: a meta-analysis," *The American Journal of Emergency Medicine*, vol. 36, no. 3, pp. 480–487, 2018.

[21] H. Shi, Y. Hong, J. Qian, X. Cai, and S. Chen, "Xuebijing in the treatment of patients with sepsis," *The American Journal of Emergency Medicine*, vol. 35, no. 2, pp. 285–291, 2017.

[22] J. T. Liu, X. D. Cao, and H. F. Li, "Meta-analysis of the effectiveness of Xuebijing injection in the treatment of sepsis," *Journal of Shanxi University of Chinese Medicine*, vol. 22, no. 1, pp. 12–18, 2021.

[23] J. W. Zhang, Y. L. He, Y. Shi, and M. Li, "Meta-analysis of the efficacy and safety of Xuebijing injection in the treatment of vascular endothelial injury in sepsis," *Journal of Hainan Medical University*, vol. 27, no. 12, pp. 910–917, 2020.

[24] X. S. Zhou, G. H. Tang, J. Y. Li, and Y. Ye, "Xuebijing injection reducing mortality of septic shock patients: a systematic review and meta-analysis," *Chinese Archives of Traditional Chinese Medicine*, vol. 34, no. 9, pp. 2161–2164, 2016.

[25] G. Li, Y. Xiao, J. Zhu, X. Ma, and W. Gao, "Systematic review of Xuebijing injection in treatment of septic shock," *Journal of Emergency in Traditional Chinese Medicine*, vol. 25, no. 5, pp. 834–838, 2016.

[26] Y. Xu, Y. Geng, H. Tong, H. Li, P. Wan, and L. Su, "Meta-analysis of Xuebijing injection in improving survival rate of patients with sepsis," *Clinical Journal of Traditional Chinese Medicine*, vol. 26, no. 5, pp. 456–460, 2014.

[27] N. Li, W. Jiang, L. Yu, and W. Zhang, "Systematic review of Xuebijing injection in treatment of sepsis," *Chinese Journal of Modern Drug Application*, vol. 7, no. 22, pp. 8–11, 2013.

[28] C. L. Sun, Y. Zhuang, and X. Wang, "Meta-analysis of Xuebijing injection in the treatment of sepsis," *Journal of Emergency in Traditional Chinese Medicine*, vol. 21, no. 3, pp. 411–413, 2012.

[29] J. Hu, H. Shang, J. Li, J. Li, Z. Zhang, and B. Zhang, "Injection for sepsis: a comprehensive review," *Medical Journal of Chinese People's Liberation Army*, vol. 35, no. 1, pp. 9–12, 2010.

[30] Y. J. Wu, J. Zhang, and L. J. Qi, "Clinical efficacy and safety of Xuebijing injection on sepsis: a meta-analysis," *Chinese Critical Care Medicine*, vol. 32, no. 6, pp. 691–695, 2020.

[31] K. Kubra, H. Beatrice, E. C. Steve et al., "Human metabolic response to systemic inflammation: assessment of the concordance between experimental endotoxemia and clinical cases of sepsis/SIRS," *Critical Care*, vol. 19, p. 71, 2015.

[32] J. D. Wang, "Diagnosis and treatment ideas and practice course of integrative treatment in acute critical illness," *Tianjin Journal of Traditional Chinese Medicine*, vol. 15, pp. 241-242, 1998.

[33] W. Zhang, Y. N. Zhu, and Y. Wang, "Application of Xuebijing injection in clinical application of infectious diseases," *Lishizhen Medicine and Materia Medica Research*, vol. 25, no. 5, pp. 1189–1191, 2014.

Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2021, Article ID 6350035, 15 pages
https://doi.org/10.1155/2021/6350035

Review Article

Phytochemistry and Pharmacology of *Thymus broussonetii* Boiss

Hanae Naceiri Mrabti,[1] Latifa Doudach,[2] Naoual El Menyiy,[3] Mohammed Bourhia,[4] Ahmad Mohammad Salamatullah,[5] Mohamed Reda Kachmar,[6] Omar Belmehdi,[7] Hamza El Moudden,[8] Nidal Naceiri Mrabti,[9] Hicham Harhar,[8] Nasreddine El Omari,[10] and Abdelhakim Bouyahya[11]

[1]*Laboratory of Pharmacology and Toxicology, Bio Pharmaceutical and Toxicological Analysis Research Team, Faculty of Medicine and Pharmacy, Mohammed V University in Rabat, BP 6203, Rabat, Morocco*
[2]*Biomedical Engineering Department, National School of Arts and Crafts Rabat (ENSAM), Mohammed V University in Rabat, BP 6203, Rabat, Morocco*
[3]*Faculty of Sciences Dhar El Mahraz, Sidi Mohamed Ben Abdellah University, Fez, Morocco*
[4]*Laboratory of Chemistry-Biochemistry, Environment, Nutrition and Health, Faculty of Medicine and Pharmacy, Hassan II University, Casablanca 21100, Morocco*
[5]*Department of Food Science & Nutrition, College of Food and Agricultural Sciences, King Saud University, P.O. Box 2460, Riyadh 11451, Saudi Arabia*
[6]*Faculty of Sciences, Health and Environment Laboratory, Plant Protection Team, Moulay Ismail University, Meknes, BP 11201, Zitoun, Morocco*
[7]*Biology and Health Laboratory, Department of Biology, Faculty of Science, Abdelmalek-Essaadi University, Tetouan, Morocco*
[8]*Laboratory of Materials, Nanotechnology and Environment, Department of Chemistry, Faculty of Sciences, Mohammed V University in Rabat, Rabat, Morocco*
[9]*Computer Chemistry and Modeling Team, Laboratory of Materieals, Modeling and Environmental Engineering, LIMME, Faculty of Sciences Dhar El Mehraz, Sidi Mohammed Ben Abdellah University, Fes, Morocco*
[10]*Laboratory of Histology, Embryology, and Cytogenetic, Faculty of Medicine and Pharmacy, Mohammed V University, Rabat, Morocco*
[11]*Laboratory of Human Pathologies Biology, Department of Biology, Faculty of Sciences, and Genomic Center of Human Pathologies, Faculty of Medicine and Pharmacy, Mohammed V University, Rabat, Morocco*

Correspondence should be addressed to Abdelhakim Bouyahya; boyahyaa-90@hotmail.fr

Received 4 June 2021; Revised 15 August 2021; Accepted 26 August 2021; Published 6 September 2021

Academic Editor: Dejan Stojkovic

Thymus broussonetii Boiss (*T. broussonetii*) is a rare medicinal and aromatic plant. It is widely used in traditional medicine to treat several diseases, including diarrhea, fever, cough, irritation, skin diseases, rheumatism, respiratory ailments, influenza, and digestion problems. In this review, we have critically summarized previous data on *T. broussonetii* about its phytochemistry, botanical and geographical distribution, toxicological investigation, and pharmacological properties. Using scientific research databases such as Wiley Online, SciFinder, ScienceDirect, PubMed, SpringerLink, Web of Science, Scopus Wiley Online, and Google Scholar, the data on *T. broussonetii* were collected and discussed. The presented data regrouped bioactive compounds and biological activities of *T. broussonetii*. The findings of this work showed that essential oils and extracts of *T. broussonetii* exhibited numerous pharmacological activities (*in vitro* and *in vivo*), particularly antibacterial, antifungal, antioxidant, anticancer, anti-inflammatory, insecticidal, antipyretic, antinociceptive, and immunological and behavioral effects. While toxicological studies of *T. broussonetii* essential oils and extracts are lacking, modern scientific tools revealed the presence of different classes of secondary metabolites such as terpenoids, alkaloids, flavonoids, tannins, coumarins, quinones, carotenoids, and steroids. *T. broussonetii* essential oils, especially from the aerial parts, exhibited potent antibacterial, antifungal, and antioxidant effects. An in-depth

toxicological investigation is needed to validate the efficacy and safety of *T. broussonetii* extracts and essential oils and their secondary metabolites. However, further pharmacokinetic and pharmacodynamic studies should be performed to validate its bioavailability.

1. Introduction

Thymus broussonetii Boiss (*Thymus broussonetii*) belongs to the Lamiaceae family and the genus of *Thymus*. It is a small shrub of 40 cm in height and is endemic to Morocco, Algeria, and Tunisia [1]. It is known locally in Morocco as "Zaitra," "Tazouknnit," or "Azukni" [2, 3]. *T. broussonetii* is distributed on the Atlantic coast between 20 and 400 m altitude and is mainly located in arid and semiarid bioclimatic zones [4].

It is among the plants most used in Moroccan folk medicine against various illnesses such as urinary, nervous, genital, circulatory, skin, digestive, and respiratory diseases [2, 3]. It is also used to treat diabetes [3, 5, 6], cold, cough, fever, digestive disorders, and dolorous processes [7]. Other researchers have reported the use of this plant in food as a seasoning of traditional recipes (seasoning) and to flavor tea or milk [8]. Ethnobotanical surveys are the first step to identify the plant uses for each disorder. It provides information on the part used, the method of preparation, etc. However, the lack of plant information given by researchers in many surveys was repeatedly noticed. This is the case of several researchers who reported the use of *T. broussonetii* in folk medicine without mentioning the part used, the method of preparation, or/and the traditional use [9, 10].

Several classes of bioactive compounds, including flavonoids, alkaloids, terpenoids, tannins, coumarins, quinones, steroids, and carotenoids, have been identified in essential oils (EOs) and extracts of *T. broussonetii*, which explains its biological activities [11–25].

Using *in vitro* and *in vivo* pharmacological approaches, researchers reported the potential activity of *T. broussonetii* extracts and EOs. Essential oils from the aerial parts of *T. broussonetii* showed antibacterial effects against different pathogenic bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella* sp., *Bacillus* sp., *Micrococcus luteus*, etc. Moreover, the antifungal effects of *T. broussonetii* EOs against numerous pathogenic fungi, including *Candida* sp., *Aspergillus brasiliensis*, and *Saccharomyces cerevisiae* were reported by Jamali et al. [20] and Smahane et al. [16, 20, 26]. *T. broussonetii* extracts and EOs exhibited antioxidant effects using well-known techniques such as DPPH and FRAP assays [11, 13, 20, 25, 27]. The anticancer properties of *T. broussonetii* EOs have also been reported against various tumor cell lines like P815 mastocytoma, CEM, and K-562 [12, 15, 21]. Moreover, *T. broussonetii* was revealed to exhibit anti-inflammatory activity [28], anticorrosive potential [23], insecticidal [19, 27, 29], antiparasitic [30], antipyretic [22], antinociceptive [31], immunological, and behavioral effects [31]. In addition, the acute toxicological investigations of *T. broussonetii* EOs have shown death cases and some signs of toxicity [22]. However, the mechanism of action by which the bioactive compounds of *T. broussonetii* extracts and EOs exhibited these pharmacological effects is lacking.

Due to the intensification of research on the pharmacological effects of *T. broussonetii* and its compounds in recent years, we have reviewed all studies on this plant; botanical description, geographical distribution, chemical composition, all pharmacological effects, and the prospects of *T. broussonetii*. To the best of our knowledge, this review is the first report providing a scientific database that highlighted several aspects related to *T. broussonetii* and suggested the future potential clinical applications of this plant.

2. Research Methodology *Thymus broussonetii* Boiss

In this work, data conacring botanical description, taxonomy, destruction, phytochemistry, and pharmacological activities of *T. broussonetii* were collected using different databases (Google Scholar, Web of Science, PubMed, Scopus, ScienceDirect, SpringerLink, SciFinder, and Wiley Online). The collected data were organized in several areas and highlighted. The chemical structures of T. *broussonetii* were drawn using ChemDraw Pro 8.0 software.

3. Results and Discussion

3.1. Botanical Description and Geographical Distribution. *Thymus broussonetii* is an evergreen plant that grows to a height of around 5 centimeters. Its flowers clustered toward the top of the stems in a dense ovate-cylindrical inflorescence with floral leaves broader than the leaves, often purple-colored, attenuate-sharp at the tip, ciliated at the margins and concealing the calyces, these 2-lipped, the upper shallowly toothed; pink corolla 2-3 times the length of the calyx, with a distinctly protruding narrow tube. It differs from subsp. hannonis (Maire) Morales by the subpetiolate leaves and bracts hairy only on the inner side [1]. *T. broussonetii* is an endemic plant to Morocco, Tunisia, and Algeria [1]. In Morocco, it is found in the Middle Atlantic, the High Atlas, and in the north of the kingdom [32].

3.2. Chemical Composition. The secondary metabolites produced by *T. broussonetii* were the subject of numerous studies, almost all of which have been carried out on the aerial parts of this plant. The phytochemical screening of *T. broussonetii* extracts and EOs revealed its richness in phenolic compounds, in particular terpenoids, flavonoids, and phenolic acids. Analysis of *T. broussonetii* EOs by gas chromatography (GC) identified more than sixty terpenoids (Table 1; Figure 1).

The essential oil of *T. broussonetii* is mainly composed of spathulenol, eucalyptol, 1,8-cineole, β-caryophyllene, terpinolene, camphene, limonene, myrcene, sabinene, terpineol, terpinene, *p*-cymene, o-cymene, α-thujene, α-pinene,

Table 1: Chemical composition of *T. broussonetii*.

Parts	Extracts/EOs	Compounds groups	Compounds	References
Leaves	Essential oil	Terpenoids	Borneol, *p*-cymene, carvacrol, camphene, α -terpinene, α-Pinene, trans-sabinene hydrate, caryophyllene oxide, (E)-β-caryophyllene, Bornyl acetate, carvacrol methyl ether, camphor, Linalool, cis-sabinene hydrate, 4-terpineol, p-cymen-8-ol, Thymol, trans-verbenol, 1-octen-3ol, 1,8-cineol, β-pinene	[11]
Flowers and leaves	Essential oil	Terpenoids	(E)-β-caryophyllene, γ-terpinene, *p*-cymene, carvacrol, thymol, 4-terpineol, β-pinene, terpendiol, borneol, caryophyllene oxide, geraniol formate, p-menth-1,4(8)-diene, linalyl propionate, β-cadrene, thujol, cinerone, 4-isopropy-lM-2-cyclohexane-1-ol, 1-octen-3-ol	[12]
Aerial parts	Essential oil	Terpenoids	α-pinene, *p*-cymene, carvacrol, viridiflorene, borneol, γ-terpinene, myrcene, camphene, α–thujene, aromadendrene, caryophyllene oxide, α–terpinene, β–pinene, thymol, germacrene *D*, δ-cadinene, linalool	[13]
Aerial parts	Essential oil	Terpenoids	Camphor, α–terpineol, eucalyptol, germacrene *D*, borneol, terpinen-4-ol, bicyclogermacrene, β–caryophyllene, β-bourbonene, spathulenol, δ–terpineol, bornyl acetate, caryophyllene oxide, T-muurolol, γ–cadinene, thymol, trans-sabinene hydrate, linalool, cis-sabinne-hydrate, limonene, *p*-cymene, dihydrocarvone, trans-carveol, δ–cadinene, alloaromadendrene, carvacrol	[14]
Leaves	Essential oil	Terpenoids	Carvacrol, p-cymene,γ -terpinene, thymol, β--pinene, 4-terpineol, borneol, linalyl propionate, p-menth-1,4(8)-diene, geraniol formate, cinerone, carvacrol methyl ether, 4-isopropyl-1M-2cyclohexane-1-ol,1-octen-3-ol	[15]
	Essential oil	Terpenoids	Carvacrol, p-cymene, α-pinene, α-terpinene, 3-octanol, myrcene, α-terpineol, borneol, linalyl acetate, linalool, β--pinene, methyl carvacrol, p-cymen-8-ol, p-mentha-1,4(8)-diene, limonene, camphene, γ-terpinene, thymol	[16]
Aerial parts	Essential oil	Terpenoids	Carvacrol, thymol, γ-terpinene, borneol, *p*-cymene, α-pinene, camphene, myrcene, α-terpinene, α-thujene, limonene, β-pinene, linalool	[17]
Aerial parts	Essential oil	Terpenoids	Thymol, carvacrol, borneol, viridiflorene, spathulenol, aromadendrene, camphene, α-terpineol, O-cymene, terpinene-4-ol, γ-terpinene, alloaromadendrene, γ-cadinene, α-pinene, *cis*-dihydrocarvone, trans-sabinene hydrate, α-amorphene, β-patchoulene, β-cubebene, isospathulenol	[18]
Leaves	Essential oil	Terpenoids	*p*-cymene, borneol, α-pinene, thymol, camphene, γ-terpinene, carvacrol, Ledene, Limonene, Myrcene, Aromadendrene, β-pinene, α-thujene,α-terpinene, terpinen-4-ol, dihydrocarvone, allo-Aromadendrene, β-caryophyllene, *cis*-sabinene hydrate, tricyclene, sabinene, α-phellandrene, *p*-Mentha-1,4(8)-diene, linalool, γ-muurolene, spathulenol	[19]
Aerial parts	Essential oil	Terpenoids	Carvacrol, thymol, borneol, γ-terpinene, *p*-cymene, camphene, α-pinene, myrcene, viridiflorene, α-terpinene, α-thujene, aromadendrene, β-pinene, limonene, caryophyllene oxide, tricyclene, δ-cadinene, alloaromadendrene, germacrene *D*, linalool, and limonene	[20]
Aerial parts	Essential oil	Terpenoids	Borneol, thymol, *p*-cymene, γ-terpinene, carvacrol, 4-terpineol, linalyl propionate, camphor, δ-3-carene, camphene, β-pinene, geraniol formate, p-menth-1,4(8)-diene, p-mentha-1,8-diene, 4-isopropyl-1M-2 cyclohexane-1-ol, terpinene-1-ol, carvenone, bornyl acetate, cinerone, alloaromadendrene, (E)-β-caryophyllene, α-muurolene, β-cedrene, α-cadinene, caryophyllene oxide, germacrene D	[21]
	Essential oil	Terpenoids	Thymol, borneol, carvacrol, *p*-cymene, δ- terpinene, camphene, spathulenol, myrcene, α-terpineol, aromadendrene, limonene, β--pinene, α-terpinene	[22]
Aerial parts	Essential oil	Terpenoids	Carvacrol, o-cymene, γ.-terpinene, α-pinene, thymol, (+)-4-carene, 4-terpineol, α-thujene, tau-cadinol, spathulenol, limonene, β-caryophyllene, and camphene	[23]
Aerial parts	Essential oil	Terpenoids	Thymol, α-pinene, β-caryophyllene, carvacrol, γ-terpinene, borneol	[24]

Table 1: Continued.

Parts	Extracts/EOs	Compounds groups	Compounds	References
Leaves	Methanolic extract	Alkaloids	−	[25]
	Alcohol extract	Flavonoids	+	
	Aqueous extract	Tannins	+	
	Ethanolic extract	Coumarins	+	
	Methanolic extract	Terpenoids	+	
	Petroleum extract	Quinones	+	
		Steroids	+	
	Aqueous extract	Carotenoids	+	
Stems	Methanolic extract	Alkaloids	−	
	Alcohol extract	Flavonoids	+	
	Aqueous extract	Tannins	+	
	Ethanolic extract	Coumarins	+	
	Methanolic extract	Terpenoids	+	
	Petroleum extract	Quinones	+	
		Steroids	+	
	Aqueous extract	Carotenoids	+	

camphor, bornyl acetate, borneol, thymol, linalool, and carvacrol [11–24].

Chemical variability was observed in the composition of *T. broussonetii* extracted by different methods. Zerrifi et al. [17] have found that *T. broussonetii* EOs are rich in oxygenated monoterpenes (64.5%), monoterpene hydrocarbons (29.0%), sesquiterpene hydrocarbons (5.8%), and oxygenated sesquiterpenes (0.4%), while oxygenated sesquiterpenes had the lowest percentage. The carvacrol was the main compound [17].

The same results were found by Jamali et al. [20]. For the *T. broussonetii* essential oil from Essaouira (Morocco), it consisted mainly of oxygenated monoterpenes (64.5%), while the oxygenated sesquiterpenes were poorly represented (0.4%). The main component was carvacrol (43.4%), followed by thymol (12.3%) [20].

Carvacrol (39.51%) as the main constituent was also found by Chebli et al. [23]. The other components were o-cymene (14.80%), γ-terpinene (10.32%), α-pinene (9.7%), thymol (7.9%), and 4-terpineol (3.22%) [23].

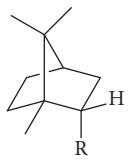
In another study, camphor (46.17%) was found to be the major component followed by α-terpineol (7.69%), eucalyptol (5.76), germacrene *D* (5.21%), and borneol (4.42%) of *T. broussonnetii* essential oil in Tamri region (Western high Atlas), Morocco [14]. In addition, linalool, γ-terpinene, *cis*-sabinene hydrate, β-caryophyllene, *p*-Menth-1,4(8)-diene, caryophyllene oxide, and carvenoneare were the main compounds identified in the essential oil of *T. broussonnetii* aerial parts [21].

In comparison with wild-harvested and cultivated *T. broussonnetii*, chromatographic analysis of their essential oil revealed the presence of 19 compounds, namely α-pinene (5.0%), *p*-cymene (5.2%), borneol (8.5%), γ-terpinene (8.9%), thymol (12.3%), and carvacrol (43.4%) for wild-harvested plants in Morocco, whereas the oil obtained from cultivated plants was characterized by a higher content of α-pinene (6.5%), *p*-cymene (7.2%), and carvacrol (60.8%) [13].

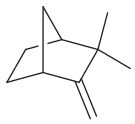
The chemical analysis of polar fraction from *T. broussonnetii* leaf extracts indicated the presence of flavonoids, tannins, coumarins, terpenoids, quinones, steroids, and carotenoids in the various extracts (aqueous extract, alcohol extract, and petroleum extract). Alkaloid compounds were not detected in the methanolic extract of plant leaves. In addition, flavonoids, tannins, coumarins, terpenoids, quinones, steroids, and carotenoids were the main compounds identified in the *T. broussonnetii* stem extracts [25].

3.3. Pharmacological Properties

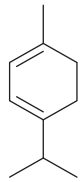
3.3.1. Antibacterial Activity. Several studies have shown the antibacterial effectiveness of different essential oils from the aerial part of *Thymus broussonetii* [28, 39, 40, 29, 41]. Table 2



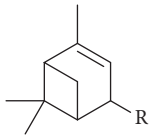
R = OH : Borneol
R = O-CO-CH3: Bornyl acetate



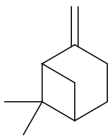
Camphene



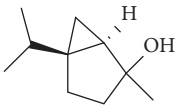
α-terpinene



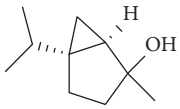
R = H : α-pinene
R = OH : Trans-verbenol



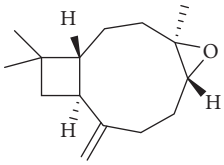
β-pinene



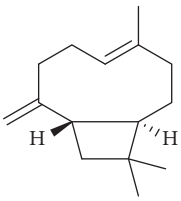
Trans-sabinene hydrate



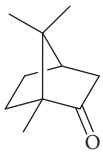
Cis-sabinene hydrate



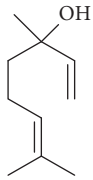
Caryophyllene oxide



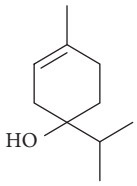
(E)-β-caryophyllene



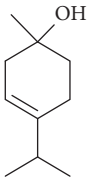
Camphor



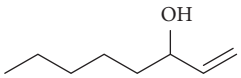
Linalool



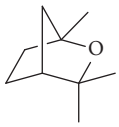
4-Terpineol



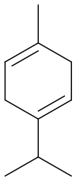
Terpinene-1-ol



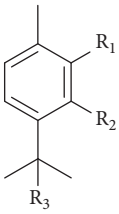
1-octen-3-ol



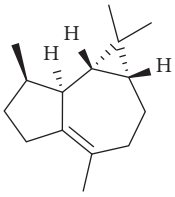
1,8-cineol



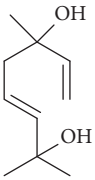
γ-terpinene



R_1 = H, R_2 = H, R_3 = H : p-cymene
R_1 = H, R_2 = OH, R_3 = H : Thymol
R_1 = OH, R_2 = H, R_3 = H : Carvacrol
R_1 = OH, R_2 = CH_3, R_3 = H : Methyl Carvacrol
R_1 = OCH_3, R_2 = H, R_3 = H : Carvacrol methyl ether
R_1 = H, R_2 = H, R_3 = OH : p-cymen-8-ol



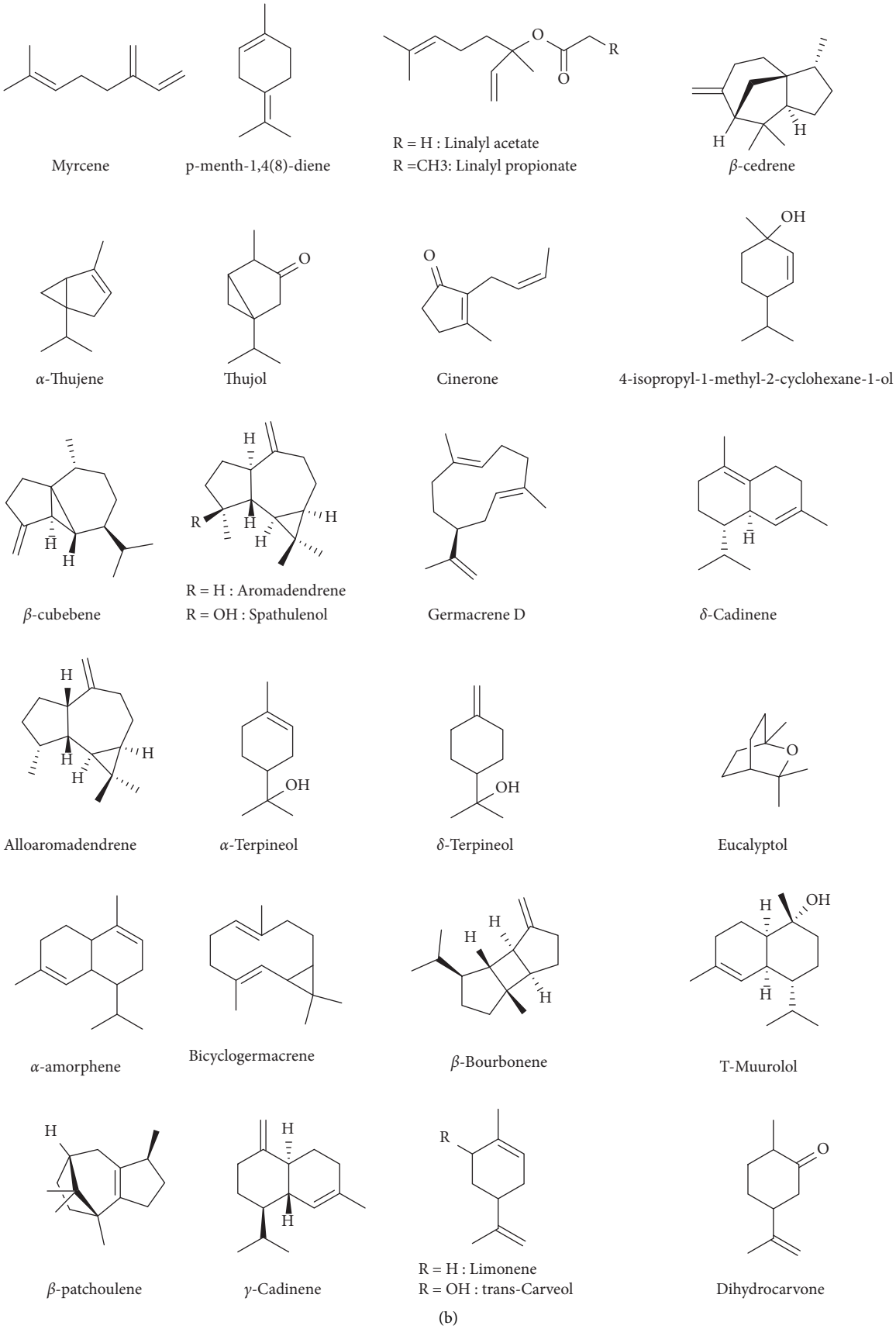
Viridiflorene



Terpendiol

(a)

Figure 1: Continued.



(b)

Figure 1: Continued.

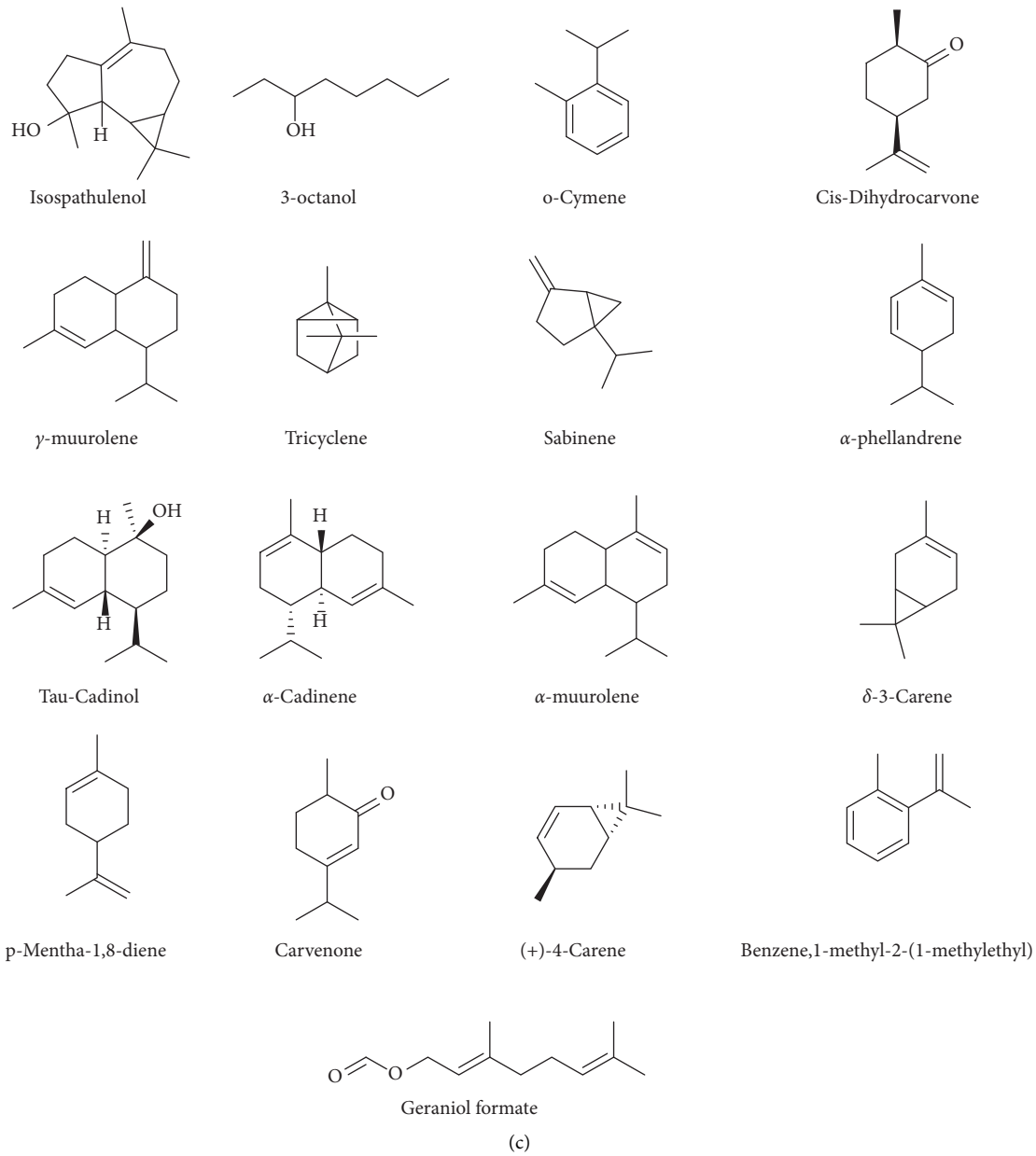


Figure 1: Chemical composition of T. *Thymus broussonetii*.

summarizes all the studies which evaluated this activity in *Thymus broussonetii*, including the plant part used, type of extract, the antibacterial test, the strains studied, and the key results. The literature screening indicated that scientists had investigated the effect of *Thymus broussonetii* against the most critical pathogenic agents belonging to Gram-negative and Gram-positive bacteria. Indeed, Lattaoui and Tantaoui-elaraki, [34] assessed the antibacterial activity of the essential oil of *T. broussonetii* aerial part against three bacteria (*Staphylococcus aureus*, *Escherichia coli*, and *Bacillus megaterium).* The result of this study showed that *T. broussonetii* essential oils inhibited the growth of all bacterial strains with MIC values of 1, 3, and 4% (v/v) against *S. aureus*, *E. coli*, and *B. megaterium*, respectively. Belaqziz et al. [33] reported the antibacterial activity of *T. broussonetti* leaf EOs using agar disc diffusion against two Gram-positive bacteria, including *S. aureus* and *Bacillus subtilis,* and four Gram-negative bacteria, namely *E. coli*, *Salmonella* sp, *Vibrio cholerae*, and *Pseudomonas aeruginosa.* The results showed that the essential oil exhibited promising antibacterial power against the strains tested; *Bacillus subtilis* ($\Phi = 33 \pm 0.4$ mm), *S. aureus* ($\Phi = 19 \pm 0.8$ mm), *Salmonella* sp. ($\Phi = 9 \pm 0.9$ mm), *Escherichia coli* ($\Phi = 21 \pm 0.1$ mm), *Vibrio cholerae* ($\Phi = 40 \pm 0.4$ mm) and *P. aeruginosa* ($\Phi = 9 \pm 0.1$ mm). In another study, El Bouzidi et al. [13] tested the antibacterial activity of essential oils obtained from both wild and cultivated *T. broussonetii* using agar disc diffusion and macrodilution methods against *Salmonella* sp. (CCMM B17), *E. coli* (CCMM B4), *E. coli* (ATCC 25922), *Bacillus cereus* (ATCC 14579), *Bacillus subtilis* (ATCC 9524), *Micrococcus luteus*

Table 2: Antibacterial effects of *T. broussonetii*.

Used parts	Extracts	Used methods	Tested strains	Key results	References
Aerial part	Essential oil	Agar disk diffusion method Broth microdilution method	*Staphylococcus aureus*	$\Phi = 42.67 \pm 1.45$ mm MIC = 0.2 μl/mL MBC = 0.6 μl/mL	[26]
			Escherichia coli	$\Phi = 29.33 \pm 0.54$ mm MIC = 1.3 μl/mL MBC = 1.3 μl/mL	
			Pseudomonas aeruginosa	$\Phi = 8.67 \pm 1.20$ mm MIC = 20 μl/mL MBC $\geq$ 80 μl/mL	
Aerial part	Essential oil	Agar disc diffusion Broth macrodilution method	*Staphylococcus aureus*	$\Phi = 35.00 \pm 1.00$ mm MIC = 0.9 mg/mL MMC = 0.9 mg/mL	[13]
			Bacillus subtilis	$\Phi = 49.67 \pm 1.53$ mm MIC = 0.23 mg/mL MMC = 0.23 mg/mL	
			Bacillus cereus	$\Phi = 48.67 \pm 1.15$ mm MIC = 0.23 mg/mL MMC = 0.23 mg/mL	
			Micrococcus luteus	$\Phi = 53.50 \pm 1.00$ mm MIC = 0.12 mg/mL MMC = 0.12 mg/mL	
			Escherichia coli 1 ATCC 25922	$\Phi = 30.17 \pm 1.00$ mm MIC = 0.90 mg/mL MMC = 0.90 mg/mL	
			E. coli 2 CCMM B4	$\Phi = 29.67 \pm 1.53$ mm MIC = 0.90 mg/mL MMC = 0.90 mg/mL	
			Enterobacter cloacae	$\Phi = 27.33 \pm 0.58$ mm MIC = 0.90 mg/mL MMC = 0.90 mg/mL	
			Salmonella sp.	$\Phi = 31.67 \pm 1.53$ mm MIC = 0.90 mg/mL MMC = 0.90 mg/mL	
			Staphylococcus aureus	$\Phi = 34.83 \pm 1.04$ mm MIC = 0.91 mg/mL MMC = 0.91 mg/mL	
			Bacillus subtilis	$\Phi = 49.00 \pm 1.00$ mm MIC = 0.23 mg/mL MMC = 0.23 mg/mL	
			Bacillus cereus	$\Phi = 47.33 \pm 1.15$ mm MIC = 0.23 mg/mL MMC = 0.23 mg/mL	
			Micrococcus luteus	$\Phi = 53.67 \pm 1.15$ mm MIC = 0.12 mg/mL MMC = 0.12 mg/mL	
			Escherichia coli 1 ATCC 25922	$\Phi = 27.5 \pm 1.53$ mm MIC = 0.91 mg/mL MMC = 0.91 mg/mL	
			E. coli 2 CCMM B4	$\Phi = 29.33 \pm 1.53$ mm MIC = 0.91 mg/mL MMC = 0.91 mg/mL	
			Enterobacter cloacae	$\Phi = 23.33 \pm 1.53$ mm MIC = 1.82 mg/mL MMC = 1.82 mg/mL	
			Salmonella sp.	$\Phi = 31.33 \pm 1.53$ mm MIC = 0.91 mg/mL MMC = 0.91 mg/mL	

Table 2: Continued.

Used parts	Extracts	Used methods	Tested strains	Key results	References
Aerial part	Essential oil	Agar diffusion method	*Bacillus subtilis* *Staphylococcus aureus* *Salmonella* sp. *Escherichia coli* *Vibrio cholerae* *Pseudomonas aeruginosa*	$\Phi = 33 \pm 0.4$ mm $\Phi = 19 \pm 0.8$ mm $\Phi = 19 \pm 0.9$ mm $\Phi = 21 \pm 0.1$ mm $\Phi = 40 \pm 0.4$ mm $\Phi = 9 \pm 0.1$ mm	[33]
Aerial part	Essential oil	Disc diffusion method	*Microcystis aeruginosa*	$\Phi = 90 \pm 0.00$ mm MIC = 0.047 mg/mL MBC = 0.095 mg/ml	[17]
Aerial part	Essential oil	Agar diffusion method	*Staphylococcus aureus*	No measurable zone of inhibition	[34]
Aerial part	Essential oil	Agar diffusion method	*Escherichia coli* *Staphylococcus aureus* *Bacillus megaterium*	No measurable zone of inhibition MIC = 1% MIC = 3% MIC = 4%	[16]

(ATCC10240), *S. aureus* (CCMM B3), and the clinically isolated strain, *Enterobacter cloacae*. Both EOs obtained from *T. broussonetii* (wild and cultivated) exhibited inhibitory activity on all the selected microorganisms, with inhibitory zones ranging between 23.33 and 53.67 mm and MIC values varied from 0.12 to 1.82 mg/mL. In fact, *Micrococcus luteus* was the most sensitive strain with MIC values of 53.50 and 53.67 mg/mL for wild and cultivated *T. broussonetii*, respectively, followed by *B. subtilis*, *B. cereus*, and *S. aureus*. However, Smahane et al. [26] investigated the inhibitory effect of *T. broussonetii* aerial part EOs against *S. aureus*, *E. coli*, and *P. aeruginosa* using disk diffusion and broth microdilution methods. The results revealed that all microorganisms tested were inhibited by essential oils with inhibitory zones ranging between 8.67 and 42.67 mm and MIC values ranged between 0.2 and 20 μg/mL.

Recently, Zerrifi and collaborators determined the *in vitro* antibacterial activity of *T. broussonetii* aerial part EOs using paper disk diffusion and microdilution methods against *Microcystis aeruginosa*. According to this study, the essential oils exhibited promising antibacterial power against the strain tested with an inhibitory zone of 90 mm, and MIC and MBC values of 0.047 and 0.095 mg/mL, respectively [17].

3.3.2. Antifungal Activity. The antifungal activity of *T. broussonetii* EOs against many fungal strains was reported in several works [13, 16, 18, 20, 26, 34]. The previous publications on the antifungal activity that studied the essential oils from aerial parts of *T. broussonetii* by different methods are summarized in Table 3.

Saad et al. [18] determined the *in vitro* antifungal efficacy of the essential oil from the aerial part against *Candida albicans* using the agar diffusion and macrodilution broth methods. Consequently, the zones of inhibition and MIC value were 38.5 mm and 0.25 μg/mL, respectively. Moreover, Jamali et al. [20] evaluated the EOs from aerial parts of the studied plant for their antifungal action against *Candida albicans*, *Candida krusei*, *Candida glabrata*, and *Candida parapsilosis* using agar disc diffusion and microdilution methods. The results revealed a strong antifungal activity against all the fungi tested with zones of inhibition ranging from 49.33 to 51.17 mm and MIC value of 0.45 mg/mL. Using the same methods and the same fungal strains, El Bouzidi et al. [13] investigated the antifungal activity of EOs obtained from wild and cultivated *T. broussonetii*. Therefore, these oils inhibited the growth of all fungal species with MIC values of 0.45 and 0.45 mg/mL for wild and cultivated *Thymus broussonetii*, respectively. In another study, the essential oil of *T. broussonetii* was tested against two fungal strains (*Candida albicans* and *Aspergillus brasiliensis*). The results revealed a strong antifungal inhibition against *Candida albicans* with zones of inhibition of 35.67 ± 0.33 mm [26].

3.3.3. Antioxidant Activity. Different studies have evaluated the antioxidant activity of extracts and EOs from different parts of *T. broussonetii* using well-known techniques such as DPPH and FRAP assays [11, 13, 20, 25, 27] (Table 4). Indeed, Jamali et al. [20] investigated the antioxidant activity of the essential oils from aerial parts of *T. broussonetii*, and the results showed that the essential oil exhibited an interesting anti-DPPH ($IC_{50} = 97.48 \pm 2.24$ μg/mL) and a high reducing power ($EC_{50} = 167.86 \pm 1.46$ μg/ml) compared with the standard antioxidants, quercetin, and BHT with IC_{50} values of 1.07 ± 0.01 and4.21 ± 0.08 μg/mL, respectively, for DPPH and with EC_{50} values of 2.29 ± 0.1 and 7.09 ± 0.1 μg/mL, respectively, for FRAP. In another study, the wild and cultivated *T. broussonetii* EOs were tested for their antioxidant activity by DPPH and ferric ion reduction assays. The results showed an interesting antioxidant effect of the wild and cultivated *T. broussonetii* EOs with IC_{50} values of 132.23 ± 3.09 and 145.83 ± 3.47 μg/mL, respectively, for DPPH and with EC_{50} values of 167.87 ± 1.46 and 169.355 ± 2.04 μg/mL, respectively, for FRAP [13]. Moreover, Ouariachi et al. [11] demonstrated that the essential oils from *T. broussonetii* possessed high antioxidant activity using DPPH ($IC_{50} = 90$ μg/mL). On the other hand, Ahlam et al. [25] reported the antioxidant activity of the aqueous and methanol extracts from leaves and stems of *T. broussonetii* using FRAP and DPPH methods. The results revealed that both extracts exhibited a good antioxidant activity with FRAP capacity values

Table 3: Antifungal activity of *T. broussonetii*.

Used parts	Extracts	Used methods	Tested strains	Key results	References
Aerial parts	Essential oil	Agar disc diffusion Broth microdilution method	*Candida albicans*	Φ = 50.00 ± 1.00 mm MIC = 0.45 mg/mL MMC = 0.45 mg/mL	[20]
			Candida krusei	Φ = 49.67 ± 1.53 mm MIC = 0.45 mg/mL MMC = 0.45 mg/mL	
			Candida glabrata	Φ = 49.33 ± 1.53 mm MIC = 0.45 mg/mL MMC = 0.45 mg/mL	
			Candida parapsilosis	Φ = 51.17 ± 0.76 mm MIC = 0.45 mg/mL MMC = 0.45 mg/mL	
	Essential oil	Agar disc diffusion Broth microdilution method	*Candida albicans*	Φ = 50.00 ± 1.00 mm MIC = 0.45 mg/mL MMC = 0.45 mg/mL	[13]
			Candida krusei	Φ = 49.67 ± 1.53 mm MIC = 0.45 mg/mL MMC = 0.45 mg/mL	
			Candida glabrata	Φ = 49.33 ± 1.53 mm MIC = 0.45 mg/mL MMC = 0.45 mg/mL	
			Candida parapsilosis	Φ = 51.17 ± 0.76 mm MIC = 0.45 mg/mL MMC = 0.45 mg/mL	
			Candida albicans	Φ = 49.67 ± 1.15 mm MIC = 0.46 mg/mL MMC = 0.46 mg/mL	
			Candida krusei	Φ = 47.33 ± 1.53 mm MIC = 0.46 mg/mL MMC = 0.46 mg/mL	
			Candida glabrata	Φ = 48.50 ± 0.50 mm MIC = 0.46 mg/mL MMC = 0.46 mg/mL	
			Candida parapsilosis	Φ = 50.00 ± 1.00 mm MIC = 0.46 mg/mL MMC = 0.46 mg/mL	
Aerial parts	Essential oil	Agar diffusion method macrodilution broth method	*Candida albicans*	Φ = 38.5 ± 0.70 mm MIC = 0.25 μg/mL	[18]
Aerial parts	Essential oil	Agar diffusion method	*Candida albicans*	Slightly more sensitive in presence of 0.2% oil	[34]
			Aspergillus niger	Lower sensitivity relatively resistant	
Aerial parts	Essential oil	Agar diffusion method	*Saccharomyces cerevisiae*	MIC = 3%	[16]
			Candida albicans	MIC = 3%	
			Zygorrhynchussp	MIC = 4%	
			Aspergillus niger	MIC = 3%	
Aerial part	Essential oil	Agar disk diffusion method Broth microdilution method	*Candida albicans*	Φ = 35.67 ± 0.33 mm MIC = ND MBC = ND	[26]
			Aspergillus brasiliensis	Φ = ND MIC = ND MBC = ND	

ranging between 0.105 ± 0.021 and 1.579 ± 0.014 mg/mL and anti-DPPH power with IC_{50} values ranging between 0.132 ± 0.034 and 7.665 ± 0.411 mg/mL. The highest activity was observed in methanol extract from stems with EC_{50} and IC_{50} values of 0.105 ± 0.021 and 0.132 ± 0.034 mg/mL, respectively. On the other hand, essential oil showed a DPPH-radical-scavenging activity with IC_{50} = 13.24 ± 0.06 mg/mL [27].

3.3.4. Anticancer Activity. The anticancer properties of *T. broussonetii* have also been studied. Indeed, some investigations tested the efficiency of *T. broussonetii* essential oils

Table 4: Antioxidant effects of *T. broussonetii*.

Used parts	Extracts	Used methods	Key results	References
Leaves	Aqueous extract	DPPH	$IC_{50} = 22.61 \pm 1.022$ mg/mL	[25]
	Methanol extract		$IC_{50} = 6.484 \pm 0.190$ mg/mL	
Stems	Aqueous extract		$IC_{50} = 7.665 \pm 0.411$ mg/mL	
	Methanol extract		$IC_{50} = 0.132 \pm 0.034$ mg/mL	
Leaves	Aqueous extract	FRAP	$EC_{50} = 0.597 \pm 0.013$ mg/mL	
	Methanol extract		$EC_{50} = 1.579 \pm 0.014$ mg/mL	
Stems	Aqueous extract		$EC_{50} = 0.489 \pm 0.011$ mg/mL	
	Methanol extract		$EC_{50} = 0.105 \pm 0.021$ mg/mL	
Aerial parts	Essential oil	DPPH	$IC_{50} = 13.24 \pm 0.06$ mg/mL	[27]
Aerial parts	Essential oil	DPPH	$IC_{50} = 90\ \mu g/mL$	[11]
Aerial parts (wild)	Essential oil	DPPH	$IC_{50} = 132.23 \pm 3.09\ \mu g/mL$	[13]
		FRAP	$EC_{50} = 167.87 \pm 1.46\ \mu g/mL$	
Aerial parts (cultivated)	Essential oil	DPPH	$IC_{50} = 145.83 \pm 3.47\ \mu g/mL$	
		FRAP	$EC_{50} = 169.355 \pm 2.04\ \mu g/mL$	
Aerial parts (wild)	Essential oil	DPPH	$IC_{50} = 97.48 \pm 2.24\ \mu g/mL$	[20]
		FRAP	$EC_{50} = 167.86 \pm 1.46\ \mu g/mL$	

on many cell lines [12, 15, 21] (Table 5). Ait M'Barek et al. [15] evaluated the antiproliferative effect of *T. broussonetii* EOs from stem and leaves on human ovarian adenocarcinoma IGR-OV1 parental cell line OV1/P. The results showed that the EOs tested inhibited the proliferation of this adenocarcinoma with an IC_{50} value of 0.40 ± 0.02 (%v/v).

Moreover, *Thymus broussonetii* EOs extracted from flowers and leaves have been tested by Jaafari et al. [21] on the P815 mastocytoma cell line using MTT assay. In this study, the essential oils exhibited an important dose-dependent cytotoxic effect against the P815 cell line ($IC_{50} = 0.016\%$).

In another study, the authors evaluated the cytotoxic activity of essential oils from two chemotypes of *T. broussonetii* against five tumor cell lines, namely P-815 (murine mastocytoma), K-562 (human chronic myelogenous leukemia), CEM (acuteT lymphoblastoid leukemia), and MCF 7 (human breast adenocarcinoma) and its counterpart resistant to gemcitabine (MCF -7 gem) using MTT assay. Consequently, cell viability showed a cell proliferation inhibition by the tested products in a dose-dependent manner with IC_{50} values ranging between 3.1 and 17.5% (v/v). Additionally, cell cycle analysis detected cell cycle arrest at S and G0/G1 phases in cells. This considerable activity might be due to the high content of thymol and carvacrol known for their promising anticancer effects *via* numerous mechanisms of action such as angiogenesis, inhibition of cell migration, autophagy, apoptosis, and cell cycle arrest [35, 36].

3.3.5. Anti-Inflammatory Activity. The antiedema effects of hexane, chloroform, and methanol extracts of *T. broussonettii* were evaluated on croton oil-induced ear edema in mice. The chloroform extract showed the highest activity, reducing the oedematous response by 47%, the ID_{50} value of the indomethacin used as the reference drug (286 g/cm^2) is three times higher than that of the chloroform extract 93 g/cm^2. The chloroform extract of *T. broussonettii* possesses an anti-inflammatory activity ascribable to its triterpenic acid content; in fact, ursolic and oleanolic acid justify the edema inhibition observed. Ursolic acid was more potent than oleanolic acid with ID_{50} values of 56 and 132 g/cm^2 corresponding to 0.12 and 0.29 mol/cm^2, respectively [28] (Table 6).

3.3.6. Anticorrosive Potential. The essential oils of *T. broussonnetii* at different concentrations (ranging from 0.05 to 2 g/L) were tested against corrosion on C38 steel in 1 M medium, HCl, using electrochemical impedance spectroscopy (EIS), potentiodynamic polarization, and weight loss methods. The essential oil was found to be rich in bioactive substances, mainly carvacrol (39.51%) followed by benzene, 1-methyl-2-(1-methylethyl) (14.80%), gammaterpinene (10.32%), alpha-pinene (9.7%), thymol (7.9%), and 3-cyclohexen-1-ol, 4-methyl-1-(1-methylethyl) (3.22%). Using the EIS test, the essential oil (2 g/L) inhibited the corrosion of metals and alloys in acid solutions with a percentage of 82.35% of the inhibition efficiency. The polarization studies showed that *T. broussonnetii* EOs inhibit both anodic metal dissolution and cathodic hydrogen reduction reactions. At the highest inhibition concentration, the maximum inhibition efficiency observed indicates that many molecules were adsorbed on the metal surface. At 2 g/L, the best efficiency obtained in the presence of essential oil was 81.63%. It has been noted that the inhibition efficiency increases with increasing temperature. The highest efficiency was 90% and reached 328 K. The inhibitory mechanism was probably achieved by chemical adsorption (chemisorption) of TBS molecules on the surface of carbon steel and this indeed increases with rising temperature [23] (Table 6).

3.3.7. Insecticidal Activities. The *T. broussonetti* EOs were investigated for their insecticidal activity, using the larvae test sensibility technique. The chemical analysis by GC-MS showed that the major compounds of *T. broussonetii* essential oil were p-cymene (21.0%), borneol (16.5%), α-pinene (11.8%), and thymol (11.3%). The EOs of this plant proved larvicidal effectiveness against the fourth instar larvae of *Culex pipiens* and were significantly higher at the dose of 0.125 ppm compared to the control. The lethal concentration 50 (LC_{50}) during

Table 5: Anticancer effects of *T. broussonetii*.

Parts used	Extracts	Used methods	Cell lines	Key results	References
Leaves and stems	Essential oils	Crystal violet assay	The parental human ovarian adenocarcinoma cell line IGR-OV1 (OV1/P)	$IC_{50} = 0.40 \pm 0.02\%$(v/v)	[15]
Flowers and leaves	Essential oils (variety: TbA)	MTT assay	P815 mastocytoma cell line	$IC_{50} = 4.7\%$(v/v)	[12]
			CEM	$IC_{50} = 3.6\%$(v/v)	
			K-562	$IC_{50} = 10\%$(v/v)	
			MCF -7	$IC_{50} = 10\%$(v/v)	
			MCF -7 gem	$IC_{50} = 8.9\%$(v/v)	
	Essential oils (variety: TbB)	MTT assay	P815 mastocytoma cell line	$IC_{50} = 8.5\%$(v/v)	
			CEM	$IC_{50} = 3.1\%$(v/v)	
			K-562	$IC_{50} = 13.5\%$(v/v)	
			MCF -7	$IC_{50} = 15.4\%$(v/v)	
			MCF -7 gem	$IC_{50} = 17.5\%$(v/v)	
Flowers and leaves	Essential oils	MTT assay	P815 mastocytoma cell line	$IC_{50} = 0.016\%$(v/v)	[21]

Table 6: Other pharmacological activities of *T. broussonetii*.

Activities	Used parts	Extracts	Experimental approaches	Key results	References
Anti-inflammatory activity	Leaves	n-hexane	Croton oil ear test in mice inhibition of the croton oil-induced ear edema in mice	Edema reduction = 9%	[28]
		Chloroform		Edema reduction = 47%	
		Chloroform + methanol		Edema reduction = 16%	
		Methanol		Edema reduction = -5%	
Anticorrosive activity	Aerial parts	Essential oils	Loss measurements and electrochemical techniques	82.35% inhibition efficiency at a dose of 2 g/L	[23]
Insecticidal activity	Aerial parts	Essential oils	Fourth instar larvae of *Culex pipiens*	$LC_{50} = 0.23$	[19]
Antiparasitic activity	Aerial parts	Essential oils	Oral administration (20 g/animal) at the time of infection and thereafter for several days	Absence of intracerebral cysts No anomalies	[30]
Antipyretic activity	Stem	Water, butanol, and ethyl acetate	Yeast-induced fever in rats	Significantly reduced the temperature in febrile rats	[37]
Acute toxicity	Aerial parts	Essential oils	Swiss mice (25–35 g)	$LD_{50} = 2.66$ g/kg	[22]
Antinociceptive activity	Leaves and stem	Water	Chemical and thermal models (*in vivo*)	Writhing inhibition = 88.9%	[31]
		Ethyl acetate and butanol		Writhing inhibition = 69% Writhing inhibition = 62.8%	
Insecticidal activity	Aerial parts	Essential oils	Effect against adults of *Tribolium castaneum* herbst	$LD_{50} = 0.08\ \mu l/cm^2$ $LD_{90} = 0.19\ \mu l/cm^2$	[29]
Insecticidal activity	Aerial parts	Essential oils	Effect against *Tribolium castanum* pest foodstuffs	$TL_{50} = 1.5\ \mu l/cm^2$	[27]
Immunological and behavioral activities	Leaves and stem	Water, butanol, and ethyl acetate	Tested the neurostimulant effects of the extracts	Increased (*in vivo*) the number of leukocyte categories studied	[31]

exposure of the insect population to EOs at 24 hours was 0.23, and the effective toxicity on *C. pipiens* larvae was associated with the thymol compound of thyme oil [19] (Table 6).

3.3.8. Antipyretic Activity. At a dose of 200 mg/kg b.w., *T. broussonetii* aqueous, butanol, and ethyl acetate extracts were investigated *in vivo* for their antipyretic effect on yeast-induced fever. In normothermic rats, the extracts were tested to determine whether the antipyretic activity is related to a hypothermic effect. Indeed, all extracts significantly reduced rectal temperature in febrile animals. However, they did not induce hypothermia in normal rats. Besides, an inhibition of platelet aggregation has been observed by acting in the same way as NSAI drugs. Furthermore, extracts of *T. broussonetii* contain many types of compounds such as triterpenes, saponins, tannins, flavonoids, and several salicylates. The presence of these compounds can enhance this antipyretic activity [22] (Table 6).

3.3.9. Antinociceptive. The immunostimulatory and neurotropic antistress effects of extracts (aqueous, ethyl acetate, and butanolic extracts) and EOs of *T. broussonetii* were evaluated at three doses. Therefore, the aqueous and ethyl

acetate extracts showed the best results. In fact, thyme extracts increased the number of leucocyte categories studied, in particular polynuclear cells, total lymphocytes, TCD4+, TCD8+, and NK cells. It has been suggested that intraperitoneal administration of *T. broussonetii* extracts has a potent direct effect on leucocytes *in vivo*. In contrast, this assumes that the two extracts partially prevent stress-induced disturbances in the rate of leukocytes. The ethyl acetate extract inhibited the increase in polynuclear cells caused by stress, increased lymphocytes, and decreased polynuclear counts in the stressed mice treated with the aqueous extract compared to the stressed mice [31].

T. broussonetii was investigated to study the behavioral effects using the light/dark box test. At 12 mg/kg, the aqueous extract increased the number of transitions and the number of traversed squares and decreased the time spent in the dark compartment. The ethyl acetate extract increased both the number of traversed squares and the number of transitions without affecting the time spent in the dark compartment. The aqueous extract exerted an anxiolytic effect on the animals, while it could rather enhance locomotor and exploratory activities. The improvement in animal activity observed in the light/dark box after treatment with the aqueous extract is rather due to its anxiolytic-like effect and the ethyl acetate extract improved exploratory and locomotor activities in mice (Table 6).

3.3.10. Antiparasitic Activity. In another work, the effect of *T. broussonetii* EOs was assessed on the experimental transmission of *Toxoplasma gondii* cysts in mice. These oils were administered orally (20 μg/animal) at the infection time and thereafter for several days. In mice given the essential oils, no cyst was observed. In addition, no disorder was noted in the control animals given the thyme EOs [30] (Table 6).

3.3.11. Insecticidal Activity. The insecticidal activity of *T. broussonetii* EO was screened using the contact toxicity assay. The oil proved insecticidal effectiveness against *Tribolium castaneum* Herbst. After 24 h of treatment, the LD_{50} and LD_{90} were 0.08 and 0.19 μl/cm^2, respectively. These results suggest that the contents of thyme EOs, in particular those obtained from the genus *Thymus*, have a good botanical bioinsecticide potential against *Tribolium castaneum* Herbst [29].

The insecticidal activity of the EO of this plant was examined against *Tribolium castanum* by the contact toxicity assay. The essential oil exhibited the highest insecticidal activity with a median lethal time (TL_{50}) of 1.5 μL/cm^2 with LT_{50} (lethal time required to kill 50% of the exposed insects) values of 30,36 (24,62–38,48) at a dose of 1 μl/cm^2 and 4,81(3,8–5,99) at a dose of 1,5 μl/cm^2, respectively and a LT_{90} (lethal time required to kill 90% of the exposed insects) of 222,78(138,62–475,59) at a dose of 1 μl/cm^2 and 16,07 (11,4–30,08), respectively. The *Thymus broussonnetii* Boiss EO could act as a substitute for biopesticide and reduce the harmful impact of chemical insecticides on the environment and humans [27] (Table 6).

3.3.12. Immunological and Behavioral Effects. The antinociceptive effect of aqueous, butanol, and ethyl acetate extracts of *T. broussonetii* was studied using thermal and chemical nociception models and naloxone (a nonselective opioid antagonist) to determine the role of the opioid system in the antinociceptive activity of these extracts. To determine the phytoconstituents of the extracts tested, phytochemical screening was carried out, which revealed the presence of tannins in all the extracts. Quinones, saponins, and flavonoids were detected in butanol and ethyl acetate extracts, while terpenes were only identified in the ethyl acetate extract [31].

The butanol and aqueous extracts showed an antinociceptive effect in both phases of formalin (50–300 mg/kg), tail immersion, and writing tests. At the same time, only the nociceptive response of the second phase was significantly reduced by the ethyl acetate extract (100–300 mg/kg). In the first and second phases, the aqueous extract was the most effective, with ED_{50} values of 177 (147–200) and 134 (95–170) mg/kg, respectively. The aqueous extract (200 mg/kg) showed a potent effect and significantly reduced the number of writhes induced by acetic acid, with 88.9% of writhes inhibition compared to those of ethyl acetate (69%) and butanol (63%) extracts. These obtained proved that *T. broussonetii* contains active compounds (polar and nonpolar) having antinociceptive activity with distinct mechanisms of action [31] (Table 6).

3.4. Toxicological Investigations. An acute toxicity screening was carried out for *T. broussonetii* EOs in order to verify their harmlessness to avoid a possible overdose and to properly determine the toxicological profile of the *T. broussonetii* species. This was assessed using the Leitchfield and Wilcoxon method, and the effective lethal dose (LD_{50}) was measured. Subsequently, signs of toxicity such as diarrhea, convulsion, piloerection, motor coordination, and behavioral changes (excitation and twitches) were determined. For the groups receiving the dose of 1 g/kg, the change in body weight was also determined. On the other hand, thymol (36.7%) and borneol (21.9%) were the two major compounds, followed by *p*-cymene (7.6%) and β-pinene (0.7%). At a dose of 2 mg/kg, some cases of death and signs of toxicity were recorded. The LD_{90}s and LD_{50}s were estimated to be 7.31 (5.64–13.54) and 4.47 (3.6–6.72) g/kg, respectively [22].

4. Conclusion and Perspectives

Here, the phytochemistry, toxicology, and pharmacological properties of *T. broussonetii* were highlighted. Phytochemical studies of this species showed its richness in numerous bioactive compounds, exhibiting important biological effects. Pharmacological investigations confirmed the safety of this plant. However, these investigations must be further investigated using several toxicological reports at several different doses and time periods. Pharmacological biology explorations demonstrated that *T. broussonetii* essential oils and extracts exhibit important and remarkably antimicrobial, anticancer and, anti-inflammatory properties.

These investigations were conducted using *in vitro* approaches, and therefore, further *in vivo* examinations should be performed to explore the pharmacological properties of T. broussonetii importantly. Moreover, mechanisms related to the biological effects of *T. broussonetii* and its bioactive compounds should also be explored to validate their pharmacodynamic actions.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

References

[1] M. Valverde, *R. El Género Thymus L (Labiatae) En Africa*, Springer, Berlin, Germany, 1993.

[2] H. Ouhaddou, H. Boubaker, F. Msanda, and A. El Mousadik, "An ethnobotanical study of medicinal plants of the agadir ida ou tanane province (southwest Morocco)," *Journal of Applied Bioscience*, vol. 84, pp. 7707–7722, 2014.

[3] S. Belhaj, N. Chaachouay, and L. Zidane, "Ethnobotanical and toxicology study of medicinal plants used for the treatment of diabetes in the high Atlas central of Morocco," *Journal of Pharmacy & Pharmacognosy Research*, vol. 9, pp. 619–662, 2021.

[4] A. Abbad, R. Belaqziz, K. Bekkouche, and M. Markouk, "Influence of temperature and water potential on laboratory germination of two Moroccan endemic thymes: Thymus maroccanus ball. And Thymus broussonetii Boiss," *African Journal of Agricultural Research*, vol. 6, pp. 4740–4745, 2011.

[5] H. O. H. Ouhaddou, A. Alaoui, and A. Sezgin, "Ethnobotanical survey of medicinal plants used for treating diabetes in agadir ida outanane region, southwestern Morocco," *Arabian Journal of Medicinal and Aromatic Plants*, vol. 6, pp. 72–86, 2020.

[6] S. Skalli, R. Hassikou, and M. Arahou, "An ethnobotanical survey of medicinal plants used for diabetes treatment in rabat, Morocco," *Heliyon*, vol. 5, Article ID e01421, 2019.

[7] J. Bellakhdar, *Pharmacopée Marocaine Traditionnelle*, Ibis Press, Newburyport, MA, USA, 1997.

[8] M. Tbatou, A. Belahyan, and R. Belahsen, "Wild edible plants traditionally used in the countryside of El jadida, coastal area in the center of Morocco," *Life Sciences Leaflets*, vol. 75, pp. 28–48, 2016.

[9] H. Briguiche and L. Zidane, "Ethnobotanical study of medicinal plants from el-jadida city (Morocco)," *Lazaroa*, vol. 37, pp. 145–151, 2016.

[10] R. Mehdioui and A. Kahouadji, "Etude ethnobotanique auprès de La population riveraine de La forêt d'Amsittène: cas de La commune d'Imi n'Tlit (province d'Essaouira)," *Bulletin de l'Institut Scientifique, Section Sciences de la Vie*, vol. 29, pp. 11–20, 2007.

[11] E. m. E. Ouariachi, E. mokhtar, I. Hamdani et al., "Chemical composition and antioxidant activity of essential oils of Thymus broussonetii Boiss. And Thymus algeriensis Boiss. From Morocco," *Asian Pacific Journal of Tropical Disease*, vol. 4, no. 4, pp. 281–286, 2014.

[12] A. Jaafari, H. A. Mouse, L. A. M'Bark et al., "Differential antitumor effect of essential oils and their major components of Thymus broussonettii: relationship to cell cycle and apoptosis induction," *Herba Polonica*, vol. 55, pp. 36–50, 2009.

[13] L. El Bouzidi, C. A. Jamali, K. Bekkouche et al., "Chemical composition, antioxidant and antimicrobial activities of essential oils obtained from wild and cultivated Moroccan Thymus species," *Industrial Crops and Products*, vol. 43, pp. 450–456, 2013.

[14] H. Boubaker, "Chemical characterization and antifungal activities of four Thymus species essential oils against postharvest fungal pathogens of citrus," *Industrial Crops and Products*, vol. 7, 2016.

[15] L. Ait M'Barek, H. Ait Mouse, A. Jaâfari et al., "Cytotoxic effect of essential oil of thyme (Thymus broussonettii) on the IGR-OV1 tumor cells resistant to chemotherapy," *Brazilian Journal of Medical and Biological Research*, vol. 40, pp. 1537–1544, 2007.

[16] A. Tantaoui-elaraki, N. Lattaoui, A. Errifi, and B. Benjilali, "Composition and antimicrobial activity of the essential oils of thymus broussonettii, T. zygisandT. Satureioides," *Journal of Essential Oil Research*, vol. 5, no. 1, pp. 45–53, 1993.

[17] S. E. A. Zerrifi, A. Kasrati, E. M. Redouane et al., "Essential oils from Moroccan plants as promising ecofriendly tools to control toxic cyanobacteria blooms," *Industrial Crops and Products*, vol. 143, Article ID 111922, 2020.

[18] A. Saad, M. Fadli, M. Bouaziz, A. Benharref, N.-E. Mezrioui, and L. Hassani, "Anticandidal activity of the essential oils of Thymus maroccanus and Thymus broussonetii and their synergism with amphotericin B and fluconazol," *Phytomedicine*, vol. 17, no. 13, pp. 1057–1060, 2010.

[19] R. Belaqziz, R. Harrak, A. Romane, and K. Oufdou, "Antimicrobial and insecticidal activities of the endemic Thymus broussonetti Boiss. and Thymus maroccanus ball," *Records of Natural Products*, vol. 8, 2010.

[20] C. A. Jamali, L. El Bouzidi, K. Bekkouche et al., "Chemical composition and antioxidant and anticandidal activities of essential oils from different wild Moroccan Thymus species," *Chemistry and Biodiversity*, vol. 9, no. 6, pp. 1188–1197, 2012.

[21] A. Jaafari, H. A. Mouse, E. M. Rakib et al., "Chemical composition and antitumor activity of different wild varieties of Moroccan thyme," *Revista Brasileira de Farmacognosia*, vol. 17, no. 4, 2007.

[22] K. Elhabazi, R. Aboufatima, A. Bensalah et al., "Acute toxicity of essential oils of two moroccan endemic species," *Thymus Broussonetii and Thymus Leptobotrys*, vol. 6, 2012.

[23] H. Chebli, M. Zaafrani, A. Batah et al., "Chemical composition and green anticorrosive potential of Thymus broussonnetii Boiss subsp. broussonnetii essential oils in hydrochloric acid medium," *Journal of Bio- and Tribo-Corrosion*, vol. 5, no. 1, p. 13, 2019.

[24] S. Belmalha, G. Echchgadda, J. Ibijbijen et al., "Characterizing the major morphological traits and chemical compositions in nine species of wild thyme from Morocco," *European Journal of Scientific Research*, vol. 145, p. 14, 2017.

[25] S. Ahlam, B. Fatima, O. Mohammed, A. Youssef, M. Lhou, and R. Abderrahmane, "Phytochemical screening and antioxidant activity of four moroccan thymus species: T. Leptobotrys Murb." *Aromatic Plants*, vol. 12, 2015.

[26] B. Smahane, B. Mounyr, B. Faisl, M. Stephane, M. Sghir, and B. Dalila, "Antimicrobial activities of essential oil of five plant species from Morocco against some microbial strains," *International Journal of Pharmacognosy and Phytochemical Research*, vol. 8, p. 6, 2016.

[27] A. El Hamdaoui, Y. Elmaati, A. Bouglad et al., "Contribution à La Caractérisation et à La Valorisation de Deux Espèces de Thym de l'arganeraie Du Sud Ouest Marocain," *Actes 3ème Congrès Int. L'Arganier*, vol. 8, pp. 1901–1906, 2015.

[28] H. Ismaili, S. Sosa, D. Brkic et al., "Topical anti-inflammatory activity of extracts and compounds from Thymus broussonettii," *Journal of Pharmacy and Pharmacology*, vol. 54, no. 8, pp. 1137–1140, 2002.

[29] C. Alaoui-Jamali, A. Kasrati, D. Leach, and A. Abbad, "Étude comparative de l'activité insecticide des huiles essentielles des espèces de thyms originaires du Sud-Ouest marocain," *Phytothérapie*, vol. 16, no. 5, pp. 268–274, 2018.

[30] A. Dahbi, B. Bellete, P. Flori et al., "The effect of essential oils from Thymus broussonetii Boiss on transmission of Toxoplasma gondii cysts in mice," *Parasitology Research*, vol. 107, no. 1, pp. 55–58, 2010.

[31] K. Elhabazi, A. Dicko, F. Desor, A. Dalal, C. Younos, and R. Soulimani, "Preliminary study on immunological and behavioural effects of Thymus broussonetii Boiss., an endemic species in Morocco," *Journal of Ethnopharmacology*, vol. 103, no. 3, pp. 413–419, 2006.

[32] M. Fennane, M. I. Tattou, and B. Valdés, "Catalogue des plantes vasculaires rares, menacées ou endémiques du maroc," *Herbarium Mediterraneum Panormitanum*, 1998.

[33] R. Belaqziz, R. Harrak, A. Romane, K. Oufdou, and M. A. E. ElFels, "Antimicrobial and insecticidal activities of the endemic Thymus broussonetti Boiss. and Thymus maroccanus ball," *Records of Natural Products*, vol. 4, p. 230, 2010.

[34] N. Lattaoui and A. Tantaoui-Elaraki, "Comparative kinetics of microbial destruction by the essential oils ofThymus broussonettii, T. zygisandT. Satureioides," *Journal of Essential Oil Research*, vol. 6, no. 2, pp. 165–171, 1994.

[35] A. Bouyahya, O. Belmehdi, A. Benjouad et al., "Pharmacological properties and mechanism insights of Moroccan anticancer medicinal plants: what are the next steps?" *Industrial Crops and Products*, vol. 147, Article ID 112198, 2020.

[36] H. Elbe, G. Yigitturk, T. Cavusoglu, T. Baygar, M. Ozgul Onal, and F. Ozturk, "Comparison of ultrastructural changes and the anticarcinogenic effects of thymol and carvacrol on ovarian cancer cells: which is more effective?" *Ultrastructural Pathology*, vol. 44, no. 2, pp. 193–202, 2020.

[37] K. Elhabazi, R. Aboufatima, A. Zyad et al., *Study on the Antipyretic Activity of Thyme (Thymus Broussonetii) in Experimental Rats*, Vol. 7, ResearchGate, Berlin, Germany, 2012.

[38] A. Pakdemirli, C. Karaca, T. Sever et al., "Carvacrol alters soluble factors in HCT-116 and HT-29 cell lines," *Turkish Journal of Medical Sciences*, vol. 50, pp. 271–276, 2020.

Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2021, Article ID 5548404, 13 pages
https://doi.org/10.1155/2021/5548404

Review Article

A Review on Health Benefits of *Malva sylvestris* L. Nutritional Compounds for Metabolites, Antioxidants, and Anti-Inflammatory, Anticancer, and Antimicrobial Applications

Seyyed Mojtaba Mousavi,[1] Seyyed Alireza Hashemi,[2] Gity Behbudi,[3] Sargol Mazraedoost,[4] Navid Omidifar,[5] Ahmad Gholami,[4,6] Wei-Hung Chiang,[1] Aziz Babapoor,[3] and Nelson Pynadathu Rumjit[7]

[1]*Department of Chemical Engineering, National Taiwan University of Science and Technology, Taipei, Taiwan*
[2]*Department of Mechanical Engineering, Center for Nanofibers and Nanotechnology, National University of Singapore, Singapore*
[3]*Department of Chemical Engineering, University of Mohaghegh Ardabili, Ardabil, Iran*
[4]*Biotechnology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran*
[5]*Department of Pathology, Shiraz University of Medical Sciences, Shiraz, Iran*
[6]*Pharmaceutical Sciences Research Center, Shiraz University of Medical Sciences, Shiraz, Iran*
[7]*Nanotechnology and Catalysis Research Centre (NANOCAT), Institute for Advanced Studies (IAS), University of Malaya (UM), Kuala Lumpur, Malaysia*

Correspondence should be addressed to Ahmad Gholami; gholami@sums.ac.ir

Received 23 February 2021; Revised 28 June 2021; Accepted 6 August 2021; Published 16 August 2021

Academic Editor: Dejan Stojkovic

The utilization of medicinal plants and their derivatives in treating illnesses is more appropriately recognized as herbal remedy than traditional medicine. For centuries, medicinal herbs have been used for the treatment of diseases in many countries. *Malva sylvestris* L. is a kind of mallow derived from Malvaceae species and is recognized as common mallow. This amazing plant has antimicrobial, hepatoprotective, anti-inflammatory, and antioxidant properties and is considered as one of the most promising herbal medicinal species. This plant's traditional use in treating many diseases and preparing pharmaceutical compounds can show us how to know in depth the plant origin of drugs used to produce antibiotics and other therapeutic agents.

1. Introduction

Malva sylvestris L. (*M. sylvestris*) is one of the medicinal plants commonly recognized as common mallow in Europe, Iran, Pakistan, and India. *M. sylvestris* is a biennial-perennial herbaceous plant commonly found in North Africa, Europe, and Southwest Asia [1, 2]. The plant generally grows in moist areas, for instance, near marshes, ditches, oceans, riverbanks, and meadows [3]. Due to the softening properties of this plant, the Romans and ancient Greeks used it as a softener [4, 5]. Traditionally, these medicinal plants have been used to treat several infections and diseases, such as cold, burn, cough, tonsillitis, bronchitis, digestive problems, eczema, and cut wounds under different weather conditions [6]. As a natural product, *M. sylvestris* leaves and flowers showed various therapeutic effects. Figure 1 shows some of the medicinal applications of this plant.

Fluid extracts of *M. sylvestris* leaves and flowers are used to treat inflammatory diseases of mucous membranes, cystitis, and diarrhea [8]. This plant derives its restoration competencies from the mucilage and flavonoids located in the vegetation and leaves. Young leaves, shoots, flowers, and fruits are consumed in salads, soups, or boiled root vegetables. Flowering flora in the mallow

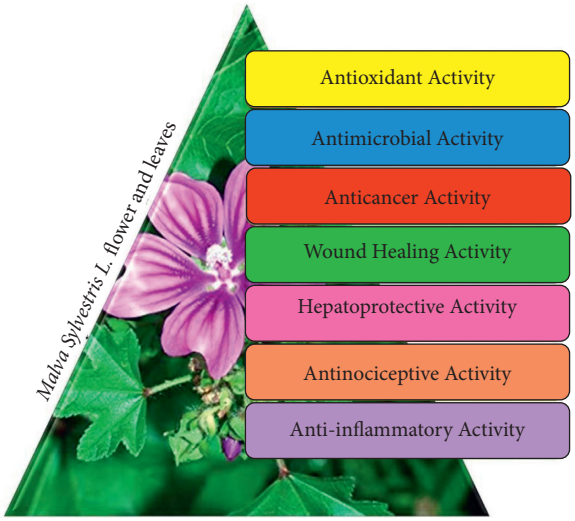


Figure 1: Flower of *M. sylvestris* and different biological activities [7].

family (Malvaceae) or hibiscus generally include the *Malva* and *Hibiscus* species. *Hibiscus* species comprise the swamp-rose mallow (*Hibiscus moscheutos*); another type of rose mallow (*Hibiscus militaris*), a shrub that grows to a peak of 2 m; and superb rose mallow (*Hibiscus grandiflorus*), with ample white to purplish flowers. Herbal medicine is one of the oldest treatment practices followed by humans. In the last 30 years, medicine specialists focus on the use of medicinal herbs in preventing and treating diseases. Among the numerous species used in traditional medicine, Malvaceae family is more prominent due to its diverse applications, and its consumption can be traced back to 3000 years ago.

The marshmallow (*Althea officinalis*), generally found in swamplands or marshes near the sea, is indigenous to North America and local to Europe and North Africa. Recently, its root has been used to make sweets. *Malva* plant in India, with a maximum height of up to 40 cm, is prescribed for the treatment of cough and cold due to respiratory problems involved and for the treatment of gastrointestinal problems [9]. This drug is used in Brazil to treat bronchitis, wounds, colitis, and hemorrhoids [10]. The chemicals in the leaf of *Malva*, which has many vitamins, allow for faster recovery by secreting certain analgesics to reduce pain and discomfort [11].

Medicinal plants have been frequently used to treat a variety of human diseases. Over the last century, the use of vegetation in medication, hematology, oncology, and immunology has affected the identity of natural composites: codeine, taxol, vinblastine, morphine, and cocaine, among others. The results of several studies have shown that *Malva* extract contains different compounds, including phenolic derivatives, flavonoids, terpenoids, catalase enzymes, sulfite oxidase, fatty acids, and certain strolls (specifically essential fatty acids such as omega-3 and omega-6), beta carotene, and vitamins C and E, which have anti-inflammatory and antioxidant properties [12–15]. Therefore, it can protect the kidney against injuries due to renal toxicity resulting from the cisplatin and vanadium system [16]. Extensive research shows that this plant, with different chemical compounds, can minimize liver damage caused by carbon tetrachloride. *M. sylvestris* has antimicrobial, antinociceptive, hepatoprotective, wound-healing, anticancer, anti-inflammatory, and potent antioxidant properties (Figure 2). Also, this plant contains many valuable compounds such as strong antioxidants and carbohydrates and unsaturated fatty acids. Tannins, flavonoids, phenolic compounds, and ascorbic acid found in the *Malva* plant are used to treat most cancers and for wound-healing [2, 7, 9, 17].

1.1. Phytochemistry. The prevalence of using plant antioxidants, considering their use in various research and applied aspects of antioxidants, especially the valuable compounds underlying phenolic induction with its groups with free radical absorption, plays an essential role in spreading its use as an oxidation preventive agent [18]. In the study conducted by Nawwar et al. [19], the phenol carboxylic and free organic acids were methylated. By using the following formula, the contents of components were calculated:

$$C\,(\text{mg/kg}) = K_1 \times K_2 \times 1000, \quad (1)$$

where $K_1 = A_1/A_2$ (A_1 and A_2 are the peak areas of the test and standard compounds, respectively) and K_2 is the mass of the internal standard (μg) added to the sample. The component combination of organic acids is shown in Table 1.

A total of 13 organic acids extracted from the leaves of *M. sylvestris* are known, including malonate (1284.4 mg/kg), malate (3510.0 mg/kg), oxalate (4170.7 mg/kg), fumarate (6924.8 mg/kg), and citrate (13133.2 mg/kg). These compounds contribute to developing the immunostimulant and antioxidant properties for *M. sylvestris* and their preparations based on these natural compounds [5, 20]. It is proven that these flavonoids structures, along with other phenolic compounds, are present in higher amounts in the *M. sylvestris* flowers and have more effective antioxidant properties, as given in Tables 1 and 2. The antioxidant property was found to be more profound in flower extracts of *M. sylvestris* based on the results of the (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical method (DPPH assay) and ferric reducing antioxidant power assay (FRAP assay). The researchers further discovered more flavonoids and phenolic contents and antioxidants in leaves than in leafy flower stems and flowers when 95% ethanol was removed [21].

A major phytoalexin found in *M. sylvestris* was 2-methyl-3-methoxy-5,6-dihydroxy-1,4-naphthoquinone, known as malvone (Figure 2). Figure 3 shows some flavonoids that have a significant therapeutic effect.

1.2. Carbohydrate Content in M. sylvestris. Research has shown that most carbohydrates in plant materials derived from polysaccharides demonstrate an unknown mechanism during antioxidant activity. In animal experiments, these polysaccharides, especially pectins, are mainly found in plant tissues, show antioxidant and antidiabetic properties, and even adjust blood insulin, as given in Table 3.

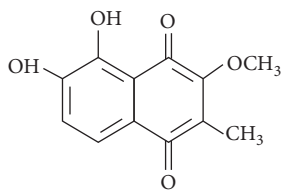


Figure 2: Chemical framework of malvone A, a phytoalexin found in *M. sylvestris* [7].

Table 1: Quantitative contents of organic acids in leaves of *M. sylvestris* [20].

Acid	Retention time (min)	Content (mg/kg)
Oxalic	8.88	4170.7
Malonic	11.13	1284.4
Fumaric	11.97	6924.8
Succinic	12.95	644.9
Benzoic	13.96	60.1
Glutaric	15.51	37.7
Phenylacetic	16.62	103.6
Salicylic	16.93	219.0
Malic	21.32	3510.0
Citric	28.46	13133.2
Vanillic	31.33	84.3
Ferulic	38.99	397.7
p-Coumaric	39.73	65.9

Table 2: *In vitro* antioxidant activity of *M. sylvestris* flowers and leaves, complete flavenoid content, and total phenolic content (TPC) [21].

Sample	TPC (mg GAE/g FW)	Total flavonoids (mg QE/g FW)	FRAP	DPPH
			mM TE/g FW	
Mallow leaves	1.42 ± 0.14	0.76 ± 0.19	4.04 ± 0.85	3.88 ± 0.51
Mallow flowers	6.32 ± 0.13	1.45 ± 0.21	6.01 ± 0.54	5.98 ± 0.43

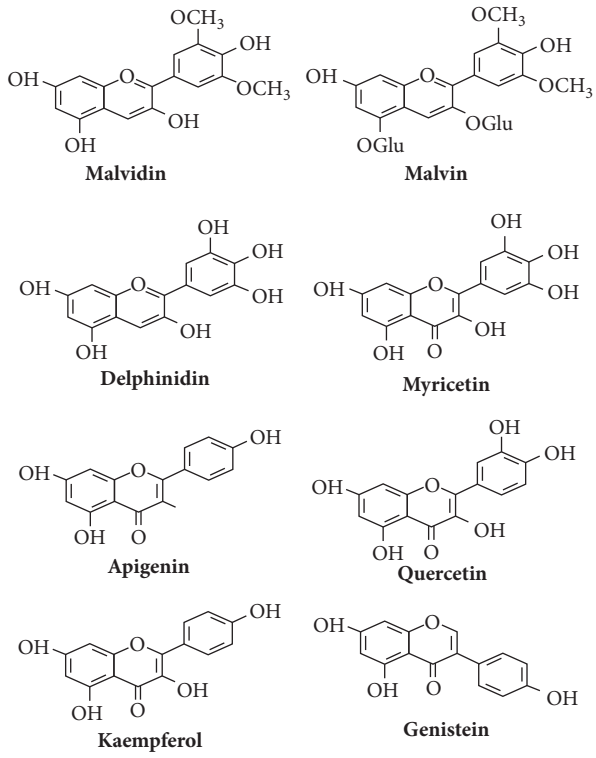


Figure 3: Some flavonoids of *M. sylvestris* [7].

The leaves are the richest in flavonoids, and this explains their therapeutic properties in traditional medicine.

1.3. Mucilages. The mucilages contain trehalose, galactose, sucrose, glucose, fructose, mannose, rhamnose, galacturonic, and glucuronic acid, but 2″-O-a-(4-O-methyl-a-d-glucuronosyl)-xylotriose, raffinose, fucose, xylose, arabinose, and uronic acid have also been found in *M. sylvestris*. It is considered an essential antimicrobial agent due to its resistance to the pathogen *Verticillium dahliae* [22].

1.4. Pigments. Qualitative analysis of acetone extracts from *M. sylvestris* has been done using chromatography. These assessments approve the presence of xanthophylls, chlorophyll B, and chlorophyll A [23].

1.5. Fatty Acids/Sterols. In *M. sylvestris* leaves, the presence of the stigmasterol, g-sitosterol, and the steroid campesterol has been reported [22]. The plant growth status affects the qualitative and quantitative constituents of these materials. Lipids exist separately in the flowering stems, immature fruits, flowers, and leaves [2]. These include tricosanoic acid, heneicosanoic acid (C20:3n3 + C21:0), lignoceric acid, 14-eicosadienoic acid, cis-11, behenic acid, arachidic acid,

Table 3: *In vitro* antioxidant activity, total flavenoid content, and total phenolic content of *M. sylvestris* leaves and flowers [21].

Sample	Fructose	Glucose	Sucrose	Reducing sugars	Total soluble carbohydrates
Mallow leaves	0.88	0.61	0.46	2.1	42.9
Mallow flowers	2.03	0.93	0.21	5.5	47.0

eicosenoic acid, a-linolenic acid, linoleic acid, heptadecanoic acid, palmitoleic acid, pentadecanoic acid, oleic acid, stearic acid, myristic acid, palmitic acid, myristoleic acid, lauric acid, capric acid, caprylic acid, and caproic acid. Extracts from leaf upon rapid cure with methanol and acetyl chloride contain 0.47% lipids and linolenic acid (42.21%). Because of the availability of indispensable fatty acids such as omega-3 and omega-6, *M. sylvestris* plays a pivotal role as a nutraceutical food. The consumption of omega-3 fatty acid compounds can prevent many diseases, such as coronary artery disease, diabetes, and cancer.

1.6. Chemical Elements. Assessment of the leaves of *M. sylvestris* has shown the presence of essential and nonessential metallic elements, halogens, and nonmetals. Analysis was performed using plasma optical emission spectrometry (ICP-OES), and the presence of Zr, Zn, U, Tl, Sr, Pb, Ni, Na, Mn, Mg, Sn, La, K, Si, Fe, Cu, Cr, Co, Ca, Bi, Ba, B, and Al was also shown [24]. *M. sylvestris* has exhibited a considerable ability to accumulate substantial metals (Zn, Pb, Ni, Cu, and Cd) from soils rich in these materials. Thus, it is crucial to address this issue in affected populations living in hazardous zones [25].

1.7. Vitamins. One of the natural properties of *M. sylvestris* is the human cell supplementation using ascorbic acids (vitamin C) and tocopherols (vitamin E). Vitamin E is considered a remarkable cancer prevention agent of the tocopherols in the human body [2, 26].

1.8. Enzymes. In the oxidative degradation of sulfur-containing amino acids, sulfite oxidase as an enzyme plays an integral role in ending the reaction (Figure 4). The absence of this enzyme might lead to death. Sulfite oxidase has additionally been discovered in the leaves of *M. sylvestris* and has been found in numerous bacteria and animal species [26–29]. Various phenolic derivatives have been found in extracts from different parts of *M. sylvestris* [26, 27].

1.9. Pharmacological Activity. M. sylvestris has been reported for use in the therapy of oral diseases. Anti-inflammatory and antimicrobial effects on the antimicrobial outcomes of ethanolic extracts from *M. sylvestris* stems were investigated in contrast to methicillin-resistant *Staphylococcus aureus* through biofilm adherence/formation tests and planktonic growth [30].

The biofilm foundation method showed that ethanolic stem extracts had medium activity in planktonic growth tests against *S. aureus* with bounded bacteriostatic effects [30–32]. Ethanolic extracts obtained from the inflorescences and leaves of *M. sylvestris* have a significant impact on

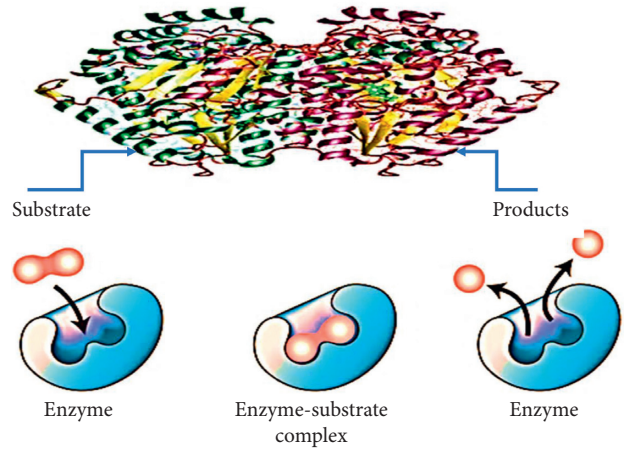


Figure 4: Mechanism of enzyme activity.

Helicobacter pylori. This bacterial strain plays an essential role in treating peptic ulcers and gastric cancers [33].

Hence, in the agar, diameter inhibition zones, crude methanolic extracts did not significantly inhibit strains of *Saccharomyces cerevisiae*, *Bordetella bronchiseptica*, *Candida albicans*, *Serratia marcescens*, *B. pumilus*, *B. cereus, B. subtilis*, *M. luteus*, *S. epidermidis*, *K. pneumonia*, *S. aureus*, *E. coli*, *P. fluorescens*, and *P. aeruginosa* [34–36]. The useability of *M. sylvestris* in mice considering the aqueous extract as an anti-inflammatory agent has been investigated. Research has shown that this type of extract might significantly reduce inflammation. The hydroalcoholic extract received from *M. sylvestris* leaves exhibited an anti-inflammation effect on croton oil-induced swelling in the ears of mice. The extract outcome has been proven by these facts [37].

The pharmacological activities of *M. sylvestris* are summarized in Table 4. Claimed patents are listed in Table 5. Other details related to the medicinal use of *M. sylvestris* are given in Table 6.

1.10. Traditional Uses of Malva Species. The traditional-ethnobotanical uses of *M. sylvestris* are given in Table 7.

Gas chromatography and mass spectroscopy analyses were carried out on compounds found in methanolic leaf extracts of *M. sylvestris*; results are shown in Table 8 [7].

1.11. Nutritional Values of Different Parts of the Plant. The investigation of the dietary arrangement of each one of those parts is necessary. The plant pieces and corresponding nutritional values are given in Table 9. Besides, Table 10 summarizes the sugar content in different parts of *M. sylvestris*.

Table 4: *M. sylvestris* pharmacological activities.

Activity	Models	Extract/pharmaceutical preparations	Findings
Biochemical profile	Extract intake in rats by drinking water	Aqueous extract from aerial parts	Bodyweight dosages (400 and 800 mg/kg) resulted in a significant rise in serum triglycerides, while other lipids, liver enzyme parameters, and glycaemic (alanine and aspartate transaminases, alkaline phosphatase, lactate dehydrogenase) were unaffected [35]
Bioadhesive mucous membranes	*Ex vivo* system (mucous membranes prepared buccal region tissue from killed pigs)	Aqueous extracts (flowers)	Less bioadhesion for epithelial tissue. Not feasible to correlate rehydration effects in this study, anti-irritative and anti-inflammatory [38]
Antiaging	Quantitative reverse transcriptase-PCR (polymerase chain reaction) and DNA macro array	Extract from seed	The rise in antioxidant gene expression [39]
Antimicrobial	Sequential dilution of plant extracts mixed with 1 ml of DPPH	Methanolic extracts (seeds), dichloromethane, and *n*-hexane	Antioxidant properties by thin-layer chromatography (TLC) qualitative plates test. For the DPPH test, no low activities for methanolic and *n*-hexane extracts were observed, and there was no activity for dichloromethane extract [40]
Anticancer	MTT test	Hydroalcoholic leaves extract	Notable proliferative reduction of A375 and B16 cancer cell lines [41]
Acetylcholinesterase (AChE)	The activity of enzymes evaluated at visible wavelengths	Ethanolic extract, essential oil fraction, decoction, and from aerial portions	No inhibitory observed through the use of the ethanolic extract, and 25% inhibited using 5 mg/ml of plant decoction; 28% of AChE inhibition by 0.1 mg/ml of essential oil [18]

Table 5: Pharmacological activities of *M. sylvestris* proclaimed in patents.

Activities	Extract/formulations	Findings
Skin whitening	*M. sylvestris* and other plant extracts	High pigmentation inhibition effect and excellent skin whitening [42]
Anti-inflammatory	Flowers' hydroalcoholic extract and associations	Antiulcer by topical application and anti-inflammatory [7]

Table 6: Other related medicinal uses of *M. sylvestris*.

General use	Parts used	Preparation	Specific use
Vaginal disorders	Flowers and leaves	Decoction	Vaginal itching [43]
Pain	Root and leaves	The vapor of decoction (*M. sylvestris* association)	Lumbar ache [44]
Urological disorders	Fruit	Infusion	Irritation of urinary organs, protector of bladder mucous [45]
Respiratory complaints	Fruit	Infusion	Cough [46]
	Aerial parts	Decoction	Respiratory diseases, cough, sore throat, bronchitis [47, 48]
	Leaves/flowers	Leaves/flowers	Pectoral asthma, spasmolytic, expectorant, cough, and emollient [49, 50]
	Whole plant	Infusion	Chronic bladder ulcer, bladder pains [46, 51, 52]
Inflammation	Leaves, flowers, and whole plant	A crushing plant	Rheumatism, the local application against arthritis [53]
Haemorrhoidal	Leaves	Vapour, infusion	Antihaemorrhoidal [54, 55]
Dermatological ailment	Flowers and leaves Decoction	Infusion, decoction Emollient	Astringent, acne [49, 56] Roots [57]

Table 6: Continued.

General use	Parts used	Preparation	Specific use
Menstrual pains	Roots	Decoction	Menstrual pain [58, 59]
	Flowers	Infusion	Dysmenorrhoea [60]
Gastrointestinal disturbance	Whole plant	Decoction	Laxative effects or depurative, against abdominal pains [61]
Other relevant uses	Roots	Decoction	Fever, abortion, weakness, hypertension, and menstrual pain [58, 59, 62]
	Flowers/leaves	Infusion	Soothing, sedative [49, 63]

Table 7: Traditional uses of *M. sylvestris*.

Country/ region	Used part/s	Use/s (reference)
Iran	Different parts	Cough, expectorant, clear the lung, lubricant, swellings, laxative [64], respiratory diseases of animals, immunomodulation [65]
Pakistan	Leaves	Unspecified method: relaxing activity, gastric mucus, anti-inflammatory, indigestion, diuretic, bladder ulcer [66]
Algeria	Flower	Infusion: antiseptic for the reproductive system, to treat canker sores, colds, constipation, asthma, otitis, colic, abdominal pain, astringent, antiseptic, softening, insect bites, swelling, boils, abscesses [67]
Turkey	Roots	Infusion: abortive [68]
Europe	Aerial parts	Constipation, diarrhea, rumination, tympanism, abdominal colic [69]
Italy	Leaf, root, flower	Leaves decoction or infusion: bronchitis, weight loss, cold, cough, cystitis, belly pain. Crushed leaves: toothache, whitlow [70]
Cyprus	Leaves	Consumed and cooked daily: antidiabetic [71]
India	Aerial parts Different parts of the whole plant	Stimulates the uterus, intestines, ulceration of urinary bladder, cough, enlargement of the spleen, jaundice, sore throat, anti-inflammatory, cooling, mucilaginous [72]. Eaten twice a day to strengthen weak eyesight [73, 74]
Brazil	Unspecified	Infusion: tonsillitis wound, rheumatism, uterine inflammation, boil, diuretic, cleanser [75]
Slovakia	Aerial parts	Food [76]
Syria	Flowers, leaves	Used as a laxative, respiratory infections, cough, mouth wash [77]
Portugal	Unspecified	Unspecified method: treatment of infections [61]
Spain	Aerial parts	Infusion: *Urtica dioica* stings and fever, bruises, wounds, laxative [62, 78], cold, kidney malfunction, dysmenorrhoea, gastralgia [60]
Morocco	Roots, leaves	Urinary or respiratory disorders, cataplasm or decoction [79]
Costa Rica	Whole plant	Unspecified method: ornamental [80]
Poland	Fruits	Eaten raw, immature
Lebanon	Flowers, leaves	Used to treat arthritis and rheumatism [66]

Table 8: Major phytochemical compounds detected in the methanolic extract of *M. sylvestris*.

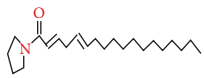
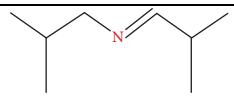
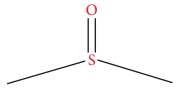
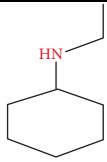
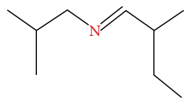
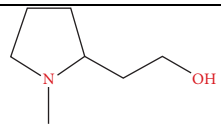
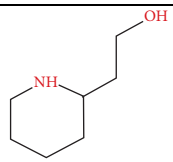
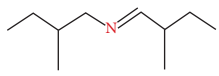
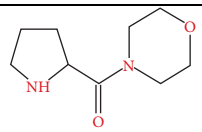
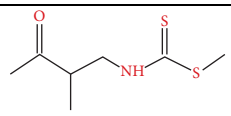
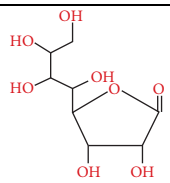
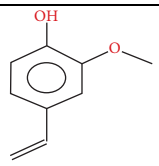
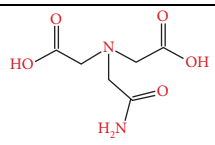
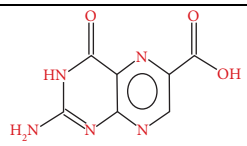
Serial no.	RT (min)	Molecular weight	Exact mass	Chemical structure	MS fragment ions	Pharmacological actions
1.	3.396	333	333.303165		55,81,98,113,150,220,264,333	Unknown
2.	3.218	127	127.1360993		57,84,112	Unknown
3.	3.476	78	78.013936		63,78	Anti-inflammatory and antioxidant
4.	3.590	127	127.1360993		55,71,84,98,127	Anti-inflammatory and antioxidant

Table 8: Continued.

Serial no.	RT (min)	Molecular weight	Exact mass	Chemical structure	MS fragment ions	Pharmacological actions
5.	3.877	141	141.15175		57,69,98,113,126	Antistereochemistry
6.	4.306	129	129.115364		55,84,98,129	Unknown
7.	4.449	129	129.115364		56,84,98,128	Antimicrobial, antimalarial, antibacterial
8.	4.563	155	155.167399		56,70,84,98,113,127,140,154	Antimicrobial activity
9.	4.649	184	184.121178		56,70,86,114,142	Antimicrobial activity
10.	5.215	191	191.043856		57,85,143,191	Antibacterial activity
11.	6.057	238	238.068868		61,73,84,112,127,142,159,189,220	Antibacterial activity
12.	7.041	150	150.06808		51,77,89,107,135	Antioxidant, antimicrobial, and anti-inflammatory
13.	8.025	190	190.058971		71,101,127,146,172,190	Unknown
14.	7.916	207	207.039239		57,69,105,149,163,207	Anticancer, antiviral

Gas chromatography-mass spectrometry (GC-MS) evaluation is a practical approach used for countless functions with the most excellent sensitivity and specificity. A volume of 1 μL methanol extract of *Malva sylvestris* was infused into the GC-MS and inspected typically for 45 minutes. The period since the infusion was made (initial time) to when washing occurred is referred to as the retention time (RT) [81, 82]. Helium fuel containing an eluent was used as a carrier [83].

1.12. Antioxidant Activity. *M. sylvestris* has antiradical properties due to high phenolic contents and is capable of preventing oxidation. Flavenoid compounds in this plant have high inhibitory power. These plants are also free of complications in comparison to chemical drugs [84]. The production of different oxygen species over the body's antioxidants causes oxidative stress. Evidence suggests that stress is one of the essential factors of aging in brain function, liver disease, cardiovascular disorders, and cancer [85].

Table 9: Energetic value (Kcal/100 g of dry weight), macronutrients' composition (g/100 g of dry weight), and moisture (g/100 g of fresh weight) of different *M. sylvestris* components [2].

	Leafy flowered	Immature fruits	Flowers	Leaves
Energy	372.43 ± 1.08 b	393.45 ± 4.41 a	372.02 ± 2.13 b	359.72 ± 1.10 c
Reduced sugars	10.46 ± 0.70 b	2.09 ± 0.12 d	13.95 ± 0.16 a	6.22 ± 0.49 c
Ash	10.76 ± 0.04 b	12.83 ± 0.78 a	10.54 ± 0.30 b	13.53 ± 0.11 a
Fat	3.09 ± 0.27 b	8.96 ± 0.22 a	2.84 ± 0.37 b	2.76 ± 0.40 b
Proteins	14.26 ± 0.44 a	3.26 ± 0.25 d	8.50 ± 0.51 c	12.25 ± 1.01 b
Carbohydrates	71.89 ± 0.35 c	74.96 ± 1.10 b	78.12 ± 0.44 a	71.46 ± 0.81 c
Moisture	77.26 ± 1.34 a	45.60 ± 0.97 d	72.49 ± 1.89 c	76.30 ± 0.54 b

Table 10: Sugar composition (g/100 g of dry weight) of numerous *M. sylvestris* components (mean ± SD; $n = 3$).

Leafy flowered stems	Immature fruits		Flowers	Leaves
Raffinose	*Nd*	0.26 ± 0.03 a	*Nd*	*Nd*
Trehalose	3.09 ± 0.03 a	*Nd*	1.47 ± 0.06 c	2.67 ± 0.11 b
Sucrose	3.30 ± 0.10 a	0.11 ± 0.03 d	2.47 ± 0.05 c	3.97 ± 0.03 b
Glucose	4.74 ± 0.18 b	1.52 ± 0.07 d	7.36 ± 0.13 a	3.15 ± 0.43 c
Fructose	3.53 ± 0.18 b	0.40 ± 0.03 d	8.72 ± 0.14 a	1.82 ± 0.23 c
Total sugars	14.67 ± 0.49 b	2.30 ± 0.10 d	20.02 ± 0.26 a	11.61 ± 0.51 c

In each row, different letters mean significant differences ($p < 0.05$) [2].

1.13. Anti-Inflammatory Activity. Several research groups have investigated *M. sylvestris* anti-inflammatory activity [35]. Their results support the notion that the compound malvidin 3-glucoside seems to be primarily accountable for this effect, and *M. sylvestris* leaves possess topical anti-inflammatory properties. The results of studies on the antimicrobial properties of *M. sylvestris* indicate that the plant also has antibacterial and antiviral activity against many human pathogens [86].

1.14. Anticancer Activity. Cancer is a generic term for a significant group of diseases that can affect any part of the body. Based on the report of World Health Organization (WHO), cancer is a leading cause of death universally. Reports show that *M. sylvestris* possesses anticancer properties. Daniela et al. [41] demonstrated cytotoxic activity of *M. sylvestris* leaf extracts on murine using an MTT assay and human cancer cell lines. The biological test found that *M. sylvestris* extracts significantly decrease cancer cell lines (Figure 5) [5, 41, 87, 88].

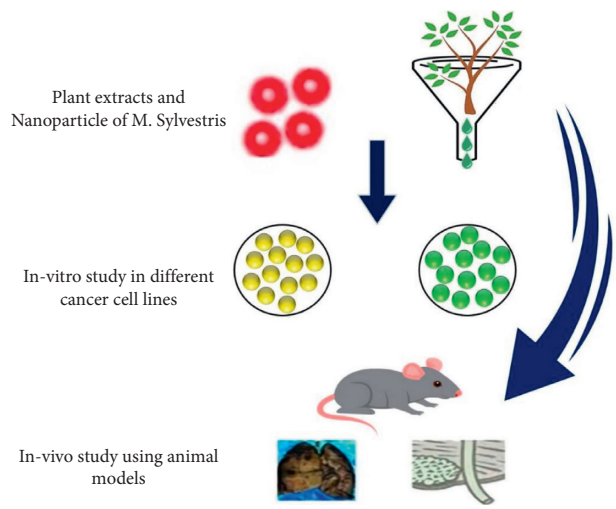


Figure 5: *In vivo* and *in vitro* anticancer activities of *M. sylvestris* against different types of cancer [41].

1.15. Wound Healing Activity. The topical application of the ethanolic hydroalcoholic extract of *Malva* leaves in a dose-dependent manner increases the rate of contraction of skin ulcers and reduces the duration of its repair process in rats. On the other hand, fiber plants are responsible for producing and secreting collagen. Protein collagens are a central extracellular matrix, which leads to an increase in the ability of wound edges to bind to each other.

1.16. Hepatoprotective Activity. The liver should be physiologically involved in all vital functions of the body. Any malfunction in the liver causes a set of disorders that can cause irreparable damage to this member; influential factors such as oxidative stress, free radicals, chemicals, viruses, and medicines can cause liver tissue degradation [89–91]. The literature confirmed the presence of antioxidant compounds in *M. sylvestris*. These compounds, in turn, remove the free radicals and help protect tissues, especially in the liver [92].

1.17. Antiosteoporosis Activity. Because of the imbalance between osteoblast and osteoclast activities, osteoporosis leads to weakening bone strength and elevation of fracture risk [93, 94]. *M. sylvestris* aqueous extracts can induce the activity of the signaling pathways and affect the osteoblast in an osteoclast difference [12, 86].

1.18. Antinociceptive Activity. The antinociceptive activity of *M. sylvestris* aqueous extracts was assessed against traditional pain models in mice by Esteves et al. [10]. Extensive

antinociceptive activity was demonstrated in the writhing test (76.4% of inhibition), as well as inhibition of inflammation (46.6%) and neurogenic (61.8%) phases of the formalin model. Their outcomes suggest that *M. sylvestris* possesses stimulating substances, which act as antinociceptive agents.

1.19. Antimicrobial Activity. *M. sylvestris* performs antimicrobial activities against various bacterial and fungal species. The disc diffusion method has reported the antimicrobial activity of *M. sylvestris* extracts against different bacterial species. The researchers found that *M. sylvestris* has moderate activity against selected microorganisms associated with typical antibiotics [95].

De Souza et al. [96] studied the antimicrobial activity of *M. sylvestris* aerial part extracts against *C. Albicans, S. aureus, M. luteus, Bacillus subtilis, S. epidermidis, E. coli,* and *S. cerevisiae* [97]. Their study reported that ethanol extracts of *M. sylvestris* were active against *P. aeruginosa*, *B. subtilis*, and *E. coli*, whereas methanol extracts showed activity only against *S. cerevisiae* [98]. Their results demonstrated that *M. sylvestris* extracts inhibited the *in vitro* microbial activity. Other studies showed that the seed oil inhibits the growth of all microorganisms tested except the Gram-negative bacteria *P. aeruginosa* [99–101].

1.20. Preventive Effect of M. sylvestris on Urinary Toxicity after Radiation Therapy in Prostate Cancer. *M. sylvestris* has a preventive effect on urinary toxicity after radiation therapy in prostate cancer in terms of relieving the pain related to external beam radiation therapy- (EBRT-) induced urinary toxicity. Up-to-date radiotherapy techniques, for instance, three-dimensional conformal radiation therapy (3D-CRT) and intensity-modulated radiation therapy (IMRT), can reduce genitourinary and gastrointestinal toxicity induced by EBRT [102].

1.21. Antifungal Assay. The antifungal activities of the plant extracts were the same against *Penicillium* spp., *C. Albicans*, *Aspergillus niger*, *Candida kefir*, and *Sclerotinia sclerotiorum* by the circle dissemination technique. Amphotericin B (10 μg) was considered a positive control, and the plates were cultured at 30°C for 48 hours. The minimal inhibitory concentrations (MICs) of the concentrates against the test microorganisms were controlled by the agar diffusion strategy [3, 103, 104].

1.22. Healing of Atopic Dermatitis. *M. sylvestris* is the most common dermatological ailment treatment, for example, atopic dermatitis; however, conventional therapeutics, such as corticosteroids and antihistamines, have no effects [105]. Natural agents, which generally have no extensive side effects, could be used to determine its efficacy. In this study, its effectiveness in treating atopic dermatitis was assessed and it could topically be used as an effective cream to reduce the dermatitis symptoms in children.

2. Conclusion

This review showed the significance of *M. sylvestris* as a medicinal herb and functional food. Findings indicate that relatively extensive research has been carried out on chemical compounds and pharmacological effects, as well as different aspects of the *Malva* plant. *M. sylvestris* is an important resourceful plant because of its effective medicinal properties. Studies have proven its potential for health benefits due to its antioxidant activity, anti-inflammatory activity, anticancer activity, wound-healing activity, hepatoprotective activity, antinociceptive activity, and antimicrobial activity. The leaves, flowers, and roots are used for medicinal reasons. Herein, one-of-its-kind organic activities of *M. sylvestris* L., traditional uses, main phytochemical compounds detected in methanolic extracts, and pharmacological activities of *M. sylvestris* were reviewed.

Data Availability

All the data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

References

[1] F. Alizadeh and A. Khodavandi, "Malva sylvestris inhibits Candida albicans biofilm formation," *Journal of Herbmed Pharmacology*, vol. 6, 2016.

[2] L. Barros, A. M. Carvalho, and I. C. F. R. Ferreira, "Leaves, flowers, immature fruits and leafy flowered stems of *Malva sylvestris*: a comparative study of the nutraceutical potential and composition," *Food and Chemical Toxicology*, vol. 48, no. 6, pp. 1466–1472, 2010.

[3] S. M. Razavi, G. Zarrini, G. Molavi, and G. Ghasemi, "Bioactivity of *Malva sylvestris L.*, a medicinal plant from Iran," *Iranian journal of basic medical sciences*, vol. 14, no. 6, pp. 574–9, 2011.

[4] P. M. Guarrera, "Traditional phytotherapy in Central Italy (Marche, Abruzzo, and latium)," *Fitoterapia*, vol. 76, no. 1, pp. 1–25, 2005.

[5] S. Mazraedoost and G. Behbudi, "Nano materials-based devices by photodynamic therapy for treating cancer applications," *Advances in Applied NanoBio-Technologies*, vol. 2, no. 3, pp. 9–21, 2021.

[6] A. G. Pirbalouti, S. Azizi, A. Koohpayeh, and B. Hamedi, "Wound healing activity of *Malva sylvestris* and Punica granatum in alloxan-induced diabetic rats," *Acta Poloniae Pharmaceutica*, vol. 67, no. 5, pp. 511–516, 2010.

[7] J. C. Gasparetto, C. A. F. Martins, S. S. Hayashi, M. F. Otuky, and R. Pontarolo, "Ethnobotanical and scientific aspects of *Malva sylvestris L.*: a millennial herbal medicine," *Journal of Pharmacy and Pharmacology*, vol. 64, no. 2, pp. 172–189, 2012.

[8] A. Farina, A. Doldo, V. Cotichini et al., "HPTLC and reflectance mode densitometry of anthocyanins in *Malva silvestris L.*: a comparison with gradient-elution reversed-phase HPLC," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 14, no. 1-2, pp. 203–211, 1995.

[9] N. Yeole, P. Sandhya, P. Chaudhari, and P. Bhujbal, "Evaluation of *Malva sylvestris* and Pedalium murex mucilage as suspending agent," *International Journal of PharmTech Research*, vol. 2, no. 1, pp. 385–389, 2010.

[10] P. F. Esteves, A. Sato, M. A. Esquibel, F. De Campos-Buzzi, A. V. Meira, and V. Cechinel-Filho, "Antinociceptive activity of *Malva sylvestris L*," *Latin American Journal of Pharmacy*, vol. 28, no. 3, pp. 454–456, 2009.

[11] C. Ana Maria, *Etnobotánica del Parque Natural de Montesinho Plantas, Tradición y Saber Popular en un Territorio del Nordeste de Portugal*, Universidad Autónoma de Madrid, Madrid, Spain, 2005.

[12] B. Benso, M. Franchin, A. P. Massarioli et al., "Anti-inflammatory, anti-osteoclastogenic and antioxidant effects of Malva sylvestris extract and fractions: in vitro and in vivo studies," *PLoS One*, vol. 11, no. 9, p. e0162728, 2016.

[13] C. Martins, M. Campos, A. Irioda, D. Stremel, A. Trindade, and R. Pontarolo, "Anti-inflammatory effect of Malva sylvestris, Sida cordifolia, and Pelargonium graveolens is related to inhibition of prostanoid production," *Molecules*, vol. 22, no. 11, p. 1883, 2017.

[14] S. M. Mousavi, S. A. Hashemi, M. Zarei et al., "Data on cytotoxic and antibacterial activity of synthesized Fe_3O_4 nanoparticles using *Malva sylvestris*," *Data in brief*, vol. 28, p. 104929, 2020.

[15] A. Gholami, S. Shahin, M. Mohkam, N. Nezafat, and Y. Ghasemi, "Cloning, characterization and bioinformatics analysis of novel cytosine deaminase from *Escherichia coli* AGH09," *International Journal of Peptide Research and Therapeutics*, vol. 21, no. 3, pp. 365–374, 2015.

[16] Z. Mohamadi Yarijani, H. Najafi, D. Shackebaei, S. H. Madani, M. Modarresi, and S. V. Jassemi, "Amelioration of renal and hepatic function, oxidative stress, inflammation and histopathologic damages by *Malva sylvestris* extract in gentamicin induced renal toxicity," *Biomedicine & Pharmacotherapy*, vol. 112, p. 108635, 2019.

[17] M. Wichtl, *Herbal Drugs and Phytopharmaceuticals*, CRC Press, Boca Roton, FL, USA, 1994.

[18] O. Veshkurova, Z. Golubenko, E. Pshenichnov et al., "Malvone A, a phytoalexin found in Malva sylvestris (family Malvaceae)," *Phytochemistry*, vol. 67, no. 21, pp. 2376–2379, 2006.

[19] M. A. M. Nawwar and J. Buddrus, "A gossypetin glucuronide sulphate from the leaves of *Malva sylvestris*," *Phytochemistry*, vol. 20, no. 10, pp. 2446–2448, 1981.

[20] I. I. Terninko and U. E. Onishchenko, "Component composition of organic acids in leaves of Malva sylvestris," *Chemistry of Natural Compounds*, vol. 49, no. 2, pp. 332-333, 2013.

[21] N. Petkova, A. Popova, and I. Alexieva, "Antioxidant properties and some phytochemical components of the edible medicinal *Malva sylvestris L*," *Journal of Medicinal Plants*, vol. 7, no. 1, pp. 96–99, 2019.

[22] M. D. G. T. D. Toledo, Y. Alquini, and T. Nakashima, "Caracterização anatômica das folhas de Cunila microcephala Benth. (Lamiaceae)," *Revista Brasileira de Ciencias Farmaceuticas*, vol. 40, no. 4, pp. 487–493, 2004.

[23] S. Redžić, N. Hodžić, and M. Tuka, "Plant pigments (antioxidants) of medicinal plants *Malva sylvestris L.* and *Malva moschata L.*(Malvaceae)," *Bosnian Journal of Basic Medical Sciences*, vol. 5, no. 2, pp. 53–58, 2005.

[24] U. Hiçsönmez, F. S. Ereeş, C. Özdemir, A. Özdemir, and S. Cam, "Determination of major and minor elements in the *Malva sylvestris L.* from Turkey using ICP-OES techniques," *Biological Trace Element Research*, vol. 128, no. 3, pp. 248–257, 2009.

[25] D. Desideri, M. A. Meli, and C. Roselli, "Determination of essential and non-essential elements in some medicinal plants by polarised X ray fluorescence spectrometer (EDPXRF)," *Microchemical Journal*, vol. 95, no. 2, pp. 174–180, 2010.

[26] H. Najafi, Z. Mohamadi Yarijani, S. Changizi-Ashtiyani et al., "Protective effect of *Malva sylvestris L.* extract in ischemia-reperfusion induced acute kidney and remote liver injury," *PLoS One*, vol. 12, no. 11, p. e0188270, 2017.

[27] B. A. Ganai, A. Masood, and M. A. Baig, "Isolation, purification and partial characterization of sulphite oxidase from Malva sylvestris," *Phytochemistry*, vol. 45, no. 5, pp. 879-880, 1997.

[28] S. N. Abootalebi, A. Saeed, A. Gholami et al., "Screening, characterization and production of thermostable alpha-amylase produced by a novel thermophilic Bacillus megaterium isolated from pediatric intensive care unit," *Journal of Environmental Treatment Techniques*, vol. 8, no. 3, pp. 952–960, 2020.

[29] A. Gholami, F. Mohammadi, Y. Ghasemi, N. Omidifar, and A. Ebrahiminezhad, "Antibacterial activity of SPIONs versus ferrous and ferric ions under aerobic and anaerobic conditions: a preliminary mechanism study," *IET Nanobiotechnology*, vol. 14, no. 2, pp. 155–160, 2019.

[30] N. J. Willey and K. Fawcett, "Inter-taxa differences in root uptake of 103/106Ru by plants," *Journal of Environmental Radioactivity*, vol. 86, no. 2, pp. 227–240, 2006.

[31] N. Goudarzian, Z. Sadeghi, S. M. Mousavi, S. A. Hashemi, and N. Banaei, "Chemical constituent and determination of antimicrobial and antifungal activities of Ulva lactuca species obtained from Iranian Gheshm Island," *International Journal of Scientific Engineering and Research*, vol. 8, pp. 1275–1279, 2017.

[32] A. Gholami, S. Rasoul-Amini, A. Ebrahiminezhad et al., "Magnetic properties and antimicrobial effect of amino and lipoamino acid coated iron oxide nanoparticles," *Minerva Biotecnologica*, vol. 28, no. 4, pp. 177–186, 2016.

[33] S. Ahmadi, "The importance of silver nanoparticles in human life," *Advances in Applied NanoBio-Technologies*, vol. 1, no. 1, pp. 5–9, 2020.

[34] A. Magro, M. Carolino, M. Bastos, and A. Mexia, "Efficacy of plant extracts against stored products fungi," *Revista Iberoamericana De Micologia*, vol. 23, no. 3, pp. 176–178, 2006.

[35] N. Sleiman and C. Daher, "Malva sylvestris water extract: a potential anti-inflammatory and anti-ulcerogenic remedy," *Planta Medica*, vol. 75, no. 9, p. PH10, 2009.

[36] M. Mohkam, S. Rasoul-Amini, D. Shokri et al., "Characterization and in vitro probiotic assessment of potential indigenous Bacillus strains isolated from soil rhizosphere," *Minerva Biotechnologica*, vol. 28, no. 1, pp. 19–28, 2016.

[37] C. R. Wilson, "Incidence of weed reservoirs and vectors of tomato spotted wilt tospovirus on Southern Tasmanian lettuce farms," *Plant Pathology*, vol. 47, no. 2, pp. 171–176, 1998.

[38] J. Schmidgall, E. Schnetz, and A. Hensel, "Evidence for bioadhesive effects of polysaccharides and polysaccharide-containing herbs in an ex vivo bioadhesion assay on buccal membranes," *Planta Medica*, vol. 66, no. 1, pp. 48–53, 2000.

[39] S. Talbourdet, N. S. Sadick, K. Lazou et al., "Modulation of gene expression as a new skin anti-aging strategy," *Journal of Drugs in Dermatology: Journal of Drugs in Dermatology*, vol. 6, no. 6 Suppl, pp. s25–33, 2007.

[40] Y. Kumarasamy, M. Byres, P. J. Cox, M. Jaspars, L. Nahar, and S. D. Sarker, "Screening seeds of some Scottish plants for free radical scavenging activity," *Phytotherapy Research*, vol. 21, no. 7, pp. 615–621, 2007.

[41] A. Daniela, E. Pichichero, L. Canuti et al., "Identification of phenolic compounds from medicinal and melliferous plants and their cytotoxic activity in cancer cells," *Caryologia*, vol. 60, no. 1-2, pp. 90–95, 2007.

[42] A. Yagi, "14 bioactivity of aloe arborescens preparations," *Aloes: The genus Aloe*, vol. 333, 2004.

[43] A. M. Scherrer, R. Motti, and C. S. Weckerle, "Traditional plant use in the areas of monte vesole and ascea, cilento national park (Campania, Southern Italy)," *Journal of Ethnopharmacology*, vol. 97, no. 1, pp. 129–143, 2005.

[44] R. Motti, V. Antignani, and M. Idolo, "Traditional plant use in the phlegraean fields regional park (Campania, Southern Italy)," *Human Ecology*, vol. 37, no. 6, pp. 775–782, 2009.

[45] E. Miraldi, S. Ferri, and V. Mostaghimi, "Botanical drugs and preparations in the traditional medicine of West Azerbaijan (Iran)," *Journal of Ethnopharmacology*, vol. 75, no. 2-3, pp. 77–87, 2001.

[46] M. Ishtiaq, W. Hanif, M. A. Khan, M. Ashraf, and A. M. Butt, "An ethnomedicinal survey and documentation of important medicinal folklore food phytonims of flora of samahni valley, (Azad kashmir) Pakistan," *Pakistan Journal of Biological Sciences*, vol. 10, no. 13, pp. 2241–2256, 2007.

[47] M. R. González-Tejero, M. Casares-Porcel, C. P. Sánchez-Rojas et al., "Medicinal plants in the Mediterranean area: synthesis of the results of the project Rubia," *Journal of Ethnopharmacology*, vol. 116, no. 2, pp. 341–357, 2008.

[48] S. Al-Qura'n, "Ethnopharmacological survey of wild medicinal plants in Showbak, Jordan," *Journal of Ethnopharmacology*, vol. 123, no. 1, pp. 45–50, 2009.

[49] S. Rehecho, I. Uriarte-Pueyo, J. Calvo, L. A. Vivas, and M. I. Calvo, "Ethnopharmacological survey of medicinal plants in nor-yauyos, a part of the landscape reserve nor-yauyos-cochas, Peru," *Journal of Ethnopharmacology*, vol. 133, no. 1, pp. 75–85, 2011.

[50] R. Joshi, P. Satyal, and W. Setzer, "Himalayan aromatic medicinal plants: a review of their ethnopharmacology, volatile phytochemistry, and biological activities," *Medicines*, vol. 3, no. 1, p. 6, 2016.

[51] M. Leonti, L. Casu, F. Sanna, and L. Bonsignore, "A comparison of medicinal plant use in Sardinia and Sicily-De materia medica revisited?" *Journal of Ethnopharmacology*, vol. 121, no. 2, pp. 255–267, 2009.

[52] A. Touwaide, N. G. De Santo, and G. Aliotta, "The origins of Western herbal medicines for kidney diseases," *Advances in Chronic Kidney Disease*, vol. 12, no. 3, pp. 251–260, 2005.

[53] A. Nelly, D.-D. Annick, and D. Frederic, "Plants used as remedies antirheumatic and antineuralgic in the traditional medicine of Lebanon," *Journal of Ethnopharmacology*, vol. 120, no. 3, pp. 315–334, 2008.

[54] M. Ballero, F. Poli, G. Sacchetti, and M. C. Loi, "Ethnobotanical research in the territory of Fluminimaggiore (South-Western Sardinia)," *Fitoterapia*, vol. 72, no. 7, pp. 788–801, 2001.

[55] A. Pieroni, S. Nebel, C. Quave, H. Münz, and M. Heinrich, "Ethnopharmacology of liakra: traditional weedy vegetables of the Arbëreshë of the Vulture area in Southern Italy," *Journal of Ethnopharmacology*, vol. 81, no. 2, pp. 165–185, 2002.

[56] A. De Natale and A. Pollio, "Plants species in the folk medicine of Montecorvino Rovella (Inland Campania, Italy)," *Journal of Ethnopharmacology*, vol. 109, no. 2, pp. 295–303, 2007.

[57] P. M. Guarrera, G. Salerno, and G. Caneva, "Folk phytotherapeutical plants from Maratea area (Basilicata, Italy)," *Journal of Ethnopharmacology*, vol. 99, no. 3, pp. 367–378, 2005.

[58] M. C. Loi, F. Poli, G. Sacchetti, M. B. Selenu, and M. Ballero, "Ethnopharmacology of ogliastra (Villagrande Strisaili, Sardinia, Italy)," *Fitoterapia*, vol. 75, no. 3-4, pp. 277–295, 2004.

[59] Ş. Kültür, "Medicinal plants used in Kırklareli Province (Turkey)," *Journal of Ethnopharmacology*, vol. 111, no. 2, pp. 341–364, 2007.

[60] G. Benítez, M. R. González-Tejero, and J. Molero-Mesa, "Pharmaceutical ethnobotany in the western part of Granada province (southern Spain): ethnopharmacological synthesis," *Journal of Ethnopharmacology*, vol. 129, no. 1, pp. 87–105, 2010.

[61] M. Idolo, R. Motti, and S. Mazzoleni, "Ethnobotanical and phytomedicinal knowledge in a long-history protected area, the Abruzzo, lazio and molise national park (Italian Apennines)," *Journal of Ethnopharmacology*, vol. 127, no. 2, pp. 379–395, 2010.

[62] J. A. González, M. García-Barriuso, and F. Amich, "Ethnobotanical study of medicinal plants traditionally used in the Arribes del Duero, Western Spain," *Journal of Ethnopharmacology*, vol. 131, no. 2, pp. 343–355, 2010.

[63] M. L. Leporatti and S. Ivancheva, "Preliminary comparative analysis of medicinal plants used in the traditional medicine of Bulgaria and Italy," *Journal of Ethnopharmacology*, vol. 87, no. 2-3, pp. 123–142, 2003.

[64] A. Ameri, G. Heydarirad, J. Mahdavi Jafari, A. Ghobadi, H. Rezaeizadeh, and R. Choopani, "Medicinal plants contain mucilage used in traditional Persian medicine (TPM)," *Pharmaceutical Biology*, vol. 53, no. 4, pp. 615–623, 2015.

[65] A. Ghorbani, M. Aghjeh Qeshlagh, and S. Karamati Jabehdar, "Folk herbal veterinary medicines of Tehran Watershed (Iran)," *Journal of Herbal Drugs (An International Journal on Medicinal Herbs)*, vol. 6, no. 1, pp. 31–39, 2015.

[66] A. Azab, "Malva: food, medicine and chemistry," *European Chemical Bulletin*, vol. 6, no. 7, pp. 295–320, 2017.

[67] S. Boubakr, L. Ali, M. Zoheir, H. Zahra, D. Mohamed, and A. Boukeur, "Global journal of medicinal plant research," *Global Journal of Medicinal Plant Research*, vol. 3, no. 5, pp. 1–16, 2015.

[68] G. Bulut and E. Tuzlacı, "An ethnobotanical study of medicinal plants in Bayramiç," *Marmara Pharmaceutical Journal*, vol. 19, no. 3, pp. 268–282, 2015.

[69] M. Mayer, C. R. Vogl, M. Amorena, M. Hamburger, and M. Walkenhorst, "Treatment of organic livestock with medicinal plants: a systematic review of European ethnoveterinary research," *Complementary Medicine Research*, vol. 21, no. 6, pp. 375–386, 2014.

[70] B. Menale and R. Muoio, "Use of medicinal plants in the south-eastern area of the partenio regional park (Campania, Southern Italy)," *Journal of Ethnopharmacology*, vol. 153, no. 1, pp. 297–307, 2014.

[71] H. Z. Toklu, "Herbal medicine use among diabetes mellitus patients in Northern Cyprus," *Journal of Medicinal Plants Research*, vol. 7, no. 22, pp. 1652–1664, 2013.

[72] A. Javed, M. Usman, S. M. Haider, B. Zafar, and K. Iftikhar, "Potential of indigenous plants for skin healing and care," *American Scientific Research Journal for Engineering,*

Technology, and Sciences (ASRJETS), vol. 51, no. 1, pp. 192–211, 2019.

[73] G. Hassan, T. Ahmad, and R. Mohi-Ud-Din, "An ethnobotanical study in Budgam district of Kashmir valley: an attempt to explore and document traditional knowledge of the area," *International Research Journal of Pharmacy*, vol. 4, pp. 201–204, 2013.

[74] N. Kumar, Z. Wani, and S. Dhyani, "Ethnobotanical study of the plants used by the local people of Gulmarg and its allied areas, Jammu & Kashmir, India," *International Journal of Current Research in Biosciences and Plant Biology*, vol. 2, no. 9, pp. 16–23, 2015.

[75] I. G. C. Bieski, F. Rios Santos, R. M. De Oliveira et al., "Ethnopharmacology of medicinal plants of the pantanal region (Mato Grosso, Brazil)," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 272749, 2012.

[76] L. Luczaj, "Ethnobotanical review of wild edible plants of Slovakia," *Acta Societatis Botanicorum Poloniae*, vol. 81, no. 4, 2012.

[77] A. Alachkar, A. Jaddouh, M. S. Elsheikh, A. R. Bilia, and F. F. Vincieri, "Traditional medicine in Syria: folk medicine in Aleppo governorate," *Natural Product Communications*, vol. 6, no. 1, p. 84, 2011.

[78] M. Parada, E. Carrió, and J. Vallès, "Ethnobotany of food plants in the Alt emporda region (Catalonia, Iberian Peninsula)," *Journal of Applied Botany and Food Quality*, vol. 84, no. 1, pp. 11–25, 2011.

[79] H. Ouhaddou, H. Boubaker, F. Msanda, and A. El Mousadik, "An ethnobotanical study of medicinal plants of the Agadir Ida Ou Tanane province (Southwest Morocco)," *Journal of Applied Biosciences*, vol. 84, no. 1, pp. 7707–7722, 2014.

[80] M. Kappelle, G. Avertin, M. E. Juárez, and N. Zamora, "Useful plants within a campesino community in a Costa Rican montane cloud forest," *Mountain Research and Development*, vol. 20, no. 2, pp. 162–171, 2000.

[81] N. Altaee, M. J. Kadhim, and I. H. Hameed, "Characterization of metabolites produced by *E. coli* and analysis of its chemical compounds using GC-MS," *International Journal of Current Pharmaceutical Review and Research*, vol. 7, no. 6, pp. 13–19, 2017.

[82] M. Y. Hadi and I. H. Hameed, "Uses of gas chromatography-mass spectrometry (GC-MS) technique for analysis of bioactive chemical compounds of lepidium sativum: a review," *Research Journal of Pharmacy and Technology*, vol. 10, no. 11, pp. 4039–4042, 2017.

[83] M. Jawad Kadhim, G. J. Mohammed, and I. Hadi Hameed, "In vitro antibacterial, antifungal and phytochemical analysis of methanolic extract of fruit Cassia fistula," *Oriental Journal of Chemistry*, vol. 32, no. 3, pp. 1329–1346, 2016.

[84] W. Marouane, A. Soussi, J.-C. Murat, S. Bezzine, and A. El Feki, "The protective effect of Malva sylvestris on rat kidney damaged by vanadium," *Lipids in Health and Disease*, vol. 10, no. 1, p. 65, 2011.

[85] N. Jaradat, M. Abualhasan, and I. Ali, "Comparison of antioxidant activities and exhaustive extraction yields between wild and cultivated cyclamen persicum, Malva sylvestris and Urtica pilulifera leaves," *Journal of Applied Pharmaceutical Science*, vol. 5, no. 4, pp. 101–106, 2015.

[86] B. Benso, P. L. Rosalen, S. M. Alencar, and R. M. Murata, "Malva sylvestris inhibits inflammatory response in oral human cells. An in vitro infection model," *PLoS One*, vol. 10, no. 10, p. e0140331, 2015.

[87] A. Abbaszadegan, A. Gholami, Y. Ghahramani et al., "Antimicrobial and cytotoxic activity of cuminum cyminum as an intracanal medicament compared to chlorhexidine gel," *Iranian Endodontic Journal*, vol. 11, no. 1, pp. 44–50, 2016.

[88] S. Mazraedoost and G. Behbudi, "Basic nano magnetic particles and essential oils: biological applications," *Journal of Environmental Treatment Techniques*, vol. 9, no. 3, pp. 609–620, 2021.

[89] A. Azarang, O. Farshad, M. M. Ommati et al., "Protective role of probiotic supplements in hepatic steatosis: a rat model study," *BioMed Research International*, vol. 2020, Article ID 5487659, 2020.

[90] N. Omidifar, A. Nili-Ahmadabadi, A. Gholami, D. Dastan, D. Ahmadimoghaddam, and H. Nili-Ahmadabadi, "Biochemical and histological evidence on the protective effects of *Allium hirtifolium boiss* (Persian shallot) as an herbal supplement in cadmium-induced hepatotoxicity," *Evidence-Based Complementary and Alternative Medicine: ECAM*, vol. 2020, Article ID 7457504, 2020.

[91] A. Gholami, F. Emadi, M. Nazem et al., "Expression of key apoptotic genes in hepatocellular carcinoma cell line treated with etoposide-loaded graphene oxide," *Journal of Drug Delivery Science and Technology*, vol. 57, p. 101725, 2020.

[92] L. Hussain, J. Ikram, K. Rehman, M. Tariq, M. Ibrahim, and M. S. H. Akash, "Hepatoprotective effects of *Malva sylvestris L.* against paracetamol-induced hepatotoxicity," *Turkish Journal of Biology*, vol. 38, no. 3, pp. 396–402, 2014.

[93] N. Montazeri-Najafabady, Y. Ghasemi, M. H. Dabbaghmanesh et al., "Exploring the bone sparing effects of postbiotics in the post-menopausal rat model," *BMC Complementary Medicine and Therapies*, vol. 21, no. 1, p. 155, 2021.

[94] N. Montazeri-Najafabady, Y. Ghasemi, M. H. Dabbaghmanesh, P. Talezadeh, F. Koohpeyma, and A. Gholami, "Supportive role of probiotic strains in protecting rats from ovariectomy-induced cortical bone loss," *Probiotics and Antimicrobial Proteins*, vol. 11, no. 4, pp. 1145–1154, 2019.

[95] B. Dulger and A. Gonuz, "Antimicrobial activity of certain plants used in Turkish traditional medicine," *Asian Journal of Plant Sciences*, vol. 3, no. 1, pp. 104–107, 2004.

[96] G. Coelho De Souza, A. P. S. Haas, G. L. Von Poser, E. E. S. Schapoval, and E. Elisabetsky, "Ethnopharmacological studies of antimicrobial remedies in the south of Brazil," *Journal of Ethnopharmacology*, vol. 90, no. 1, pp. 135–143, 2004.

[97] L. L. Cogo, C. L. B. Monteiro, M. D. Miguel et al., "Anti-Helicobacter pylori activity of plant extracts traditionally used for the treatment of gastrointestinal disorders," *Brazilian Journal of Microbiology*, vol. 41, no. 2, pp. 304–309, 2010.

[98] C.-L. Cheng and Z.-Y. Wang, "Bacteriostasic activity of anthocyanin of Malva sylvestris," *Journal of Forestry Research*, vol. 17, no. 1, pp. 83–85, 2006.

[99] C. Walter, Z. K. Shinwari, I. Afzal, and R. N. Malik, "Antibacterial activity in herbal products used in Pakistan," *Pakistan Journal of Botany*, vol. 43, pp. 155–162, 2011.

[100] S. M. Mousavi, S. A. Hashemi, M. Zarei et al., "Recent progress in chemical composition, production, and pharmaceutical effects of kombucha beverage: a complementary and alternative medicine," *Evidence-Based Complementary and Alternative Medicine*, vol. 2020, Article ID 4397543, 2020.

[101] A. Gholami, S. A. Hashemi, K. Yousefi et al., "3D nanostructures for tissue engineering, cancer therapy, and gene delivery," *Journal of Nanomaterials*, vol. 2020, Article ID 1852946, 2020.

[102] S. M. Razavi and G. Zarrini, "Bioactivity of aviprin and aviprin-3″-O-glucoside, two linear furanocoumarins from Apiaceae," *Russian Journal of Bioorganic Chemistry*, vol. 36, no. 3, pp. 359–362, 2010.

[103] M. Nabavizadeh, A. Abbaszadegan, A Gholami et al., "Chemical constituent and antimicrobial effect of essential oil from Myrtus communis leaves on microorganisms involved in persistent endodontic infection compared to two common endodontic irrigants: an in vitro study," *Journal of Conservative Dentistry: Journal of Computational Dynamics*, vol. 17, no. 5, pp. 449–453, 2014.

[104] A. Abbaszadegan, S. Sahebi, A. Gholami et al., "Time-dependent antibacterial effects of aloe vera and Zataria multiflora plant essential oils compared to calcium hydroxide in teeth infected with *Enterococcus faecalis*," *Journal of Investigative and Clinical Dentistry*, vol. 7, no. 1, pp. 93–101, 2016.

[105] M. Meysami, M. H. Hashempur, M. Kamalinejad, and M. Emtiazy, "Efficacy of short term topical *Malva sylvestris L.* Cream in pediatric patients with atopic dermatitis: a randomized double-blind PlaceboControlled clinical trial," *Endocrine, Metabolic & Immune Disorders-Drug Targets*, vol. 20, 2020.

Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2021, Article ID 6814215, 14 pages
https://doi.org/10.1155/2021/6814215

Review Article

Potential and Prophylactic Use of Plants Containing Saponin-Type Compounds as Antibiofilm Agents against Respiratory Tract Infections

I. Irem Tatli Cankaya [1] **and E. Inci Somuncuoglu** [2]

[1] *Hacettepe University, Faculty of Pharmacy, Department of Pharmaceutical Botany, Sıhhiye, Ankara, Turkey*
[2] *Department of Health Services, Ministry of Health, Ankara, Turkey*

Correspondence should be addressed to I. Irem Tatli Cankaya; itatli@hacettepe.edu.tr

Received 11 April 2021; Revised 5 June 2021; Accepted 14 July 2021; Published 26 July 2021

Academic Editor: Gokhan Zengin

Epidemic diseases have been observed in every period of human history, and the treatment process has taken time. Causative microorganisms reproduce as biofilm and contribute to the emergence of various infectious diseases. The process that starts with respiratory disorders causes serious lung infections due to bacteria and viruses that accumulate and multiply. The biofilms are difficult to eliminate and show increased resistance to available antimicrobial agents. There is a need to identify and develop potential resources used in treatment. The search for novel biological agents from plants is gaining popularity due to the high abundance, accessibility with consequent lower cost for discovery, and lesser side effects and toxicity. Saponins found in some plants can be alternative to antibiotics, with antimicrobial activities. This review focused on the potency of saponin-containing plants with antimicrobial properties as antibiofilm agents against these infections. For this purpose, keywords were scanned in Web of Science, Scopus, and Google academics databases, and the related literature was compiled. Approximately, 25 plant taxa belonging to 18 families traditionally used in the treatment of respiratory diseases are listed. These taxa mostly belong to Fabaceae, Asteraceae, Apiaceae, and Asparagaceae families, respectively. Most of these taxa have antibacterial, antifungal, antitussive, and anti-inflammatory activities. Especially, plants with antibiofilm activity that can be effective against many microorganisms are compiled in this study. These plants can prevent or treat upper respiratory tract diseases caused by bacteria due to the phytochemicals they contain, especially saponins.

1. Introduction

Microbial biofilms can be defined as surface-bound microorganism communities that form on living and inanimate solid surfaces and grow embedded in a matrix of extracellular polymeric materials. They are imagined as a significant virulence factor causing permanent chronic and repetitive infections; they are extremely durable to antimicrobials/antibiotics and host immune defenses. This situation seriously complicates treatment options. Approximately 75% of bacterial infections contain biofilms maintained by an extracellular matrix. Various reasons, such as limited diffusion of antibiotics into the biofilm matrix, expression of multidrug efflux pumps, type IV secretion systems, reduced permeability, and the effect of antibiotic modifying enzymes cause biofilm resistance [1–4].

In recent studies, it has been dealt with *Staphylococcus aureus* and *S. epidermidis* biofilms more specifically. Although they are often described as common species and permanent colonizers of human oromucosal membranes, they may often be opportunistic pathogens compared to premature newborns and immunocompromised patients. They are among the most extensive sources of infection in permanent medical implanted instruments, such as catheters. They can cause serious chronic infections [5, 6]. In addition, *Pseudomonas aeruginosa* is Gram-negative bacteria recognized as a lethal and resistant bacterium that can create critical infections in immunocompromised patients

and is also one of the main causes of nosocomial infections, septic shock, bronchopneumonia, and wound infections. When the bacteria form a biofilm, it is highly rough to devastate the infection. The bacteria are then more resistant to antibiotic treatment and the host immune response. This adaptability may have provided multiple drug resistance properties [7, 8].

Candida albicans is the best known and the most effective opportunistic fungal pathogen of humans and animals. It creates an extensive microflora in the gastrointestinal and genitourinary tracts of 70% of people. However, in some cases, *C. albicans* can be pathogenic for patients with critical illness, immune deficiency, and even healthy people [9, 10]. The incidence of candidiasis has increased in recent years, especially in immunocompromised patients. Reports indicate that *Candida*'s antifungal drug resistance is possible [11].

Prevention of biofilm is imagined as the main drug target in the treatment of a variety of bacterial and fungal infections, and the pharmacological improvement of these drugs is currently being widely investigated.

Antibiotic-resistant bacteria cannot be controlled or destroyed by antibiotics. They can survive or even reproduce in the presence of an antibiotic. The most critical concern with antibiotic resistance is that some bacteria have resistant to almost all antibiotics that are readily available. As a precaution, national and international principles and guidelines have been developed for the use of antibiotics and administration based on clinical evidence. Nevertheless, the number of antibiotics used and effective in this sense is very low. Antibiotics such as rifampin are widely used together with other antibiotics to cure *S. aureus* infections and seem promising in the treatment of infections; however, the data that support this application are limited and more precise data are not available [12].

Recently, various green nonlethal strategies for biofilm control have been improved. A promising alternative is to investigate naturally occurring plant-derived compounds that can block biofilm formation. Historically, plant extracts and bioactive components have been precious sources of natural products that play a central role in avoiding and curing ailments and help protect human health. Traditional medicinal plants are widely used for healthcare and cure of ailments for 2000 years by most of the world. It is also widely accepted because of the perception that they are safe and have a long history in folk medicine for treating ailments from ancient times [3]. Plants have improved sophisticated defense mechanisms to keep alive in their ecosystems and are therefore a large source of pharmaceutical compounds. The most interesting application area for medicinal plant extracts is inhibition of growth and a decrease in the number of pathogens. Only 1% of 500,000 plant species found globally have been studied phytochemically, mainly in terms of antimicrobial and antibiofilm activities. Various plant extracts containing 54 plants and common food products, 6 Florida plant extracts against *P. aeruginosa*, 168 plant extracts against *S. aureus*, 13,000 fractions of 167 plant genera, 6 herbal plants, and a few grapefruit derived flavonoids against *Escherichia coli* strains have been studied in relation to the control of pathogenic biofilms [13]. Furthermore, antibacterial properties and mainly biofilm prevention research from plant-based natural products are supported by the fact that phytochemicals are less toxic to the environment [7]. Some of the plants with antimicrobial effects containing saponin compounds had possible antibiofilm activities against isolated nosocomial bacteria; it can be an alternative to control microbial biofilm formation or can be used as a model for research of novel drugs [3]. Considering the above, this review focused on the biofilm-forming microorganisms, attempts to and control against these infections, and the antibiofilm potency of saponin-containing plants having antimicrobial properties.

2. Secondary Metabolites from Medicinal Plants: Saponins

Saponins (Latin word "*sapo*") have a distinctive foaming characteristic when diluted in aqueous solution and include steroidal or triterpenoid aglycones attached to one or more sugar units. They have significant biological characteristics such as cytotoxic, hemolytic, molluscicidal, anti-inflammatory, antifungal, antiyeast, antibacterial, and antiviral activities, and chemical structures that present natural nonionic detergent properties. Saponins can be divided into two groups, depending on the nature of the aglycone skeletons: steroidal and triterpenoid structures. The biological characteristics of saponins are based on the aglycone structure and the number of sugar chains covered. Their therapeutic potential against eukaryotic cells is linked with cell membrane permeabilizing characteristics complexing with cholesterol [14].

Saponins can impair the permeability of the bacterial outer membrane. About 90% of the surface of the Gram-negative bacteria cell wall outer membranes that do not contain natural cholesterol are covered with lipopolysaccharide (LPS). Saponins can interact with the lipid A part of Proteus LPSs, thereby increasing the permeability of the bacterial cell wall because of their detergent-like properties. Theoretically, this activity can facilitate influx of antibiotics through the bacterial cell wall membrane. Lipid A-saponin complexes can assist the intake of antibiotics (colistin and ampicillin) into naturally resistant bacterial cells. Previously, in the presence of 15 μg/mL saponin, colistin or ampicillin has been shown to reduce the number of cells in the laboratory strains of *Proteus mirabilis* S1959 and R45. To eliminate pathogenic Gram-positive and Gram-negative bacterial strains, various types of methods were studied, such as bacteriophage treatment, prevention of bacterial adhesion, and enhancing bacterial cell wall permeability. Therefore, saponin abilities and the concentrations to stimulate hemolytic and cytotoxic effects against eukaryotic cells, to interfere with antibiotics against bacterial cells, and their influences on the growth of bacterial and fungal strains are medically desired condition [14].

3. The Antibiofilm Effects of Plants with Antimicrobial Properties Containing Saponin-Type Compounds

3.1. Agave sisalana Perrine (Asparagaceae). *Agave sisalana* is native to Southern Mexico and largely cultivated in many countries. Leaf juice is traditionally used in Northern Morocco as a wash for treating skin diseases, and for pulmonary tuberculosis, syphilis, and jaundice. This species was found to have potent antimicrobial activity against several Gram positive, Gram negative, and fungus such as *Staphylococcus aureus*, *E. coli*, and *Bacillus cereus* [15]. *Agave* collected from different agricultural fields was to screen for in vitro antibiofilm and antiquorum sensing activities. The activity of the ethanolic extract of *Agave sisalana* on inhibiting biofilm formation was tested using the crystal violet method widely used by microbiologists. The extract has the highest antibiofilm activity against the test microorganism with an 87.5% reduction in biofilm formation. A low extract concentration may be necessary to prevent initial attachment of the biofilm, while a higher concentration will disrupt the preformed biofilm [16].

3.2. Anacardium occidentale L. (Anacardiaceae). *Anacardium occidentale* (cashew) is a plant species that spreads in most tropical and subtropical countries and has long been utilized in traditional medicine for treating various infectious ailments. The bark is utilized for asthma, inflammatory diseases, and wound healing treatments, to detoxify a snake bite, and for fevers, a laxative, to get rid of intestinal parasites, and to cure diabetes in Brazil and African countries [5, 17]. In Brazil, physicians prescribed the hydroethanolic extract of stem bark of titled plant as drops for dilution in water before application. Therefore, the antibacterial and antibiofilm activity of the extract at different concentrations was investigated. The concentrations of 122, 61, 30.5, and 15.2 mg/ml in the tests displayed the activity on all planktonic cells (*S. aureus* and *S. epidermis*), while reduced concentrations influenced several strains. For biofilms, 122, 61, and 30.5 mg/ml concentrations were active against all strains, but this was not observed at reduced concentrations. It was also observed that strains were completely destroyed. Phytochemical analysis performed on the extract showed the presence of compounds such as tannins, saponins, flavonoids, and phenols defined as the major active components in the extract [5]. Saponin concentration was significantly high in cashew bark extract [17].

3.3. Astragalus angulosus DC. (Fabaceae). *Astragalus* species have been utilized in folk medicine for a long time. They have been used to promote and strengthen the immune system, for colds, upper respiratory tract infections, and heart diseases, as well as chronic hepatitis and other viral infections, also used as an adjunct therapy for cancer. The aqueous and ethanolic extracts of all parts of *Astragalus angulosus*, a Lebanese endemic species, were tested and evaluated to find the antibacterial and antibiofilm activities against five bacterial strains: Gram-positive bacterial strains such as *S. epidermidis*, *S. aureus*, and *Enterococcus faecalis* and Gram-negative strains such as *E. coli* and *P. aeruginosa*. Saponins, coumarins, and flavonoids were found in all extracts and parts of the plant but in variable quantities based on diverse fractions. The ethanolic extract of whole plant had the highest bacteriostatic effect at 12.78 mg/ml concentration and was the most sophisticated that exerts its impact against 3 different strains. Another extracts also had an efficacy, however at higher concentrations and each against a single strain. Concerning the antibiofilm activity, most of the extracts were able to exterminate > 50% of *S. epidermidis* preformed biofilm, where the highest activity was achieved with the fraction of flower extracted in water and 67.7% biofilm eradication at 0.2 mg/ml was achieved [6].

3.4. Atriplex tatarica L. (Amaranthaceae). The genus *Atriplex* is a plant that grows in the world's largest continuously arid region. Many plants from the genus are known to be edible and widely used in folk medicine for various ailments. These species have antimicrobial activity and active bactericidal components. Chemical research of the *Atriplex tatarica* consumed as food in several European countries including Turkey displayed flavonoids, saponins, and alkaloids as the major secondary metabolites. Their antibacterial and antibiofilm activities against *P. aeruginosa* were evaluated by the microdilution method. Patuletin 3-*O*-β-D-apiofuranosyl-(1‴$\longrightarrow$2″)-β-D-glucopyranoside as a flavonoid compound has significant antibacterial activity, while atriplexogenin I (Figure 1) as a triterpenoid saponin derivative has the strongest antibiofilm agent. The tested compounds displayed antibiofilm activity against *P. aeruginosa* at sublethal concentrations at 0.5, 0.25, and 0.125 of MIC values. At the concentration of 0.5 MIC, all compounds decrease biofilm formation in the range of 20.07–58.06%, while some presented activity at the lowest concentration (0.125 MIC). The most promising agent on antibiofilm activity was atriplexogenin I (56.63–7.17%) at all concentrations, while patuletin 3-*O*-β-D-apiofuranosyl-(1‴$\longrightarrow$2″)-β-D-glucopyranoside (58.06 and 12.19%) and atriplexogenin III had potential at 0.5 and 0.25 of MIC values. Streptomycin and ampicillin reduced the biofilm at 52.46–89.31% and 58.94–85.24% values in all sub-MIC tested, respectively. The outcomes indicated that especially triterpenoid derivatives presented significant biofilm inhibition of *P. aeruginosa* [8].

3.5. Bacopa monnieri (L.) Wettst. (Plantaginaceae). *Bacopa monnieri* is a widespread plant growing in tropical countries and used as a medicinal plant for centuries as a memory enhancing, nervine tonic, hepatoprotective, and cardiotonic agent [18]. The extract of the plant has shown various therapeutic effects against anxiety, gastrointestinal diseases, skin disorders, epilepsy, pyrexia, and analgesia. Bacoside A (Figure 2), a saponin compound, is recognized as the major effective component of *B. monnieri*, and demonstrated its potential as an antimicrobial and antibiofilm agent against two opportunistic pathogenic bacteria, *S. aureus* and *P. aeruginosa*. Bacoside A can be regarded as an

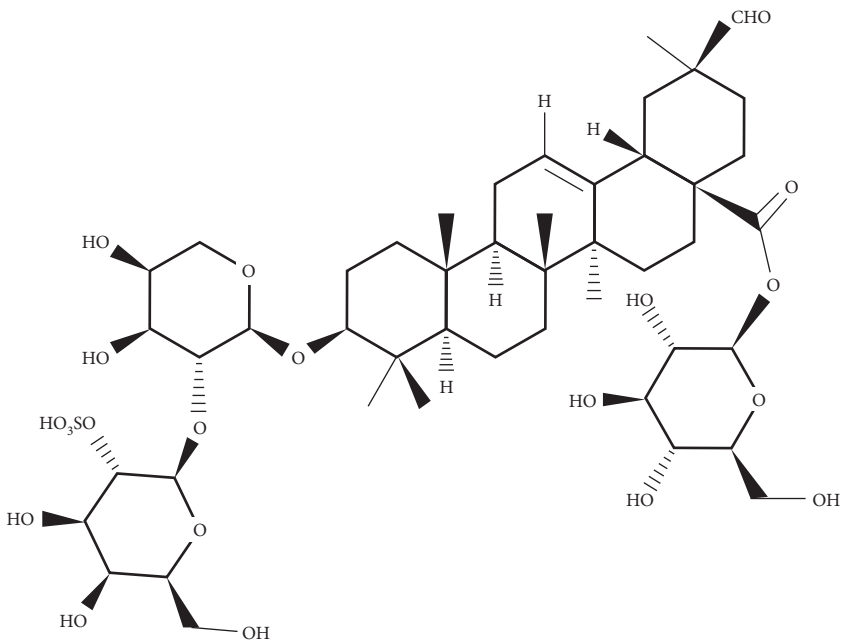


Figure 1: Chemical structure of atriplexogenin I.

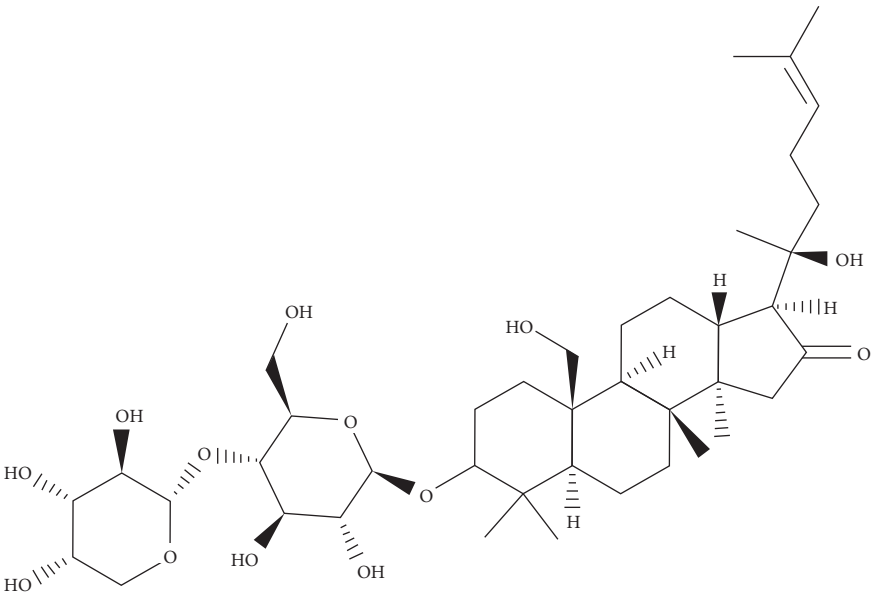


Figure 2: Chemical structure of Bacoside A.

effective antimicrobial agent at <400 μg/mL MIC value. Biofilm quantification by crystal violet assay, Bacoside A, could eliminate 88–93% (200 μg/mL) bacterial biofilm of selected bacterial strains from the biofilm substratum. Bacoside A displayed obvious evidence of biofilm dispersion due to EPS loss. Previous studies also proposed that the inhibition of EPS production leads to the elimination of bacterial biofilm. The high eradication of the bacterial biofilm after the treatment with Bacoside A was presented by SEM images qualitatively. MTT analysis also determined the percentage of mortality of bacterial cells in a treated biofilm (200 μg/mL) [19, 20].

3.6. Bellis perennis L. (Asteraceae). *Bellis perennis* is a plant native to almost all of Europe. It has been used in folk medicine for the treatment of various diseases, e.g., rheumatism, and as an expectorant [21]. The aqueous extract of the aerial parts of *Bellis perennis* that has antimicrobial, antibiofilm, and quorum-sensing inhibitory activities widely used as edible vegetables in Southwest Anatolia and contains triterpenoid saponins was studied. Antimicrobial activity was assessed against 15 bacterial strains and *Candida albicans* using the disk diffusion and broth microdilution assays. Antimicrobial and antibiofilm activity experiments demonstrated that *B. perennis* aqueous extract has moderate

antimicrobial and antibiofilm activities against *S. epidermidis* MU30, *P. aeruginosa* ATCC 27853, and *P. fluorescens* MU 181 at a concentration of 10 mg/ml. The 100 mg/ml aqueous extract of *B. perennis* presented promising antiquorum-sensing activity on *Chromobacterium violaceum* CV026 with an inhibition zone of 10 mm. Aqueous extracts of *B. perennis* blocked swarming by 9.5%. The results show that *B. perennis* may be an alternative resource for exploring beneficial ingredients in the fight against bacterial infections [22].

3.7. Calendula officinalis L. (Asteraceae). *Calendula officinalis* has traditionally been used to treat oral and pharyngeal mucositis, wounds, and burns. It is a cleansing and detoxifying plant, and the plant infusion cures chronic infections [23]. Its aqueous extract was utilized to treat skin diseases and pain and as a bactericide, and antiseptic and anti-inflammatory agents exhibited significant antibacterial effects against all pathogenic bacterial strains at 100 μg/ml concentration, especially when compared to cephotan antibiotic. *Shigella sonnei* was more susceptible to the extract than other bacteria with the highest inhibition zone (23 mm) at 100 μg/ml concentration. The concentration of 25 μg/ml of aqueous extract showed poor effect against bacterial strains, except for *S. sonnei*. It was stated that *C. officinalis* flowers' aqueous extract showed preferable antibacterial activity in comparison with other extracts from different parts (leaves, roots, and stems). They showed also remarkable antibacterial activities against *E. coli*, *P. aeruginosa*, *Enterococcus* sp., and *Staphylococcus* sp. Considering as potential antibacterial and therapeutic agents, oleanolic acid (Figure 3) and its glycosides from *C. officinalis* can be proposed. Bacterial strains (*Salmonella*, *Shigella dysenteriae*, *S. flexneri*, *S. sonnei*, and *E. coli*) had the ability to adhere to different degrees on the smooth surface of glass tubes. *Salmonella* gave adherent growth in large amounts while other bacterial isolates (*S. sonnei*, *S. flexneri*, and *S. dysenteriae*) gave adherent growth in a small amount. Aqueous extracts of *C. officinalis* have been found to reduce the adhesive growth of bacteria on glass tubes. Conversely, the extract of *C. officinalis* blocked the bacterial adhesion on the polystyrene surface and as a result caused the separation of biofilms, which caused a decrease in the absorbance values of the biofilms. These activities, recorded for *C. officinalis* flower extract, let them to be listed as potential antibiofilms and antibacterial natural agents. This may suggest that they are used as therapeutic agents to treat biofilm-related infections induced by enteric pathogens. The mechanism of the effect on the biofilm may differ for each plant extract [1].

3.8. Centella asiatica (L.) Urb. (Apiaceae), Cinnamomum zeylanicum Blume (Lauraceae), and Mentha spicata L. (Lamiaceae). *Centella asiatica* is native to Southeast Asian countries and has been traditionally used for wound healing, eczema, and burn and scar treatment and for stress and anxiety, as well as to treat periodontal disease for a long time. The inner bark of the genus *Cinnamomum* is a common herbal drug used as a spice in various countries by different cultures around the world, besides, for inflammatory diseases and diabetes, against infections (bacteria and fungi). Spearmint is native to Northern England and is widely grown in tropical and temperate regions such as Europe, South Africa, and Brazil. It is used for fevers, headache, digestive disorders, bronchitis, ulcerative colitis, and liver complaints in folk medicine. The biological activities of these species include antimicrobial and anti-inflammatory effects. The extracts of medicinal plants, *Centella asiatica*, *Mentha spicata*, and *Cinnamomum zeylanicum*, were screened against multidrug resistant *P. aeruginosa* strains for their antibacterial and antibiofilm properties. The antibiofilm activity test of methanol extracts proposed that *C. zeylanicum* and *M. spicata* extracts are potent antibiofilm agents whilst the *C. asiatica* extract is a moderate antibiofilm agent. Ethylacetate extracts of *C. zeylanicum* and *M. spicata* displayed strong antibiofilm activity. For the evaluation of the compounds responsible for antibacterial and antibiofilm activities of the selected plants, the phytochemical analysis was done according to ethnobotanical data and it was suggested that *C. asiatica* leaves, *C. zeylanicum* barks, and *M. spicata* leaves, stems, and flowers contain saponins and they are responsible for the activity [24].

3.9. Cyclamen coum Mill. and C. hederifolium Aiton (Primulaceae). *Cyclamen coum* known as a medicinal plant spreads in the forests of the Golestan area of Iran. It is known that many *Cyclamen* species are used for hemorrhoids, eczema, and wound healing. There is also information that their tubers are used as a sedative, anthelmintic, and laxative, as well as expelling digestive system worms. Tubers of the plant include large quantities of saponins (156 μ/mL). Increased levels of saponins can be obtained using different solvents and especially collected from the *n*-butanol phase. The effects of the combination of *C. coum n*-butanol extract and ciprofloxacin on the prevention of biofilm formation of *P. aeruginosa* were evaluated. Antibiotic (alone, at $6 \times$ MIC) or *n*-butanol extract of *C. coum* (alone, at $55 + 0.3$ μg/mL) remarkably impaired *P. aeruginosa* biofilm formation, but their combinations blocked more prominent biofilm formation (both antibiotic and *n*-butanol extracts of *C. coum* concentrations decreased at $3 \times$ MIC and 38 μg/mL, respectively). The *n*-butanol extract of *C. coum* had a synergistic effect against *P. aeruginosa* biofilms in combination with ciprofloxacin. It is logical to stand that the extract that contains saponins may resensitize antibiotic-resistant bacteria by preventing cell-cell communication (quorum detection) [25].

C. hederifolium Aiton is a plant with a common Mediterranean element in Çatalca and Kocaeli in Turkey. Its tubers are pounded and placed in a certain amount of water, and then the filtered water is given to the tobacco plantation and used as a pesticide to kill harmful insects. This effect is due to the fact that the saponins in its content are used to defend themselves against harmful factors that may come from the environment. In a study conducted with *C. hederifolium*, antibiofilm activity against *Staphylococcus aureus* was tested by microdilution method, and MIC values ($\leq$32 μg/ml) were determined [26].

3.10. Dioscorea panthaica Prain et Burk. (Dioscoreaceae). Traditionally, *Dioscorea panthaica* has ameliorated the symptoms of cardiovascular diseases. This medicinal endemic plant can also be utilized as a topical medicine in the treatment of infectious ailments induced by microbial pathogens such as *Lymphatic tuberculosis* and *Bacillus anthracis*. The saponin fraction from *D. panthaica* dried rhizomes (Huangshanyao saponin extract, HSE) against *C. albicans* was investigated for its antifungal effects. HSE blocks the planktonic growth, biofilm formation, and improvement of *C. albicans*. At 16–64 μg/mL, HSE reduced the viability of adherent cells on the polystyrene surfaces, the transition from yeast to filamentous growth, and generation of phospholipase and cell membrane disruption in planktonic cells. The administration of 64 μg/mL dose of HSE blocked 80% of the adhesion in comparison with drug-free control groups. It was determined that inhibitory effects against extracellular exopolysaccharide production in preformed biofilms could be provided by 64–256 μg/mL of HSE. Hyphae behave as a critical part of the infection and biofilm formation. Throughout mucosal-related infections, hyphae attack epithelial and endothelial cells, thereby causing destruction in which hydrolytic enzymes such as phospholipase act a significant role. The presence of hyphae builds the biofilm more compatible and assists *C. albicans* cells to harm epithelial cells and diffuse deep into tissues. Therefore, there comes the idea that reducing the growth of hyphae could possibly alleviate the harm to the hosts. HSE and the pure compounds, solasodin-3-*O*-D-glucopyranoside (Figure 4), and purpurin were able to prevent the growth of *C. albicans* [27].

3.11. Erica manipuliflora Salisb. (Ericaceae). The genus *Erica* L. is symbolized by more than 700 species in the world, among these species, *Erica manipuliflora* is commonly found in coastal areas in Turkey. The herbal teas of *E. arborea* and *E. manipuliflora* prepared from their aerial parts are common in Turkey as a diuretic and astringent and utilized to treat urinary tract infections. The *n*-butanol extract of *E. manipuliflora* has been reported to have significant activity against some marine biofilm bacteria (*Pseudoalteromonas marina*, *P. haloplanktis*, *P. elyakovii*, *P. porphyrae*, *P. agarivorans*, *Alteromonas genoviensis*, *Vibrio lentus*, and *Exiguobacterium homiense*). All biofilms investigated in the study were generally resistant to both antibiotics (vancomycin and tobramycin), and *E. homiense* and *A. genoviensis* were more sensitive. It has been suggested that the *n*-butanol fractions rich in flavonoids and triterpenoid saponins of the plants used in the study may be sources for the invention of new antibiofilm agents from plant sources [13].

3.12. Glycyrrhiza glabra L. (Fabaceae). *Glycyrrhiza glabra* root, grown in Eastern Anatolia, is one of the most widely used herbal remedies and a significant source of confectionery. The pharmacological characteristics linked with *G. glabra* are well documented. In accordance with the World Health Organization, it is used as a sedative to treat sore throats and as an expectorant for cough and bronchial flu. In addition, it plays a crucial role in the prophylaxis and the therapy of gastric and duodenal ulcers as an anti-inflammatory agent, as well as it has been used for more than 20 years as a treatment for chronic hepatitis. *G. glabra* extract decreases surface tension and shows antimicrobial activity against both Gram-positive and Gram-negative bacteria. The antimicrobial effects of *G. glabra* roots and leaves, especially owing to glycyrrhizin (Figure 5), have been recorded. Glycyrrhizin interacts with membrane lipids and disrupts cell membrane integrity that causes cells to abandon intracellular organelles. In addition, *G. glabra* root extract including 7–7.5% glycyrrhizin was found to be advantageous in terms of cytotoxicity. Therefore, *G. glabra* roots have a crucial effect on biofilm which could be caused by inhibition of cell integrity, causing wall and membrane damage and leakage. The ethanolic extract of *G. glabra* roots has exceptional prevention effects against the planktonic growth of *S. pyogenes* and is the most efficacious bactericidal agent that killed 99.99% of the initial bacterial load within 3 h exposure to the $2 \times$ MIC. Although *G. glabra* extract has significant antibiofilm activity, its effectiveness is lower than NaOCl and cetrimide which are the most effective agents against *E. faecalis* biofilms on dentine discs [28, 29].

3.13. Hibiscus tiliaceus L. (Malvaceae). The plant parts are utilized for cuts, tuberculosis, and conjunctivitis in the Solomon Islands. In New Guinea, the bark is utilized as cough remedy and used for tuberculosis. The leaves are utilized to treat cough, sore throat, and open wounds. A formulation made from leaves, roots, and bark is given for fever. The leaves boiled with sugar in Java are used in the treatment of cough and bronchitis. The leaves and bark are utilized in traditional Chinese medicine to treat cough and bronchitis. In Bangladesh, leaves are used in traditional medicine for fever, cough, and dry throat, while flowers are used for bronchitis, ear infections, dysentery, and chest congestion. Extracts and fractions of different parts of *Hibiscus tiliaceus* exhibited antibacterial and antibiofilm effects against *P. aeruginosa*. The chemical components found in *H. tiliaceus* have been found to vary between plant parts, and flavonoids, phenolics, steroids, and terpenoids were the main compounds that could contribute to these activities. Saponins appear to be more involved in the fruit parts of the plant. Fruits were extracted with methanol and fractionated with chloroform, ethylacetate, and methanol. While the chloroform fraction, which contains mostly nonpolar structures, was most effective in terms of antibacterial activity, all extracts and fractions have the capacity to remarkably block the biofilm formation in terms of antibiofilm activity [7].

3.14. Medicago sativa L. (Fabaceae) and Saponaria officinalis L. (Caryophyllaceae). *Medicago sativa* is a plant known since ancient times for animal breeding and nutrition. From historical documents, it is more likely to be of eastern, especially Iranian origin. The ancient Persians' acquaintance with this herb and their use are reflected in the sources of their neighbors, from the heart of the Persian kingdom eastward to China and westward, from Mesopotamia to Europe in antiquity. In folk medicine, the herbal drug is used to treat

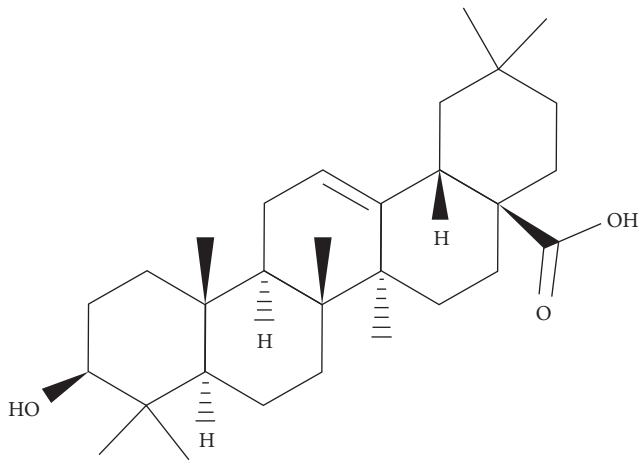


Figure 3: Chemical structure of oleanolic acid.

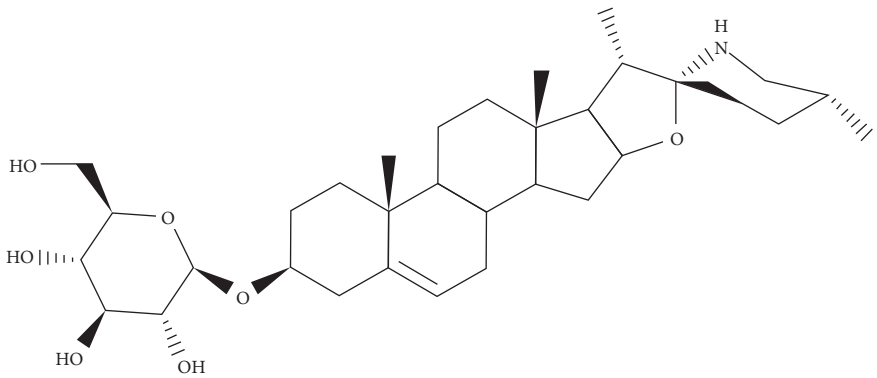


Figure 4: Chemical structure of solasodin-3-*O*-D-glucopyranoside.

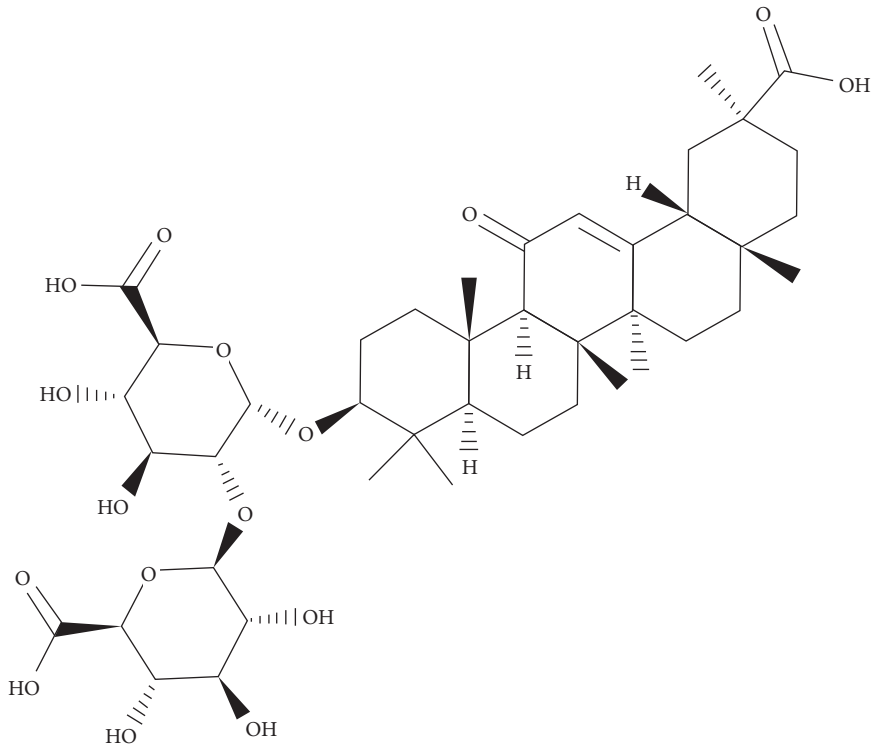


Figure 5: Chemical structure of glycyrrhizin.

diabetes and malfunctioning of the thyroid gland and cure for digestive systems [30]. Pharmacological activities of *M. sativa* saponins include hemolytic activity, cytotoxic properties, nematocidal and insecticidal activities, and an antimicrobial effect mainly against Gram-positive bacteria and some fungi, plants, and some human pathogens. *Saponaria officinalis* is a common perennial and native plant that extends throughout Europe and Asia. The uses of saponins obtained from *S. officinalis* in traditional medicine are mainly as an expectorant during the course of upper respiratory tract infections or for the treatment of skin and rheumatic lesions [10]. Medicagenic acid glycopyranosides (containing medicagenic acid as aglycones, Figure 6) are the major components of *M. sativa* extracts [31], additionally, saponariosides A (Figure 7) and B (Figure 8) are the main compounds of *S. officinalis* [32]. Since few synthetic antimycotic agents have been identified, including polyenes, azoles, echinocandins, allylamines, and 5-flucytosine, one of the lesser-known but promising agents, saponins which are surface-active phytochemicals, in this regard has been investigated. The antifungal properties against *C. albicans* with regard to antibiofilm activity and their synergistic effect with classic antimycotic of the saponins obtained from *Medicago sativa* and *Saponaria officinalis* have been studied [10]. The ability of saponin-rich extracts to inhibit *Candida* germ tube formation, which corresponds to limiting hyphal growth, is so crucial. This activity can prevent the development of invasive mycosis and fungal biofilm formation in the early stages of infection. Moreover, these extracts have been shown to have a significant effect on the reduction of *C. albicans* adhesion and biofilm generation and the elimination of mature *Candida* biofilm [33]. It was shown a significant antifungal potential of saponins obtained from the aerial parts and roots of *M. sativa* and the roots of *S. officinalis* alone or with antimycotic synergies. The ability of saponin-rich extracts to negatively affect *C. albicans* virulence factors such as germ tube formation, hyphal growth, adhesion, and biofilm formation has been indicated. These qualities of *Candida* cells play a role as new potential drug targets, and therefore, the properties of saponins seem very hopeful in the context of their potential medical application [10].

3.15. Scrophularia ningpoensis Hemsl (Scrophulariaceae). The genus *Scrophularia* mainly occurs through mountainous regions is one of the large genera of the Scrophulariaceae and used as a heart and circulatory stimulant, and diuretic. Besides, the other traditional uses of this genus include antipyretic, febrifuge, antibacterial, antierythema, anti-constipation, and antifurunculosis properties, ulcerous stomatitis, and tonsillitis treatment, as well as anti-infections' treatment in different types of disorders. The antibacterial activity of *Scrophularia ningpoensis* Hemsl extract on biofilm formation of *Klebsiella pneumonia* was investigated. The biofilm-forming capacity was conducted using the colony-forming unit test. The extract displayed considerable antibiofilm activity. Inhibition of bacterial biofilm formation was presented at the concentration of 0.75 mg/ml against *K. pneumoniae* with no signs of cytotoxicity in L929 [34].

3.16. Solidago virgaurea L. (Asteraceae). *Solidago virgaurea* is a medicinal plant widely used in Europe and other parts of the world and is known among the most researched species in its genus. The aerial parts of the plant have long been used for urinary tract ailments and as an anti-inflammatory agent in traditional medicine of different peoples [35]. In the related study, it was purposed to protect oral bacteria in healthy microflora, natural competitors of *C. albicans*, to improve a dry mouth-specific mouthwash and maintain oral mucosal hydration. Based on previous studies and the following effects of *Solidago virgaurea* saponins such as antimicrobial properties, as well as used as detergent, and hemolytic agents owing to their ability to connect membrane sterols, the antibacterial or antifungal activities of *Solidago* saponins have been studied. However, it should be noted that although saponins may interfere with membrane sterols of plants, viruses, and fungi, saponins may not exhibit significant antibacterial activity because most bacteria lack membrane sterols. Therefore, in the study, it was shown that *S. virgaurea* aqueous extracts do not have antibacterial or antifungal activity; that is, they do not inhibit bacterial growth. On the other hand, in response to the host environment, *C. albicans* yeast-hyphal transition is considerable for virulence. Thus, the second purpose of this study was to block *C. albicans* yeast-hyphal transition using *Solidago* extracts, and such an effect was observed in experiments. The extract strongly inhibited biofilm formation and decreased preformed biofilms. The percentages of reduction observed were from 95.86 to 99.46% in biofilm formation and from 76.26 to 92.37% in preformed biofilm formation. As a result, it has been shown that the aqueous extract of *S. virgaurea* creates an unfavorable environment for *C. albicans* and inhibits the yeast-hyphal transition without killing the yeast form and oral endogenous bacteria [36].

3.17. Terminalia fagifolia Mart. (Combretaceae). *Terminalia* genus consists of about 200 species widely used in folk medicine. It is located in the Brazilian Cerrado and is popularly known as "*capitão*, *capitão-do-cerrado*, *capitão-do-campo*, and *mirindiba*." All parts of *Terminalia* species are mostly given orally as decoctions or macerations to treat a wide variety of infectious diseases such as dysentery, diarrhea, cough, abdominal pain, chest pain, fever, eye infections, and respiratory infections. These species are also used externally as an antirheumatic ointment or poultice, or to heal skin infections such as acne, wound healing, and skin ulcers, and to treat itchy skin. Rodrigues de Araujo et al. [37] searched the pharmacological properties and antibacterial, antibiofilm, and cytotoxic effects of ethanol extract and its fractions of *Terminalia fagifolia* stem barks. The ethanolic extract was suspended in a mixture of H_2O/MeOH and partitioned with ethyl acetate. The organic phase was concentrated, then suspended in MeOH/H_2O. Then, it was provided aqueous and hydroalcoholic fractions. Serial dilutions (concentrations were tested at between 12.5 μg/ml and 400 μg/ml) of ethanol extract and its fractions were prepared in the MIC test. Concentrations in antibiofilm activity were tested by diluting at 1/2, 1/4, and 1/8 ratios.

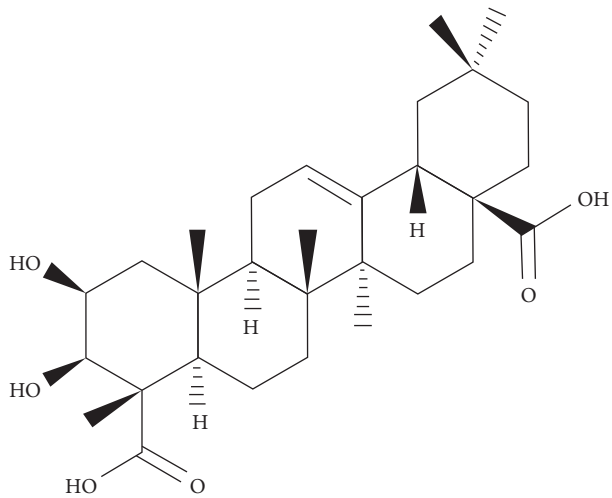


Figure 6: Chemical structure of medicagenic acid.

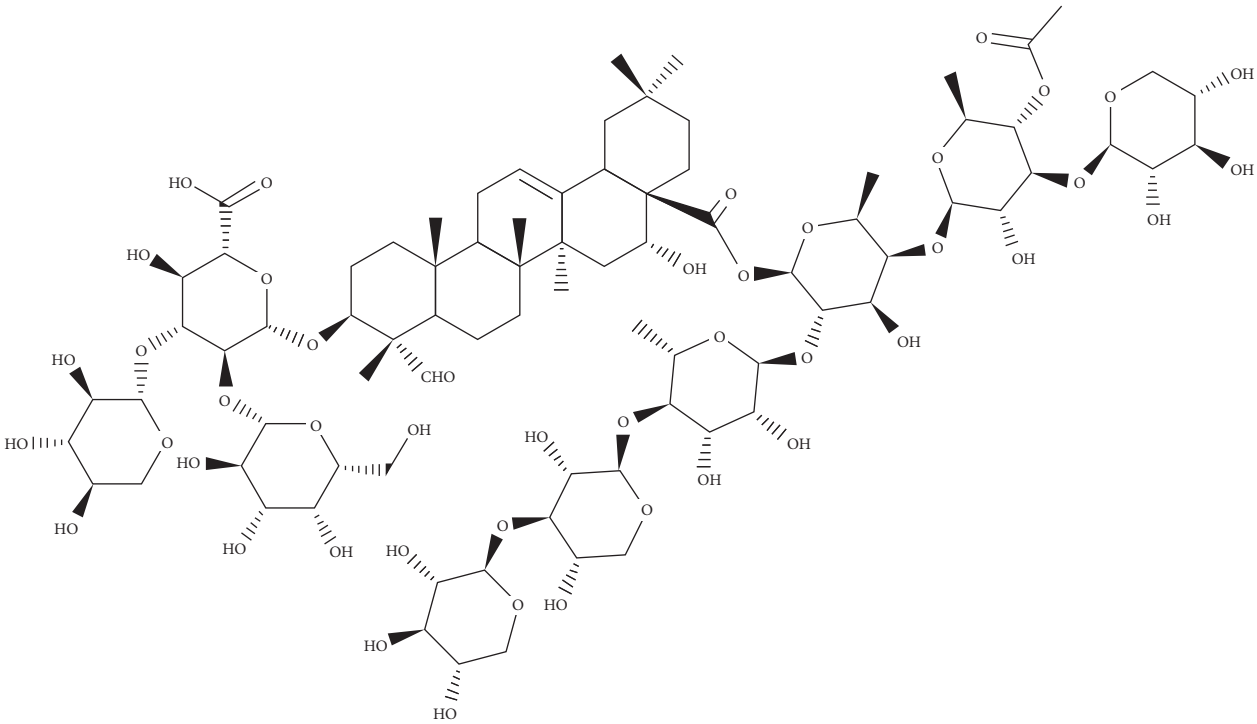


Figure 7: Chemical structure of saponariosides A.

Bacteria synthesize and release extracellular polysaccharides so that biofilm-forming bacteria adhere to surfaces. This structure not only helps with adhesion but also acts as a defense barrier. Hydrophobic bonds such as the electric charge and hydrophobicity of the surface and Van der Waals interactions are important between the organism and the material to which the organism will adhere. Extracts and fractions may have inhibited biofilm formation, leading to little-known metabolic changes. These possible changes include a reduction in the production and secretion of exopolysaccharides and a change in the electrical charge and/or hydrophobicity of the bacterial membrane. However, more specific protocols are needed to confirm the role of these fractions. The results thus obtained showed that the ethanol extracts and fractions had high antibacterial activity and that 80% of biofilm formation was inhibited for some strains. It is suggested that the presence of saponins with antimicrobial potential in the aqueous and hydroalcoholic fractions can be responsible for the activity [37].

3.18. Trifolium L. Species (Fabaceae). The genus *Trifolium* L., one of the largest genera of the Fabaceae family, attracts attention for their expectorant, analgesic, antiseptic, and antirheumatic properties, as well as containing secondary metabolites, especially saponins and flavonoids [38]. The production of saponins by plants is a remarkable part of their defense against pathogens and herbivores; however, it is well known that they have a much wider range of characteristics such as antimicrobial, hemolytic, anti-inflammatory, cytotoxic, and antitumor activity. Important and well-known virulence factors of *Candida* cells are hydrolytic enzymes such as proteases, lipases, and phospholipases. This plays a role in nutrition, adhesion to host cells, and tissue destruction. The enzymatic activity of yeasts pretreated with saponin-rich (80–98%) fractions isolated from extracts of the aerial parts of *Trifolium alexandrinum*, *T. incarnatum*, and *T. resupinatum* var. *Resupinatum* was tested. Two triterpenoid glycosides, soyasaponin Bb (soyasaponin I, Figure 9) and soyasaponin βb (soyasaponin I conjugated at the 22-position with DDMP) were previously characterized in several clover seeds and found major saponins in the three types of *Trifolium* seeds tested. Treatment of this strain with saponin fractions at 0.5 mg/mL disclosed a statistically significant reduction in the release of certain enzymes, including acid and alkaline phosphatase, naphthyl-AS-BI-phosphohydrolase, and N-acetyl-β-glucosaminidase. The production of other enzymes has also been somewhat affected [39].

3.19. Quillaja saponaria Molina (Quillajaceae) and Yucca schidigera Roezl ex Ortgies (Asparagaceae). *Quillaja saponaria* bark has been used since time immemorial by the Mapuche people, the main ethnic group of South-Central Chile to treat toothache and respiratory infections. This species is well known for its triterpene saponin content. More or less pure saponins and their specific fractions are widely used as vaccine adjuvants. Triterpenic saponins and their aglycones have been displayed to have anti-inflammatory, antiallergic, antiviral, antifungal, and cytotoxic properties [40]. *Yucca schidigera* is a medicinal plant native to Mexico and one of the major commercial sources of steroidal saponins. The main application of *Yucca* products is in animal nutrition, in particular as a feed additive. According to folk medicine, *Yucca* extracts have antiarthritic and anti-inflammatory effects [41]. The antibacterial effects of water extracts from *Yucca schidigera* and *Quillaja saponaria* containing saponins in the range of 12% to 90% were characterized. This study discovered the capacity of saponin extracts to block the adhesion and invasion of HeLa cells cultured by *E. coli*, *Yersinia enterocolitica*, *Listeria monocytogenes*, *Salmonella enterica* serovar Typhimurium, *Shigella flexneri*, and *Vibrio cholerae*. The presence of *Y. schidigera* extract during infection had the strongest antibacterial effect. Two possible mechanisms of antimicrobial activity of saponin extracts have been investigated. One of them is that some saponin molecules have an affinity for cell membrane cholesterol. In this study, it is presented that pretreatment of cells with *Y. schidigera* or *Q. saponaria* extracts can modulate cellular membrane cholesterol levels. It was also studied whether saponin extracts could modulate the activity of Ca^{2+} ion channels. The outcomes indicated that the treatment of cells with each saponin extracts, separately, for 6 or 24 hours does not produce a change in the intracellular concentration of Ca^{2+} ions. Consequently, the protective effect of saponin extracts may be owing to modulation of plasma membrane cholesterol, which leads to impaired cell membrane organization. Each of the extracts contains a mixture of individual saponins, each with a slightly different saponin structure. The extract of *Q. saponaria* contains saponins with triterpenoid aglycone skeleton, while *Y. schidigera* extract contains a saponin mixture with a steroidal aglycone skeleton. Each saponin in the *Q. saponaria* extract contains an aglycone skeleton known as quillaic acid to which branched oligosaccharides are attached. Oligosaccharides usually bind to positions C-3 and C-28 on the aglycone skeleton [42].

3.20. Ulmus rubra Muhl. (Ulmaceae). *Ulmus rubra* inner bark is registered to be known traditional healers in Canada for controlling streptococcal pharyngitis. Biofilm development is an important mechanism involved in *S. pyogenes* virulence during pharyngitis infections, providing superior survival and protection from host defense mechanisms, antibiotics, and other environmental fluctuations. Therefore, the multiple anti-*Streptococcus pyogenes* attributes involving planktonic growth inhibition, bactericidal effect, morphological disruption in cell wall/membrane, and biofilm inhibition of *U. rubra* inner bark extract were assessed. The ethanol extract of *Ulmus rubra* has significant inhibition effects against the planktonic growth of *S. pyogenes*. The effects of subinhibitory concentrations of *U. rubra* inner bark ethanol extract on biofilm formation over 72 h incubation of three *S. pyogenes* strains were quantified by MTT staining. The extract displayed inhibitory activity on biofilm formation ranging from 62.5 to 125 μg/mL. The phytochemical analysis of the extract allowed the isolation of saponins, such as oleanolic acid (Figure 4), ursolic acid (Figure 10), and betulinic acid (Figure 11) that are responsible for the activity [29].

4. Antibiofilm Effects of Natural Products

4.1. Ankaferd Blood Stopper (ABS). Ankaferd Blood Stopper (ABS) is a natural product consisting of *Thymus vulgaris*, *Urtica dioica*, *Alpinia officinarum*, *G. glabra*, and *Vitis vinifera* and does not contain inorganic or synthetic additives. The plants included in ABS have some effects on blood cells, endothelium, cell proliferation, vascular dynamics, angiogenesis, apoptosis, inflammation, or cell mediators. This product is produced by a registered Turkish company and licensed for use for dental and external and in major or minor bleeding after surgery. The main action mechanism for ABS is the formation of an encapsulated protein network that represents focal points for essential erythrocyte aggregation. The antibiofilm activity of ABS against oral streptococci was demonstrated by *in vitro* analysis and also

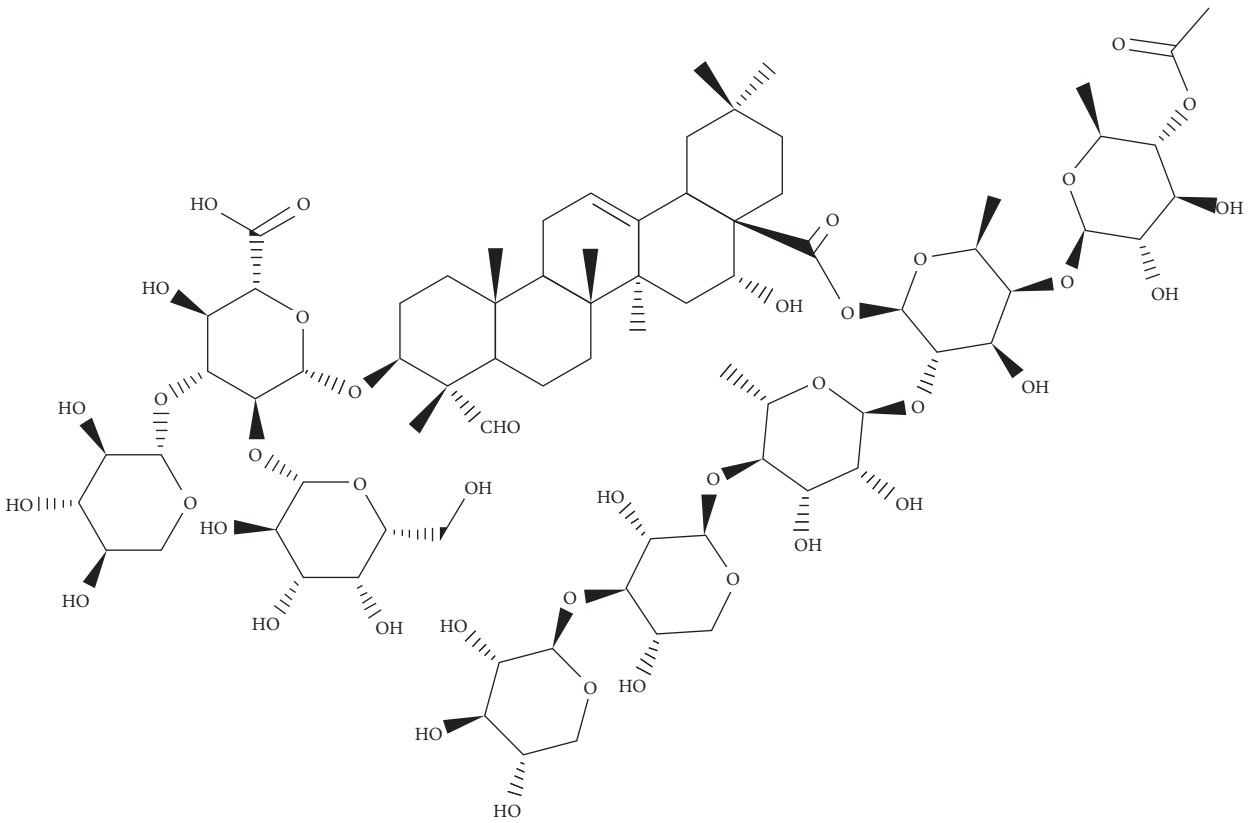


Figure 8: Chemical structure of saponariosides B.

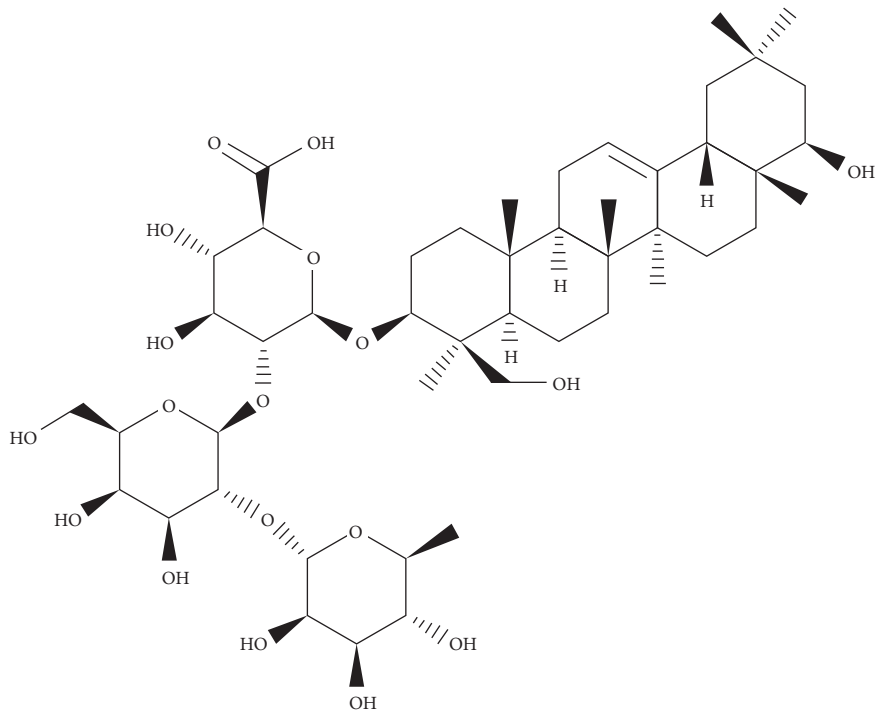


Figure 9: Chemical structure of soyasaponin Bb.

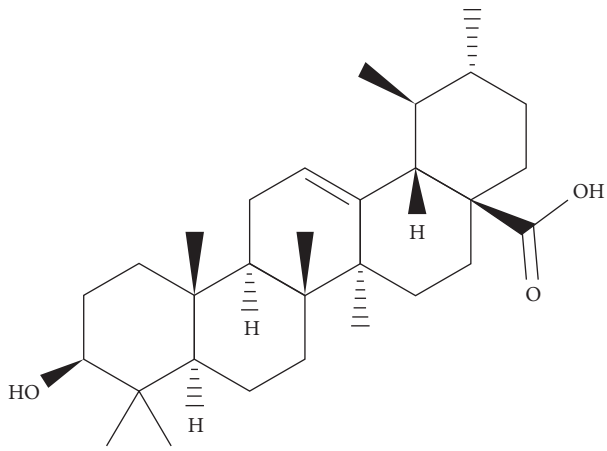


Figure 10: Chemical structures of ursolic acid.

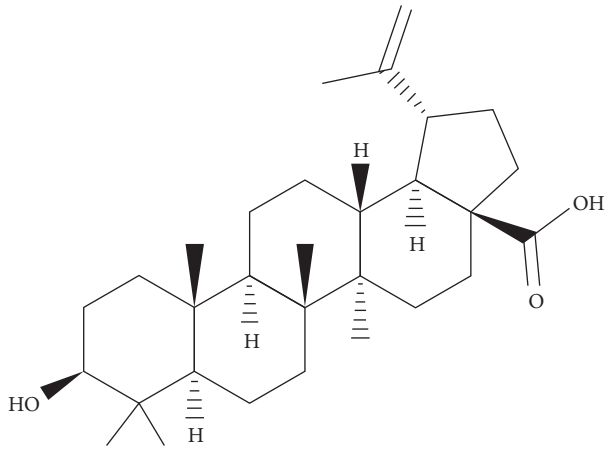


Figure 11: Chemical structures of betulinic acid.

visualized by SEM. ABS inhibited the growth of the biofilm layer of *S. aureus*, *S. sanguinis*, *S. mitis*, *S. sobrinus*, and *S. parasanguinis* by 94.48%, 87.00%, 86.57%, 82.38%, and 80.17%, respectively. Nevertheless, at the same concentration, ABS inhibited the growth of *S. oralis*, *C. albicans*, and *S. mutans* biofilm by 62.13%, 38.80%, and 7.89%, respectively. The highest antibiofilm activity of ABS with 94.48% was also monitored by SEM against biofilm formation of *S. aureus*. After ABS treatment, planktonic *S. aureus* cells could be observed, but there was no biofilm formation in the coverslips. When the chemical structures of the plants in this product were examined, it was observed that the licorice root is rich in saponin. Therefore, it is thought that the most important part of the effect is due to the surfactant compounds such as saponins in this product [43].

5. Conclusion/Future Perspectives

Treatments that inhibit planktonic bacteria and fungi have little effect on biofilms. Given the prevalence of biofilm infection, the development of antimicrobial therapies should focus on this form of growth. Biofilm models are critical for the discovery of new antimicrobial agents and to investigate their effects. One approach is to reveal agents that disrupt biofilm processes by allowing antimicrobials to attack biofilms when used in combination. The results can be divided into two categories: samples with antibacterial and antibiofilm effects; patterns containing antibiofilm but without antibacterial activities. In general, biofilm formation decreases due to the reduction of living bacteria. There appears to be a linear correlation between the capacity to block biofilm formation and bacterial growth. Compounds with antibiofilm activity but without antibacterial activity are aimed at biofilm formation, possibly by disrupting the extracellular polysaccharides matrix and the quorum-sensing mechanism, or infecting the nutrient resource without affecting bacterial growth. Although all the mentioned plants in our review show antimicrobial and antibiofilm activities, some of them (*Dioscorea panthaica*, *Glycyrrhiza glabra*, *Solidago virgaurea*, *Terminalia fagifolia*, *Quillaja saponaria*, and *Yucca schidigera*) were found to be more effective in terms of antibiofilm activity by disrupting the extracellular polysaccharide matrix.

In order to be the most medically beneficial, new agents should ideally be able to completely destroy a biofilm. To achieve this degree of activity, various strategies can be considered for the design of new drugs. High-throughput models need to be developed to economically test large molecule libraries for antibiofilm activity. Saponins are characterized by wide antimicrobial activities, and they have interesting chemical structures and properties presenting also antioxidant, anti-inflammatory, and antiapoptotic effects. The interactions between the saponin molecule and cell membrane seem complex, and due to the structural diversity of saponin molecules, it is likely that different mechanisms are involved. To fight infection, their hydrophobic components directly touch the phospholipid bilayers of the microbial cell membrane, causing an enhancement in ion permeability, leakage of vital intracellular components, or disruption and inhibition of pathogen enzyme systems and their respiration, and prevention of protein synthesis and assembly. The damage to the cell membrane might be due to the detergent-like activity of saponins. Saponins' antifungal properties are also linked to the capacity of the major components to pass through the thick fungal cell wall and to locate between the fatty acid chains of the lipid bilayers, disrupt the lipid packaging, and change the structure of the cell membrane. Another feature recommends that saponins can be utilized to promote the activity of antifungal drugs that target sterol compounds of the cytoplasmic membrane (polyenes and azoles). The effective synergistic interactions with saponin fractions and triazole-fluconazole against *Candida* strains with different sensibility were proved. Besides, saponins have made susceptible *C. albicans* more susceptible, and the resistance of *C. glabrata* strain reduced.

It will be fascinating to see how this information is applied to the discovery of antibiofilm drugs as our understanding of biofilm formation, the diseases it contributes to, and the treatment requirements continue to grow.

Abbreviations

ABS: Ankaferd Blood Stopper
EPS: Extracellular polysaccharide
HSE: Huangshanyao saponin extract
LPS: Lipopolysaccharide
MIC: Minimum inhibitory concentration
MTT: (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
SEM: Scanning electron microscope.

Conflicts of Interest

The authors declare no conflicts of interest.

References

[1] K. K. Ghaima, S. F. Rasheed, and E. F. Ahmed, "Antibiofilm, antibacterial and antioxidant activities of water extract of *Calendula officinalis* flowers," *International Journal of Biological & Pharmaceutical Research*, vol. 4, no. 7, pp. 465–470, 2013.

[2] M. R. Parsek and P. K. Singh, "Bacterial biofilms: an emerging link to disease pathogenesis," *Annual Review of Microbiology*, vol. 57, pp. 677–701, 2003.

[3] E. Sanchez, C. R. Morales, S. Castillo, C. Leos-Rivas, L. Garcia-Becerra, and D. M. Ortiz Martinez, "Antibacterial and antibiofilm activity of methanolic plant extracts against nosocomial microorganisms," *Evidence-Based Complementary and Alternative Medicine*, vol. 2016, Article ID 1572697, 9 pages, 2016.

[4] R. Vasudevan, "Biofilms: microbial cities of scientific significance," *Journal of Microbiology & Experimentation*, vol. 1, no. 3, pp. 84–98, 2014.

[5] M. V. Dias-Souza, S. Andrade, A. P. Aguiar, and A. Monteiro, "Evaluation of antimicrobial and anti-biofilm activities of *Anacardium occidentale* stem bark extract," *Journal of Natural Products*, vol. 6, pp. 198–205, 2013.

[6] H. Kanaan, M. ElMestrah, A. Sweidan, F. Assadi, A. Bazzal, and A. Chokr, "Screening for antibacterial and antibiofilm activities in *Astragalus angulosus*," *Journal of Intercultural Ethnopharmacology*, vol. 6, no. 1, pp. 50–57, 2017.

[7] Y. Andriani, H. Mohamad, K. Bhubalan, M. I. Abdullah, and H. Amir, "Phytochemical analysis, antibacterial and antibiofilm activities of Mangrove-Associated *Hibiscus tiliaceus* extracts and fractions against *Pseudomonas aeruginosa*," *Journal of Sustainability Science and Management*, vol. 12, no. 2, pp. 45–51, 2017.

[8] J. Stankovic, D. Godevac, V. Tesevic et al., "Antibacterial and antibiofilm activity of flavonoid and saponin derivatives from *Atriplex tatarica* against *Pseudomonas aeruginosa*," *Journal of Natural Products*, vol. 82, pp. 1487–1495, 2019.

[9] S. Bhattacharyya, P. Gupta, G. Banerjee, A. Jain, and M. Singh, "*In-vitro* inhibition of biofilm formation in *Candida albicans* and *Candida tropicalis* by heat stable compounds in culture filtrate of *Aspergillus flavus*," *Journal of Clinical and Diagnostic Research: JCDR*, vol. 7, pp. 2167–2169, 2013.

[10] B. Sadowska, A. Budzyńska, M. Więckowska-Szakiel et al., "New pharmacological properties of *Medicago sativa* and *Saponaria officinalis* saponin-rich fractions addressed to *Candida albicans*," *Journal of Medical Microbiology*, vol. 63, no. 8, pp. 1076–1086, 2014.

[11] A. Khodavandi, F. Alizadeh, F. Namvar, R. Mohamad, and C. Pei Pei, "Anti-Candida potential of *Allium ascalonicum* Linn: antibiofilm activity and biomolecular mechanism of action," *Journal of Pure and Applied Microbiology*, vol. 8, no. 2, pp. 349–356, 2014.

[12] B. Li and T. J. Webster, "Bacteria antibiotics resistance: new challenges and opportunities for implant-associated orthopaedic infections," *Journal of Orthopaedic Research*, vol. 36, no. 1, pp. 22–32, 2018.

[13] A. Kacar, S. Avunduk, B. Omuzbuken, and E. Aykin, "Biocidal activities of a triterpenoid saponin and flavonoid extracts from the *Erica manipuliflora* Salisb. against microfouling bacteria," *International Journal of Agricultural and Life Sciences*, vol. 2, no. 2, pp. 40–46, 2018.

[14] M. Arabski, A. Wegierek-Ciuk, G. Czerwonka, A. Lankoff, and W. Kaca, "Effects of saponins against clinical *E. coli* strains and eukaryotic cell line," *Journal of Biomedicine and Biotechnology*, vol. 2012, Article ID 286216, 6 pages, 2012.

[15] D. Tewari, Y. C. Tripathi, and N. Anjum, "*Agave sisalana*: a plant with high chemical diversity and medicinal importance," *World Journal of Pharmaceutical Research*, vol. 3, no. 8, pp. 238–249, 2014.

[16] A. A. Elmanama and M. R. Al-Reefi, "Antimicrobial, antibiofilm, AntiQuorum sensing, antifungal and synergistic

effects of some medicinal plants extracts," *IUG Journal of Natural and Engineering Studies Peer-Reviewed Journal of Islamic University-Gaza*, vol. 25, no. 2, pp. 198–207, 2017.

[17] M. O. Ojezele and S. Agunbiade, "Phytochemical constituents and medicinal properties of different extracts of *Anacardium occidentale* and *Psidium guajava*," *Asian Journal of Biomedical and Pharmaceutical Sciences*, vol. 3, no. 16, pp. 20–23, 2013.

[18] S. Shikha, M. Nidhi, and M. Upama, "*Bacopa monniera* – a future perspective," *International Journal of Pharmaceutical Sciences and Drug*, vol. 1, no. 3, pp. 154–157, 2009.

[19] F. Guzzo, M. Scognamiglio, A. Fiorentino, E. Buommino, and B. D'Abrosca, "Plant derived natural products against *Pseudomonas aeruginosa* and *Staphylococcus aureus*: antibiofilm activity and molecular mechanisms," *Molecules*, vol. 25, no. 21, pp. 5024–5048, 2020.

[20] D. Parai, E. Islam, J. Mitra, and S. K. Mukherjee, "Effect of bacoside A on growth and biofilm formation by *Staphylococcus aureus* and *Pseudomonas aeruginosa*," *Canadian Journal of Microbiology*, vol. 63, no. 2, pp. 1–28, 2016.

[21] T. Schöpke, V. Wray, B. Rzazewska, and K. Hiller, "Bellissaponins BA1 and BA2, acylated saponins from Bellis perennis," *Phytochemistry*, vol. 30, no. 2, pp. 627–631, 1991.

[22] O. Ceylan, A. Ugur, and N. Sarac, "*In vitro* antimicrobial, antioxidant, anti-biofilm and quorum sensing inhibitory activities of *Bellis perennis* L," *Journal of BioScience and Biotechnology*, vol. 2014, pp. 35–42, 2014.

[23] D. Arora, A. Rani, and A. Sharma, "A review on phytochemistry and ethnopharmacological aspects of genus *calendula*," *Pharmacognosy Reviews*, vol. 7, no. 14, pp. 179–187, 2013.

[24] M. Jahan, A. Abul Kalam Azad, and M. M. Karim, "*In vitro* antibacterial and antibiofilm activity of selected medicinal plants and spices extracts against multidrug resistant *Pseudomonas aeruginosa*," *Journal of Pharmacognosy and Phytochemistry*, vol. 7, no. 3, pp. 2114–2121, 2018.

[25] A. Abdi-Ali, M. Shafiei, F. Shahcheraghi, A. Saboora, and T. Ghazanfari, "The study of synergistic effects of *n*-butanolic *Cyclamen coum* extract and ciprofloxacin on inhibition of *Pseudomonas aeruginosa* biofilm formation," *Biological Journal of Microorganism*, vol. 3, no. 12, pp. 25–32, 2015.

[26] Ç. Aydın, C. Özay, and R. Mammadov, "Studies on *cyclamen* species distributed in Turkey," *Hacettepe University Journal of the Faculty of Pharmacy*, vol. 34, no. 2, pp. 96–112, 2014.

[27] L. Yang, X. Liu, X. Zhuang, Z. Feng, L. Zhong, and T. Ma, "Antifungal effects of saponins extract from rhizomes of *Dioscorea panthaica* Prain et Burk against *Candida albicans*," *Evidence-Based Complementary and Alternative Medicine*, vol. 2018, Article ID 6095307, 13 pages, 2018.

[28] H. E. Güldas, A. D. Kececi, E. S. Cetin, T. Ozturk, and B. Ü. Kaya, "Evaluation of antimicrobial efficacy of cetrimide and Glycyrrhiza glabra L. extract against Enterococcus faecalis biofilm grown on dentin discs in comparison with NaOCl," *Dental Materials Journal*, vol. 35, no. 5, pp. 721–727, 2016.

[29] N. Wijesundara and H. Rupasinghe, "Bactericidal and antibiofilm activity of ethanol extracts derived from selected medicinal plants against *Streptococcus pyogenes*," *Molecules*, vol. 24, no. 6, p. 1165, 2019.

[30] P. Mikaili and J. Shayegh, "*Medicago sativa*: a historical ethnopharmacology and etymological study of the alfalfa," *Research in Veterinary Science of Journal*, vol. 1, no. 9, pp. 614–618, 2011.

[31] A. E. Timbekova, M. I. Isaev, and N. K. Abubakirov, "Chemistry and biological activity of triterpenoid glycosides from *Medicago sativa*," *Advances in Experimental Medicine and Biology*, vol. 405, pp. 171–182, 1996.

[32] Z. Jia, K. Koike, and T. Nikaido, "Major triterpenoid saponins fromSaponariaofficinalis," *Journal of Natural Products*, vol. 61, no. 11, pp. 1368–1373, 1998.

[33] W. S. Sung and D. G. Lee, "*In vitro* candidacidal action of Korean red ginseng saponins against *Candida albicans*," *Biological and Pharmaceutical Bulletin*, vol. 31, no. 1, pp. 139–142, 2008.

[34] K.-D. Yook, "Antimicrobial activity and cytotoxicity test of *Scrophularia ningpoensis* Hemsl extracts against *Klebsiella pneumoniae*," *Journal of the Korea Society of Computer and Information*, vol. 21, no. 5, pp. 135–139, 2016.

[35] C. Fursenco, T. Calalb, L. Uncu, M. Dinu, and R. Ancuceanu, "Solidago virgaurea L.: a review of its ethnomedicinal uses, phytochemistry, and pharmacological activities," *Biomolecules*, vol. 10, no. 12, pp. 1619–1650, 2020.

[36] M. Chevalier, E. Medioni, and I. Prêcheur, "Inhibition of *Candida albicans* yeast-hyphal transition and biofilm formation by *Solidago virgaurea* water extracts," *Journal of Medical Microbiology*, vol. 61, no. 7, pp. 1016–1022, 2012.

[37] A. R. de Araujo, P. V. Quelemes, M. L. G. Perfeito et al., "Antibacterial, antibiofilm and cytotoxic activities of *Terminalia fagifolia* Mart. extract and fractions," *Annals of Clinical Microbiology and Antimicrobials*, vol. 14, no. 1, p. 25, 2015.

[38] T. Sabudak, N. Guler, and L. Trifolium, "Trifolium L. - a review on its phytochemical and pharmacological profile," *Phytotherapy Research*, vol. 23, no. 3, pp. 439–446, 2008.

[39] A. Budzyska, B. Sadowska, M. Wieckowska-Szakiel et al., "Saponins of *Trifolium* spp. aerial parts as modulators of *Candida albicans* virulence attributes," *Molecules*, vol. 19, no. 7, pp. 10601–10617, 2014.

[40] S. Sarkhel, "Evaluation of the anti-inflammatory activities of *Quillaja saponaria* mol. Saponin extract in mice," *Toxicology Reports*, vol. 3, pp. 1–3, 2016.

[41] P. R. Cheeke, S. Piacente, and W. Oleszek, "Anti-inflammatory and anti-arthritic effects of *Yucca schidigera*: a review," *Journal of Inflammation (London, England)*, vol. 3, pp. 6-7, 2006.

[42] A. M. Johnson, *Saponins as Agents Preventing Infection Caused by Common Waterborne pathogens. Doctor of Philosophy*, The University of Texas, Arlington, TX, USA, 2013.

[43] R. Boran, T. Baygar, N. Saraç, and A. Uğur, "Ankaferd blood stopper with antibiofilm potential successfully inhibits the extracellular matrix degradation enzymes and promotes wound healing of 3T3 fibroblasts *in vitro*," *Turkish Journal of Medical Sciences*, vol. 48, pp. 627–634, 2018.

Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2021, Article ID 9961089, 14 pages
https://doi.org/10.1155/2021/9961089

Research Article

New Evidence for *Artemisia absinthium* L. Application in Gastrointestinal Ailments: Ethnopharmacology, Antimicrobial Capacity, Cytotoxicity, and Phenolic Profile

Marija Ivanov, Uroš Gašić, Dejan Stojković, Marina Kostić, Danijela Mišić, and Marina Soković

Department of Plant Physiology, Institute for Biological Research "Siniša Stanković"—National Institute of Republic of Serbia, University of Belgrade, Bulevar Despota Stefana 142, Belgrade 11000, Serbia

Correspondence should be addressed to Marina Soković; mris@ibiss.bg.ac.rs

Received 23 March 2021; Revised 13 July 2021; Accepted 16 July 2021; Published 23 July 2021

Academic Editor: Yanting Song

Artemisia absinthium L. (Asteraceae) is traditionally used for gastrointestinal ailments and disorders linked to numerous risk factors including microbial infections. We aimed to provide contemporary evidence for its ethnopharmacological use and determine its antimicrobial capacity and mode of action, cytotoxicity, and phenolic constituents. Ethnopharmacological survey was conducted using semistructured interviews. Antimicrobial and antibiofilm capacities were determined by microdilution/crystal violet assay, respectively. Modes of action tested include estimation of exopolysaccharide production (congo red binding assay) and interference with membrane integrity (crystal violet uptake and nucleotide leakage assay). Cytotoxicity was determined using crystal violet assay. Polyphenolic profiling was done by advanced liquid chromatography/mass spectrometry (UHPLC-LTQ OrbiTrap MS). *Artemisia absinthium* in Serbia is traditionally used for gastrointestinal disorders, among others. Further study revealed high antifungal capacity of herb ethanolic extract towards range of *Candida* species (MIC 0.5–1 mg/mL) along with promising antibacterial activities (MIC 0.25–4 mg/mL). Interference with membrane integrity could be observed as a possible antimicrobial mechanism. Antibiofilm potential can be considered as high (towards *C. krusei*) to limited (towards *P. aeruginosa*) and moderate based on reduction in exopolysaccharide content. In concentrations up to 400 μg/mL, no cytotoxicity was observed towards HaCaT and HGF-1 cell lines. Polyphenolic analysis revealed twenty-one different constituents. *A. absinthium* usage as a gastrointestinal ailment remedy has been confirmed *in vitro* by its antimicrobial capacity towards microorganisms whose presence is linked to the diseases and associated complications and noncytotoxic nature of the natural product. The observed activities could be attributed to the present phenolic compounds.

1. Introduction

Artemisia absinthium L. (Asteraceae), wormwood, is perennial shrubby medicinal plant. This plant is used in shampoos, face serums, masks, essences, and other cosmetology products along with abundant utilization in food industry as the main flavoring ingredient in alcoholic beverage absinth [1]. The essential oil obtained from this species is rich in bioactive chemical constituents such as *cis*-epoxy-*o*-cimene, chrysanthenol, and chrysanthenyl acetate [2] while herb extracts are rich sources of different biomolecule classes such as flavonoids, coumarins, and fatty acids [1].

Its aerial parts are traditionally used worldwide for digestive discomforts and gastrointestinal (GI) ailments, and due to antimicrobial and diuretic properties [3] with randomized controlled trials confirming its bioactivity in Crohn's disease [4]. Ranges of microorganisms are found to have a role in the Crohn's disease etiology including Streptococci, *Pseudomonas aeruginosa*, and *Listeria monocytogenes* [5]. Patients suffering from Crohn's disease and other GI ailments are also more frequently colonized by *Candida* spp. compared to healthy individuals [6].

The GI disease presence is being linked to a range of factors including chronic sinonasal diseases [7] with

tonsillectomies seen as a risk factor for inflammatory bowel disease [8]. The link between oral disease and GI ailments is the ability of bacteria to translocate from oral cavity into the GI tract by hematogenous and enteral routes giving the opportunity for oral microbes to cause a range of GI diseases [9]. Even pathogens, such as *Klebsiella pneumoniae,* which were until recently referred to mainly as upper respiratory tract colonizers, have now emerged as notable GI tract-associated microorganisms [10]. Biofilm forming abilities of pathogenic microorganisms have been linked to the onset of GI diseases such as colorectal cancer [11] and inflammatory bowel disease [12].

Our study aimed to investigate the ethnopharmacological use of wormwood among Serbian population and to provide modern evidence for the uses currently practiced in Serbia. Therefore, this study aimed to examine the effects of *A. absinthium* ethanol extract on a range of pathogens associated with GI disorders as well as their impact on biofilm forming abilities of microbial species. Chemical analysis was performed in order to reveal bioactive constituents of the medicinal herb.

2. Materials and Methods

2.1. Ethnopharmacological Survey on the Use of A. absinthium—Current Update. The survey was performed using semistructured questionnaires via a face-to-face interview and circulating these questionnaires among cross section of people above 20 years of age; 117 filled-up reports were collected throughout Serbia. A questionnaire in Serbian language was prepared about the use of *Artemisia absinthium* L. (Asteraceae) by the local people from different parts of Serbia, as described previously for the plant species [13]. All the respondents were aware of the present investigation and have signed the informed consent. The survey was conducted during three months. The survey covered different age groups of both sexes, whose gender, age, educational background, professional status, and knowledge on the use of *A. absinthium* were also documented. Each participant was interviewed separately to generate data on diseases, regarding the treatment through medicinal plant *A. absinthium*. The record of questionnaires used included the following information: (a) the local name, (b) part of the plant used, (c) method of preparation, (d) mode of application, and (e) ethnomedicinal uses. Fidelity level (FL) was applied for diseases or ailments that were reported [13]. It is a ratio of informants claiming the use of a plant species for a particular purpose (Np) and number of informants using the plant to treat any disease (N). It was calculated by the formula

$$\mathrm{FL\%} = \mathrm{Np}/N \times 100. \quad (1)$$

2.2. Chemicals and Materials. Acetonitrile and formic acid (both of analytical grade) were purchased from Merck (Darmstadt, Germany). Analytical standards of phenolic compounds (chlorogenic acid, esculin, rutin, narcissin, isorhamnetin-3-O-glucoside, rosmarinic acid, apigenin, chrysoeriol, and kaempferide) were supplied by Sigma-Aldrich (Steinheim, Germany).

2.3. Preparation of the Extract. Plant *Artemisia absinthium* was collected on Ramski pesak, Serbia, during 2017. Samples of the collected plant material were identified based on the scientific botanical literature and its morphological features by one of the authors (D.S.). The plant species was deposited in our local institutional herbarium under number MI2017/Aa. A plant sample was air-dried and cut into small pieces; 10 g of the plant herb was extracted using 300 mL of ethanol (absolute, for analysis, Merck, Germany) at −20°C overnight. The extract was sonicated for 15 min and filtered through the Whatman No. 4 paper. The plant residue was reextracted using another 300 mL of ethanol, and the procedure was repeated. The obtained ethanol extract was evaporated at 40°C on a rotary evaporator (Büchi R-210) to dryness, and the dried extract was dissolved in 30% ethanol (Merck, Germany).

2.4. Antimicrobial Activity

2.4.1. Microorganisms and Culture Conditions. Candida species used were clinical isolates *C. albicans* 475/15, *C. albicans* 13/15, *C. albicans* 17/15, *C. krusei* H1/16, and *C. glabrata* 4/6/15 that were obtained and maintained as described in [14]. Reference yeast strains used were *C. albicans* ATCC 10231, *C. tropicalis* ATCC 750, and *C. parapsilosis* ATCC 22019.

The following Gram-positive and Gram-negative clinical bacteria were used: *Micrococcus luteus* (dT_9/2), *Rothia mucilaginosa* (oT_22/2), *Streptococcus agalactiae* (oT_20/1), *Streptococcus anginosus* (oT_26), *Streptococcus dysgalactiae* (oT_21/2), *Streptococcus oralis* (oT_5), *Streptococcus parasanguinis* (oT_3), *Streptococcus pyogenes* (dT_14), *Streptococcus pyogenes* (IBR S004), *Streptococcus salivarius* (dT_12), *Streptococcus salivarius* (IBR S006), *Staphylococcus hominis* (oT_14/2), *Enterococcus faecalis* IBRE001, *Enterobacter cloacae* (oT_18), and *Stenotrophomonas maltophilia* (A_12) obtained and maintained as described previously [15, 16].

Resistant strains used were *Pseudomonas aeruginosa* (IBRS P001), methicillin-resistant *Staphylococcus aureus* (IBRS MRSA 011), and *Escherichia coli* (IBRS E003) previously described by Victor et al. [17].

Reference bacterial American Type Culture Collection strains used were *Listeria monocytogenes* (NCTC 7973), *Yersinia enterocolitica* (ATCC 23715), *Klebsiella pneumoniae* (ATCC 13883), *Escherichia coli* (ATCC 35210), *Salmonella enterica* (ATCC 13311), and *Enterobacter cloacae* (ATCC 35030).

Tested microorganisms are deposited at the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Siniša Stanković"—National Institute of Republic of Serbia, University of Belgrade.

2.4.2. Anticandidal Activity. Minimal inhibitory and minimal fungicidal concentration (MIC/MFC) were determined [18]. Briefly, fresh overnight yeast cultures were adjusted to a concentration 1.0×10^5 CFU/well with the use of sterile saline. The microplates were incubated at 37°C for 24 h, after which the MIC and MFC were determined. The MIC values

were considered as the lowest concentrations without microscopically observed growth. Following the serial subcultivations of 10 μL into microtiter plates containing 100 μL of broth per well, as well as subsequent incubation at 37°C for 24 h, the lowest concentrations with no visible growth were defined as the MFC values, indicating 99.5% killing of the original inoculum. Ketoconazole was used as a positive control (Sigma-Aldrich, Germany).

2.4.3. Antibacterial Activity. Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) were determined by a serial microdilution of *A. absinthium* extract in 96-well microtiter plates following the protocol described by Kostić et al. [19]. Streptomycin was used as positive control.

2.4.4. Inhibition of Biofilm Formation. Strains used for inhibition of biofilm formation assay were *C. albicans* 475/15, *C. krusei* H1/16, *Klebsiella pneumoniae* (ATCC 13883), *Streptococcus pyogenes* (IBR S004), and *Pseudomonas aeruginosa* (IBRS P001). Impact of plant extract on microbial attachment as the first stage of biofilm formation was determined as described by Smiljković et al. [20]. Microorganisms were incubated for 24 h in 96-well microtiter plates with an adhesive bottom (Sarstedt, Germany) at 37°C with MIC and sub-MIC concentrations of the compound. After 24 h, each well was washed twice with sterile PBS (phosphate-buffered saline, pH 7.4). Fixation of adhered cells was done with methanol, after which the plate was air-dried and stained with 0.1% crystal violet (BioMérieux, France) for 30 min. Wells were washed with water, air-dried, and then 100 μL of 96% ethanol (Zorka, Serbia) was added into wells to suspend all the bound stain. Absorbance was read at 595 nm with a Multiskan FC Microplate Photometer (Thermo Scientific). Percentage of inhibition of biofilm formation was calculated by using the following equation:

$$\left[\frac{(\text{A595 control} - \text{A595 sample})}{\text{A595 control}}\right] \times 100. \quad (2)$$

2.4.5. Congo Red Binding Assay. The impact of tested compound on exopolysaccharide (EPS) production by *C. albicans* 475/15 biofilm was estimated with some modifications according to the method previously published [21]. Preformed 24 h biofilms in microtiter plates were treated with *A. absinthium* extract at its MIC, 0.5 MIC, and 0.25 MIC concentrations for 24 h at 37°C. Planktonic cells were then discarded and the adhered cells were washed with PBS. Congo red (1%, w/v) was added to wells and was kept in dark for 30 min. Excess dye was removed and the bound congo red was solubilized with 200 μL DMSO. The absorbance was measured at 490 nm in a microtiter plate reader. Percentage of EPS inhibition was calculated according to the following equation:

$$\%\text{inhibition} = \frac{(\text{OD}_{490}\,(\text{control}) - \text{OD}_{490}\,(\text{sample}))}{\text{OD}_{490}\,(\text{control})} * 100. \quad (3)$$

2.4.6. Crystal Violet Uptake Assay. Alteration of membrane permeability was detected by modified crystal violet uptake assay [22]. *C. albicans* 475/15 cells were harvested by centrifugation 10,000 rpm for 5 min, washed twice, and suspended in 50 mM PBS (pH 7.4). Plant extract in its previously determined minimal inhibitory concentration was added to the cell suspension, and samples were incubated at 37°C for 30 min. Control samples were untreated *C. albicans* cells. The cells were harvested at 10,000 rpm for 5 min and suspended in PBS containing 10 μg/mL of crystal violet. The cell suspension was incubated for 10 min at 37°C and centrifuged at 10,000 rpm for 5 min, after which the OD_{595} of the supernatant was measured using a Multiskan FC Microplate Photometer (Thermo Scientific). The optical density (OD) value of crystal violet solution, which was originally used in the assay, was considered as 100% excluded. The percentage of crystal violet uptake was calculated following the formula

$$\%\text{dye uptake} = 100 - \left[\left(\frac{\text{OD of the sample}}{\text{OD value of crystal violet solution}}\right) \times 100\right]. \quad (4)$$

2.4.7. Nucleotide Leakage Assay. The impact of *A. absinthium* extract on fungal membrane permeability (nucleotide leakage) was determined according to previously published protocol [23, 24] with some modifications and compared to untreated yeast cells. The culture of *C. albicans* 475/15 incubated overnight at 37°C was washed twice and resuspended in 10 mM PBS (pH 7.4), reaching the final density of 10^8 CFU/mL. Strain was incubated with the extract at the MIC for 30 min; *C. albicans* incubated with 10 mM PBS (pH 7.4) was used as control. After incubation, cell suspensions were centrifuged at 10,000 g for 10 min and supernatant absorbance measured at 260 nm and 280 nm with Agilent/HP 8453 UV-Visible Spectrophotometer (Agilent Technologies, USA) at room temperature (25°C).

2.5. Cytotoxicity. Cytotoxic effect of *A. absinthium* alcoholic extract was determined on human gingival fibroblasts cells (HGF-1) and spontaneously immortalized keratinocyte cell line (HaCaT) using crystal violet assay as described previously [25]. The extract of *A. absinthium* was dissolved in PBS to a final concentration of 8 mg/mL. HGF-1 cells were grown in Fibroblast Basal Medium (ATCC® PCS-201-030™), while HaCaT cells were grown in high-glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 1% penicillin and streptomycin (Invitrogen), at 37°C in a 5% CO_2 incubator. Forty-eight hours before treatment, cells were seeded in a 96-well microtiter adhesive plate at a seeding density of 4×10^3 cells per well. After 48 h, the medium was removed

and the cells were treated for next 24 h with various concentration of the extract in triplicate wells. Subsequently, the medium was removed; the cells were washed twice with PBS and stained with 0.4% crystal violet staining solution for 20 min at room temperature. Afterwards, crystal violet staining solution was removed; the cells were washed in a stream of tap water and left to air dry at room temperature. The absorbance of dye dissolved in methanol was measured in a plate reader at 570 nm (OD_{570}). The results were expressed as relative growth inhibition (GI_{50}) rate (%).

2.6. UHPLC-LTQ OrbiTrap MS Analysis of Polyphenolic Compounds. Separation and identification of polyphenols in tested extract were done by using UHPLC system (Accela 600) coupled to LTQ OrbiTrap MS (Thermo Fisher Scientific, Bremen, Germany). All the method details about chromatographic separation and setting of mass detector were given in previous work [26].

Some compounds were confirmed by comparison with the appropriate standard, while other compounds were tentatively identified by high resolution mass spectrometry (HRMS) and MS^n fragmentation using appropriate literature about analysis of various *Artemisia* species [27–34].

2.7. Statistical Analysis. All the experiments were performed in three repeats. All the data were calculated as a mean ± standard error and statistically analyzed using GraphPad PRISM 6 software.

3. Results and Discussion

3.1. Ethnopharmacological Investigation on A. absinthium Use among People in Serbia. The results of ethnopharmacological survey are presented in Table 1. A total of 117 informants were interviewed. Among them, 30 were residing in northern Serbia, 25 in east Serbia, 24 in central Serbia, 21 in west Serbia, and 17 in southern Serbia. Majority of informants (67.52%) were females, while the most represented age group was between 40 and 60 years (57.26%), with secondary educational background (47.86%). Among 117 informants, 29 reported no knowledge of *A. absinthium* neither in ethnobiology as a food or drink nor in medicinal purposes. On the other hand, 88 informants reported 138 medicinal uses of *A. absinthium*. The most prevalent use of *A. absinthium* was as aperitif or appetizer in alcoholic preparations. With a fidelity level of 39.77%, GI disorders were highly ranked reported use among the local people (mostly frequently alcoholic preparations and rarely tea), followed by the use of *A. absinthium* in wound-healing (laying the plant directly on wounds or preparations based on mixture of powdered plant material and animal fat). Other uses were less frequently reported (Table 1). Our ethnopharmacological survey conducted among the people living in Serbia is in accordance with previous literature data published on the use of *A. absinthium* [3, 35]. Since one of the most frequent uses for wormwood in Serbia was the application to improve the state of GI ailments, we strived to enlighten the activity of alcoholic extract against bacteria and fungi linked to GI disorders.

3.2. Antimicrobial Capacity of A. absinthium L. and Insights into the Modes of Action. The examined extract has shown promising antifungal properties with MIC in the range 0.5–1 mg/mL. Moderately higher resistance could be observed for non-albicans *Candida* strains *C. krusei* and *C. glabrata* with MIC 1 mg/mL (Table 2).

A previous study of *A. absinthium* essential oil [36] has indicated GIC_{50} (growth inhibitory concentration for 50% of microorganisms) 0.1 mg/mL, indicating better activity than the one recorded for the extract in our study. Aqueous plant extract tested at maximal 0.5 mg/mL concentration was ineffective against *Candida parapsilosis* ATCC 22019 and *Candida albicans* ATCC 90028 [37]. The study by Valdes et al. [38] found IC_{50} of ethanol extract towards *C. albicans* to be higher than 64 μg/mL. Study of apigenin [23], a compound present in the *A. absinthium* extract (Table 3), determined MIC towards different *Candida* species in the range 0.1–0.15 mg/mL, while another constituent, rutin, exhibited MIC 0.0375 mg/mL [39], indicating it as a possible carrier of antifungal activity.

The antifungal potential of *A. absinthium* ethanolic extract determined in our study is a solid basis for further antifungal development and provides a scientific evidence for the traditional usage of *A. absinthium* for GI disorders linked to fungal overgrowth. Species belonging to the genus *Candida* are residing on the mucosal surfaces of different parts of gastrointestinal tract including oral mucosa and gut [40]. However, this colonization is not always harmless, and these fungal species can cause severe infections. Besides oral candidiasis, these yeasts are also the most common cause of the infectious esophagitis [41]. *C. glabrata* had also been linked with Crohn's disease, where it possibly induces gut inflammation [42], while the presence of *C. krusei* in the stomach has been associated with gastritis and ulcers [43].

Ethanolic extract of *A. absinthium* herb has shown excellent antibacterial potential with MIC 0.25–4 mg/mL (Table 4). The most susceptible to the extract was *Streptococcus salivarius* (dT_12) with MIC 0.25 mg/mL, while the most resistant were *L. monocytogenes* (NCTC 7973) and resistant strains of *E. coli* (IBRS E003) and *S. aureus* (IBRS MRSA 011) with MIC 4 mg/mL. Resistant strain of *P. aeruginosa* (IBRS P001) was susceptible to the treatment with 1 mg/mL of herb extract.

A recent study [44] of *A. absinthium* methanol extract has indicated MIC 2.5–1.255 mg/mL towards *Escherichia coli* ATCC 10536, while in our study it exhibited MIC 4 mg/mL towards *E. coli* (IBRS E003) resistant strain. Unlike high susceptibility of resistant *P. aeruginosa* strain recorded in our study, in the study by Boudjelal et al. [44], it did not show activity (MIC > 2.5 mg/mL) as well as the one by Khan et al. [45]. Methanolic extract studied previously by Hasannezhad et al. [46] has shown MIC 41.7 mg/mL towards *L. monocytogenes* PTCC 1298, unlike 4 mg/mL determined in our study. Methanol extract concentration 8 mg/mL reduced *Escherichia coli* ATCC 25922 growth for 51.5% [47].

Table 1: Ethnomedicinal use of wormwood in Serbia, collected data in the field.

Gender		Age group		Education	
Male	38 (32.48%)	20–40	15 (12.82%)	Primary	14 (11.96%)
Female	79 (67.52%)	40–60	67 (57.26%)	Secondary	56 (47.86%)
		60–80	35 (29.91%)	High	47 (40.17%)
Total informants	117				
Ethnomedicinal use	Citation for particular use	Plant part used (number of reports)	Preparation methods	Fidelity level (%)	
No medicinal use	29	—	—	—	
Aperitif, appetizer	46	24–leaves 22–herb	Alcoholic solution	52.27	
Gastrointestinal disorders	35	35–herb	Alcoholic solution; boiled as tea	39.77	
Wound healing	24	11–leaves 10–herb 3–flowers	Direct application of plant parts on wounded skin; lard coating with dry flowers	27.27	
Coughs	21	21–herb	Tea; alcoholic solution	23.86	
Improvement of memory	7	6–roots 1–herb	Alcoholic solution; chewing	7.95	
Preventive	5	5–herb	Tea	5.68	

Table 2: Anticandidal activity of *A. absinthium* L. ethanolic extract.

Yeasts	*A. absinthium*		Ketoconazole	
	MIC (mg/mL)	MFC (mg/mL)	MIC (μg/mL)	MFC (μg/mL)
C. albicans 475/15	0.5	1.0	3.2	6.4
C. albicans 13/15	0.5	1.0	1.6	51.2
C. albicans 17/15	0.5	1.0	1.6	51.2
C. krusei H1/16	1.0	2.0	1.6	3.2
C. glabrata 4/6/15	1.0	2.0	1.6	6.4
C. albicans ATCC 10231	1.0	2.0	1.6	6.4
C. tropicalis ATCC 750	0.5	1.0	1.6	6.4
C. parapsilosis ATCC 22019	0.5	1.0	3.2	6.4

MIC: minimal inhibitory concentration and MFC: minimal fungicidal concentration.

The ethanol extract from *A. absinthium* growing wild in Serbia has shown promising antibacterial potential towards a range of bacterial strains with eight of them having MIC $\leq$ 0.5 mg/mL. Range of bacterial species is nowadays associated with gastrointestinal ailments. Likewise, antibiotic-resistant strains of *P. aeruginosa* are frequent colonizers of intensive care units patients' GI tract [48], while colons of Crohn's disease patients have higher frequency of *E. coli* [49]. On the other hand, increased levels of *Streptococcus* species have been associated with upper gastrointestinal symptoms of functional dyspepsia [50]. Recently, *K. pneumoniae* has been highlighted as the frequent GI disease-associated pathogenic bacterium being linked to the diseases such as Crohn's disease, ulcerative colitis, and colorectal cancers [10].

Strain *C. albicans* 475/15 was selected as the reference strain for the investigations of antimicrobial mode of action. It can be observed that application of *A. absinthium* significantly affected membrane integrity (Figure 1) as observed by dramatic increase in the uptake of crystal violet (Figure 1(a)) and further confirmed by an increase in nucleotide and protein leakage (Figure 1(b)).

Previous studies conducted with apigenin, the phenolic compound present in *A. absinthium* (Table 3), proved the membrane antagonistic activity for single extract constituent [23], while the study of *A. asiatica* essential oil proved its potential to induce leakage of cell constituents [51].

Ability of the microorganisms to group into biofilms has been associated with numerous chronic diseases including those of GI tract [52]. Likewise, *E. coli* ability to establish biofilms has been linked to the presence of ulcerous colitis [53]. It has been shown for *C. albicans* that genes associated with its adhesion ability, which is the first stage of biofilm formation, have higher expression levels during the colonization of the cecum and invasion of host tissue [54].

Extract of *A. absinthium* has shown a promising potential in the means of reduction of microbial ability to establish biofilms (Figure 2). Application of extract at 0.5 MIC concentration has reduced *C. krusei* biofilm formation ability for more than 50%, with the significant reduction (>50%) observed also for MIC of extract towards *C. albicans* 475/15 and *S. pyogenes* IBR S004. The least promising was its ability to interfere with *P. aeruginosa* (IBRS P001) and *K. pneumoniae* ATCC 13883 biofilms since it caused less than 30% reduction with the highest applied concentration (MIC).

Plant extracts tested by Khan et al. [45] were not able to interfere significantly with *S. aureus* biofilm, while the

Table 3: Compounds identified in *Artemisia absinthium*.

No.	t_R, min	Compound name	Molecular formula, $[M–H]^-$	Calculated mass, $[M–H]^-$	Exact mass, $[M–H]^-$	Δ ppm	MS^2 fragments, (% Base peak)	MS^3 fragments, (% base peak)	MS^4 fragments, (% base peak)	References
1	3.75	Dihydroxybenzoic acid hexoside	$C_{13}H_{15}O_9^-$	315.07216	315.07153	2.00	**153** (100), 152 (50), 109 (15), 108 (10)	**109** (100)	—	Melguizo-melguizo et al., [32]
2	4.38	Syringic acid hexoside	$C_{15}H_{19}O_{10}^-$	359.09837	359.09778	1.64	**197** (100), 182 (10)	**182** (100), 153 (20)	—	Klick and Herrmann, [28]
3	5.23	5-O-Caffeoylquinic acid (chlorogenic acid)[a]	$C_{16}H_{17}O_9^-$	353.08781	353.08752	0.82	**191** (100), 179 (5)	173 (75), **127** (100), 111 (40), 93 (60), 85 (90)	109 (40), 99 (50), 85 (100)	Han et al., [33]
4	5.77	Esculin[a]	$C_{15}H_{15}O_9^-$	339.07216	339.07156	1.77	**179** (100), 161 (30), 135 (20)	**135** (100)	—	Han et al., [33]
5	6.17	Feruloylquinic acid	$C_{17}H_{19}O_9^-$	367.10346	367.10281	1.77	203 (15), 193 (10), **191** (100), 173 (5)	173 (25), **127** (100), 111 (40), 93 (40), 85 (80)	—	Han et al., [33]
6	6.44	Quercetin 3-O-(6″-rhamnosyl) glucoside (Rutin)[a]	$C_{27}H_{29}O_{16}^-$	609.14611	609.14545	1.08	343 (5), **301** (100), 300 (30), 271 (10), 255 (5)	273 (25), 257 (20), **179** (100), 151 (75)	151 (100)	Han et al., [33]
7	6.83	Kaempferol 7-O-(6″-rhamnosyl) hexoside	$C_{27}H_{29}O_{15}^-$	593.15119	593.15015	1.75	327 (10), 286 (20), **285** (100), 257 (5)	267 (40), **257** (100), 241 (30), 229 (40), 213 (30)	255 (10), 239 (30), 229 (100), 163 (40)	Hoffmann and Harrmann, [27]
8	6.92	Isorhamnetin 3-O-(6″-rhamnosyl) glucoside (Narcissin)[a]	$C_{28}H_{31}O_{16}^-$	623.16176	623.16077	1.59	316 (15), 315 (100), 300 (30), 271 (20), 255 (10)	301 (5), **300** (100), 287(5), 272(5), 255(5)	272 (100), 271(90), 255 (50), 243 (10), 166 (5)	Hoffmann and Harrmann, [27]
9	7.00	Apigenin 8-*C*-[6“-(3-hydroxy-3-methylglutaryl)] hexoside	$C_{27}H_{27}O_{14}^-$	575.14063	575.14032	0.54	557 (10), 513 (15), **431** (100), 341 (80), 311 (70)	341 (10), **311** (100)	283 (100)	—
10	7.00	Spinacetin 3-O-(6″-rhamnosyl)hexoside	$C_{29}H_{33}O_{17}^-$	653.17232	653.17206	0.40	346 (20), **345** (100), 330 (40), 302 (15), 287 (20)	**330** (100), 305 (5)	315 (10), 302 (100), 287 (30), 273 (5), 166 (5)	Hoffmann and Harrmann, [27]
11	7.11	Dicaffeoylquinic acid isomer 1	$C_{25}H_{23}O_{12}^-$	515.11950	515.11975	-0.49	**353** (100)	**191** (100), 179 (40), 173 (5), 135 (10)	173 (95), 171 (50), 127 (90), 111 (30), 85 (100)	Han et al., [33]
12	7.20	Isorhamnetin 3-O-glucoside[a]	$C_{22}H_{21}O_{12}^-$	477.10385	477.10339	0.96	357 (15), **315** (100), 314 (90), 299 (20), 285 (15)	**300**(100), 286(5), 272(10)	283 (5), 272 (100), 271 (60), 255 (40), 243 (10)	Hoffmann and Harrmann, [27]
13	7.25	Dicaffeoylquinic acid isomer 2	$C_{25}H_{23}O_{12}^-$	515.11950	515.11932	0.35	**353** (100), 335 (5), 299 (10), 255 (5), 203 (10)	**191** (100), 179 (65), 173 (60), 135 (20)	173 (80), 171 (20), 127 (80), 111 (20), 85 (100)	Han et al., [33]

Table 3: Continued.

No.	t_R, min	Compound name	Molecular formula, $[M–H]^-$	Calculated mass, $[M–H]^-$	Exact mass, $[M–H]^-$	Δ ppm	MS^2 fragments, (% Base peak)	MS^3 fragments, (% base peak)	MS^4 fragments, (% base peak)	References
14	7.28	Spinacetin 3-O-hexoside	$C_{23}H_{23}O_{13}^-$	507.11441	507.11395	0.91	492 (60), **345** (100), 344 (80), 330 (20), 329 (60)	**330** (100), 302 (5), 286 (10)	—	Hoffmann and Harrmann, [27]
15	7.43	Chrysoeriol 7-O-hexoside	$C_{22}H_{21}O_{11}^-$	461.10894	461.10904	−0.22	446 (10), 300 (10), **299** (100), 284 (10)	**284** (100)	256 (100)	Benyahia et al., [31]
16	7.50	Rosmarinic acid[a]	$C_{18}H_{15}O_8^-$	359.07724	359.07672	1.45	223 (10), 197 (30), 179 (40), **161** (100), 133 (10)	**133** (100)	105 (100)	Sahin et al., [30]
17	9.52	Apigenin[a]	$C_{15}H_9O_5^-$	269.04554	269.04529	0.93	269 (60), **225** (100), 201 (30), 151 (70), 149 (50)	210 (10), 197 (50), 196 (20), 183 (40), **181** (100)	—	Olennikov et al., [34]
18	9.73	Chrysoeriol[a]	$C_{16}H_{11}O_6^-$	299.05611	299.05576	1.17	285 (10), **284** (100)	284 (20), **256** (100)	256 (40), 228 (100), 160 (40)	Olennikov et al., [34]
19	11.43	Eupatorin	$C_{18}H_{15}O_7^-$	343.08233	343.08160	2.13	329 (10), **328** (100)	314 (10), **313** (100), 285 (5)	298 (100), 285 (10), 270 (15)	Rashid et al., [61]
20	11.67	Casticin	$C_{19}H_{17}O_8^-$	373.09289	373.09250	1.05	359 (10), **358** (100)	**343** (100)	328 (100), 315 (15), 300 (30), 299 (20), 284 (10)	Han et al., [33]
21	11.75	Kaempferide[a]	$C_{16}H_{11}O_6^-$	299.05611	299.05573	1.27	285 (10), **284** (100)	255 (20), 240 (15), 228 (20), 164 (25), **151** (100)	106 (100), 83 (10), 65 (5)	Lai et al., [29]

[a]Confirmed using available standards, all the other compounds were identified based on HRMS data. Bold numbers are peaks which were further fragmented in MS^3 and MS^4 experiment.

investigation of caffeoylquinic acids from *A. absinthium* proved their antibiofilm effect towards *S. aureus* and *E. faecalis* [55].

The mode of antibiofilm mechanism was investigated on the strain *C. albicans* 475/15 in order to establish whether reduction in exopolysaccharide production by the fungal cells is the reason for observed reduced biofilm capacity in previous experiment (Figure 2). Treatment with MIC of *A. absinthium* extract has reduced the exopolysaccharide bound congo red for more than 25% indicating that it might be moderately involved as the plant antibiofilm mechanism (Figure 3).

3.3. Cytotoxicity. Cytotoxicity of *A. absinthium* extracts is demonstrated in Figure 4. It could be observed that growth rate of both cell lines was not significantly affected by *A. absinthium* up to a concentration 400 μg/mL. The concentration 400 μg/mL had only effect on HaCaT cell line where growth rate was 85.47% when compared to the control. The previous studies point that concentration that caused decrease in growth rate for 50% and higher than 401 μg/mL should be considered as nontoxic [25].

Previous study of the leaves and stem ethanolic extracts [56] showed reduction in HaCaT cell viability (71.6% viability with 1000 μg/mL of extract) while range of concentrations as the one tested in our study (25–400 μg/mL) did not cause significant reduction in cell viability. Although a different methodology was used (alamarBlue assay by Moacă et al. [56] and crystal violet assay in our study), results prove noncytotoxic nature of the tested plant extract. Previous study of ethanolic extract of *Artemisia apiacea* proved its noncytotoxicity towards HaCaT in a concentration up to 200 μg/mL [57].

Table 4: Antibacterial activity of *A. absinthium* L. ethanolic extract.

Bacteria	*A. absinthium* (mg/mL)		Streptomycin (μg/mL)	
	MIC	MBC	MIC	MBC
Micrococcus luteus (dT_9/2)	0.5	1.0	6.2	12.5
Rothia mucilaginosa (oT_22/2)	0.5	1.0	12.5	25
Streptococcus agalactiae (oT_20/1)	1.0	2.0	3.1	6.2
Streptococcus anginosus (oT_26)	2.0	4.0	3.1	6.2
Streptococcus dysgalactiae (oT_21/2)	0.5	1.0	6.2	12.5
Streptococcus oralis (oT_5)	1.0	2.0	12.5	25
Streptococcus parasanguinis (oT_3)	0.5	1.0	3.1	6.2
Streptococcus pyogenes (dT_14)	2.0	4.0	3.1	6.2
Streptococcus pyogenes (IBR S004)	1.0	2.0	14.0	28.0
Streptococcus salivarius (dT_12)	0.2	0.5	6.2	12.5
Streptococcus salivarius (IBR S006)	1.0	2.0	3.8	7.6
Enterococcus faecalis (IBR E001)	0.5	1.0	50.0	100.0
Staphylococcus hominis (oT_14/2)	2.0	4.0	37.5	75.0
MRSA (IBRS MRSA 011)	4.0	8.0	100	>100
Listeria monocytogenes (NCTC 7973)	4.0	8.0	50.0	100
Enterobacter cloacae (oT_18)	0.5	1.0	37.5	75
Enterobacter cloacae (ATCC 35030)	0.5	1.0	25.0	50.0
Stenotrophomonas maltophilia (A_12)	2.0	4.0	37.5	75.0
Pseudomonas aeruginosa (IBRS P001)	1.0	2.0	50.0	100.0
Yersinia enterocolitica (ATCC 23715)	1.0	2.0	10.0	20.0
Klebsiella pneumoniae (ATCC 13883)	1.0	2.0	5.0	10.0
Salmonella enterica (ATCC 13311)	1.0	2.0	50.0	100.0
Escherichia coli (IBRS E003)	4.0	8.0	100	>100
Escherichia coli (ATCC 35210)	1.0	2.0	50.0	100

MIC: minimal inhibitory concentration and MBC: minimal bactericidal concentration. Results are in mg/mL.

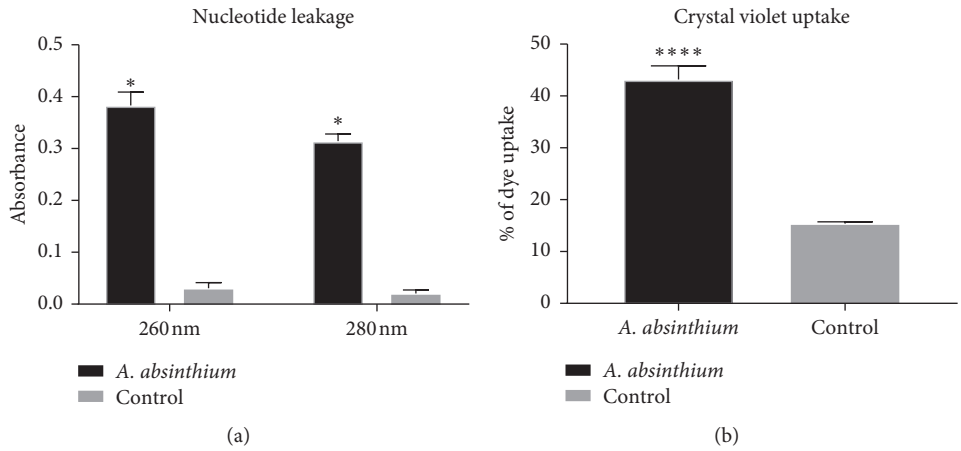


Figure 1: Destabilization of *C. albicans* 475/15 membrane after treatment with *A. absinthium* (MIC, 0.5 mg/mL) for 30 min detected by (a) crystal violet uptake (%) and (b) leakage of nucleic acids (260 nm) and proteins (280 nm). The error bars indicate standard deviations. The asterisks represent statistical significance ($^{*}p < 0.05$; $^{****}p < 0.0001$).

3.4. Chemical Profiling. This study describes the UHPLC-LTQ OrbiTrap MS polyphenolic profile of the *Artemisia absinthium* extract. A total of 21 compounds (Table 3) were identified. Nine compounds were identified using analytical standards, while the other twelve were identified by the search for their $[M–H]^-$ deprotonated molecules combined with its MS^4 fragmentations.

Examination of mass spectra revealed seven phenolic acid derivatives. The most represented compounds from this group were quinic acid derivatives: chlorogenic acid (**3**), feruloylquinic acid (**5**), two derivatives of dicaffeoylquinic acid (**11** and **13**), and rosmarinic acid (**16**).

Eight compounds from the group of flavonoid glycosides were identified. Four of them (**6**, **7**, **8**, and **10**) were marked as rutinosides, because as MS^2 base peak they give a fragment ion formed by the loss of 308 Da (rhamnosyl-hexoside). Fragmentation pathway of kaempferol 7-O-(6″-rhamnosyl) hexoside (compound **7**) was confirmed in literature [58]. It

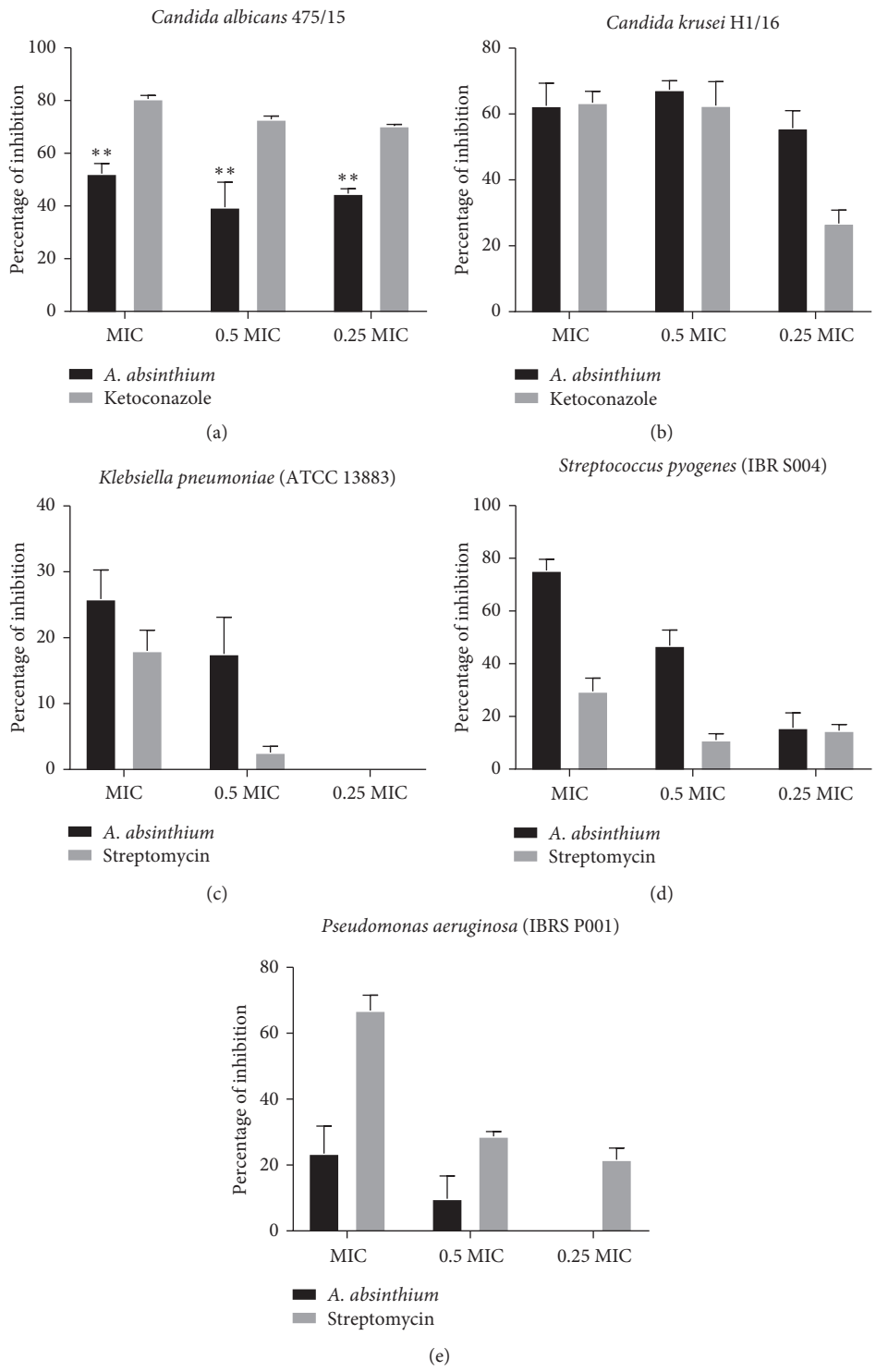


Figure 2: Percentage of inhibition of fungal and bacterial biofilm formation after treatment with *A. absinthium* in range of concentration 0.125 mg/mL–1 mg/mL. The error bars indicate standard deviations. The asterisks represent statistical significance (** $p < 0.05$).

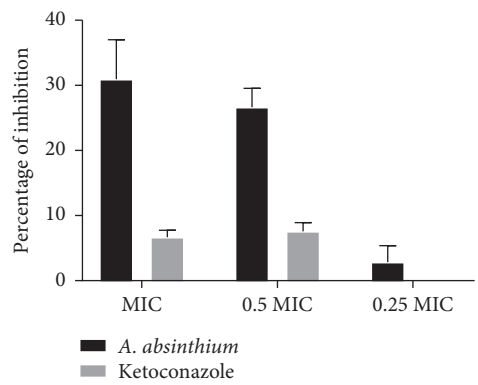


Figure 3: Estimated inhibition (%) of exopolysaccharide biofilm matrix content by congo red binding assay determined using *C. albicans* 475/15 biofilm after treatment with *A. absinthium* compared to commercial antifungal drug ketoconazole. The error bars indicate standard deviations. Statistical difference was not significant.

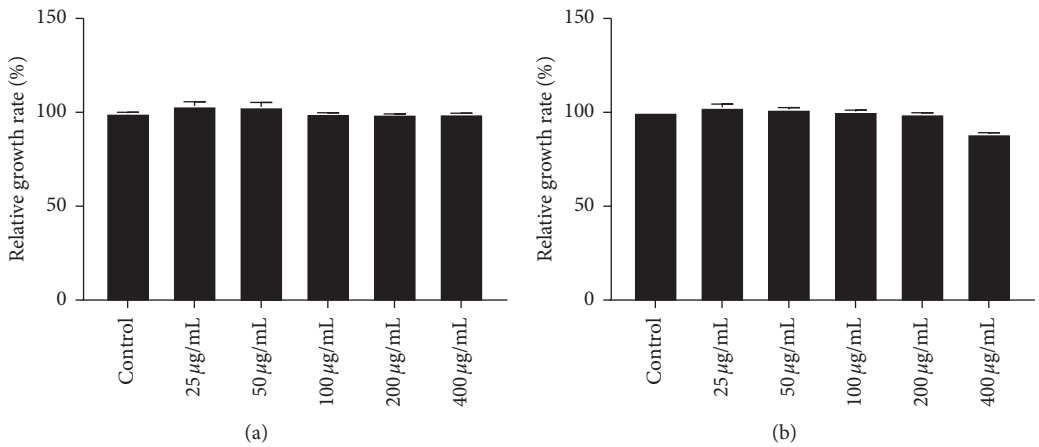


Figure 4: Cytotoxicity of *A. absinthium* alcoholic extract towards (a) HGF-1 cells and (b) HaCaT cell line, represented as relative growth rates (%) at different concentrations of the extract. The error bars indicate standard deviations among three independent replicates.

should be noted here that kaempferol 3-O-(6″-rhamnosyl) hexoside has been previously identified in some *Artemisia* species [27], but in our case the fragmentation corresponds to a 7-O derivative [58]. Namely, the position of sugar binding to flavonoids can be sensed by the abundance of the molecular ion of aglycone and their radical ion [59]. Of course, for accurate confirmation of this claim, it is necessary to isolate this compound and record its NMR spectra. Compounds **12**, **14**, and **15** were identified as hexosides of isorhamnetin, spinacetin, and chrysoeriol, respectively. The MS^2 base in the case of these three compounds is formed by a neutral loss of 162 Da. Apigenin 8-*C*-[6″-(3-hydroxy-3-methylglutaryl)]hexoside (**9**) is a compound that, in our opinion, has not yet been identified in *Artemisia* species. In the first fragmentation step, this compound loses the hexosyl group (162 Da) and thus the MS^2 base peak is formed. The MS^3 base peak is generated by homolytic sugar cleavage (120 Da) and further neutral loss of 28 Da (CO) gives MS^4 base peak. The detailed fragmentation pathway of this compound is depicted in Figure 5. Confirmation that aglycone part of this compound was vitexin (apigenin 8-*C*-glucoside) and not isovitexin (apigenin 6-*C*-glucoside) has been found in the literature [60].

Considering the five identified flavonoid aglycones, three were confirmed by comparison with standards (**17**, **18**, and **21**) and two (**19** and **20**) were tentatively identified by examination of its MS spectra. Compound **19** was previously detected in aerial parts of *Artemisia incisa* Pamp [61], while compound **20** was identified in the crude extracts and some fractions of *Folium Artemisia* Argyi, a traditional Chinese herb medicine and food supplement [33].

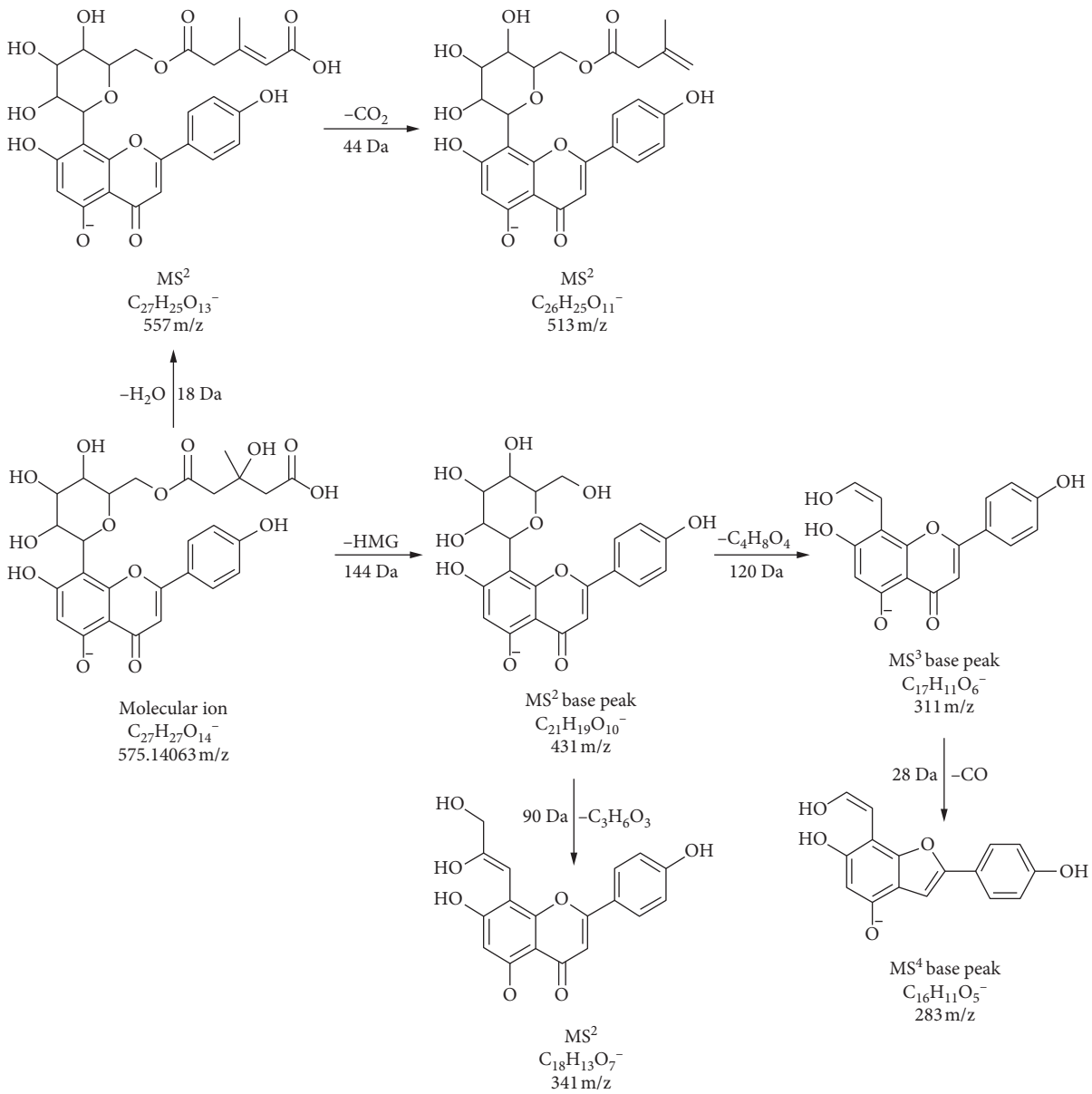


Figure 5: Fragmentation pathway of compound 9 (apigenin 8-C-[6“-(3-hydroxy-3-methylglutaryl)]hexoside).

4. Conclusions

Artemisia absinthium L. wide usage as a traditional remedy for GI diseases might be based on the ability of the herb to reduce the growth of microorganisms whose presence is linked to GI discomfort. This antimicrobial potential could be attributed to the broad spectrum of bioactive phenolic compounds present in the herb ethanol extract since for some of them identical mechanism of antimicrobial activity and wide antimicrobial spectrum has been determined previously.

Data Availability

The datasets used and analyzed in the current study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Marija Ivanov and Uroš Gašić contributed equally to this work.

Acknowledgments

This research was funded by the Serbian Ministry of Education, Science and Technological Development (contract nos. 451-03-9/2021–14/200007).

References

[1] A. Szopa, J. Pajor, P. Klin et al., "Artemisia absinthium L.-Importance in the history of medicine, the latest advances in phytochemistry and therapeutical, cosmetological and culinary uses," *Plants*, vol. 9, no. 9, p. 1063, 2020.

[2] M. Bailen, L. F. Julio, C. E. Diaz et al., "Chemical composition and biological effects of essential oils from Artemisia absinthium L. cultivated under different environmental conditions," *Industrial Crops and Products*, vol. 49, pp. 102–107, 2013.

[3] G. E. S. Batiha, A. Olatunde, A. El-mleeh et al., "Bioactive compounds, pharmacological actions, and pharmacokinetics of wormwood (Artemisia absinthium)," *Antibiotics*, vol. 9, no. 6, pp. 1–25, 2020.

[4] J. Langhorst, H. Wulfert, R. Lauche et al., "Systematic review of complementary and alternative medicine treatments in inflammatory bowel diseases," *Journal of Crohn's & Colitis*, vol. 9, no. 1, pp. 86–106, 2015.

[5] P. B. Eckburg and D. A. Relman, "The role of microbes in Crohn's disease," *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America*, vol. 44, no. 2, pp. 256–62, 2007.

[6] C. A. Kumamoto, "Inflammation and gastrointestinal Candida colonization," *Current Opinion in Microbiology*, vol. 14, no. 4, pp. 386–91, 2011.

[7] D. T. Book, T. L. Smith, J. P. Mcnamar et al., "Chronic sinonasal disease in patients with inflammatory bowel disease," *American Journal of Rhinology & Allergy*, vol. 17, no. 2, pp. 87–90, 2003.

[8] W. Sun, X. Han, S. Wu, and C. Yang, "Tonsillectomy and the risk of inflammatory bowel disease: a systematic review and meta-analysis," *Journal of Gastroenterology and Hepatology*, vol. 31, no. 6, pp. 1085–1094, 2016.

[9] S. Kitamoto, H. Nagao-Kitamoto, R. Hein, T. M. Schmidt, and N. Kamada, "The bacterial connection between the oral cavity and the gut diseases," *Journal of Dental Research*, vol. 99, no. 9, pp. 1021–1029, 2020.

[10] C. P. Kaur, J. Vadivelu, and S. Chandramathi, "Impact ofKlebsiella pneumoniaein lower gastrointestinal tract diseases," *Journal of Digestive Diseases*, vol. 19, no. 5, pp. 262–271, 2018.

[11] C. Rizzato, J. Torres, E. Kasamatsu et al., "Potential role of biofilm formation in the development of digestive tract cancer with special reference to helicobacter pylori infection," *Frontiers in Microbiology*, vol. 10, p. 846, 2019.

[12] A. Srivastava, J. Gupta, S. Kumar, and A. Kumar, "Gut biofilm forming bacteria in inflammatory bowel disease," *Microbial Pathogenesis*, vol. 112, pp. 5–14, 2017.

[13] D. Stojković, L. Barros, J. Petrović et al., "Ethnopharmacological uses of Sempervivum tectorum L. in southern Serbia: scientific confirmation for the use against otitis linked bacteria," *Journal of Ethnopharmacology*, vol. 176, pp. 297–304, 2015.

[14] M. Smiljkovic, M.-T. Matsoukas, E. Kritsi et al., "Nitrate esters of heteroaromatic compounds asCandida albicansCYP51 enzyme inhibitors," *ChemMedChem*, vol. 13, no. 3, pp. 251–258, 2018.

[15] M. M. Nikolić, K. K. Jovanović, T. L. Marković et al., "Antimicrobial synergism and cytotoxic properties of Citrus limon L., Piper nigrum L. and Melaleuca alternifolia (Maiden and Betche) Cheel essential oils," *The Journal of Pharmacy and Pharmacology*, vol. 69, no. 11, pp. 1606–1614, 2017.

[16] M. Kostić, M. Ivanov, Â Fernandes et al., "Antioxidant extracts of three russula genus species express diverse biological activity," *Molecules (Basel, Switzerland)*, vol. 25, no. 18, p. 4336, 2020.

[17] K. Victor, L. Boris, G. Athina et al., "Design, synthesis and antimicrobial activity of usnic acid derivatives," *Medchemcomm*, vol. 9, no. 5, pp. 870–882, 2018.

[18] M. Ivanov, A. Kannan, D. Stojković et al., "Revealing the astragalin mode of anticandidal action," *EXCLI Journal*, vol. 19, pp. 1436–1445, 2020.

[19] M. Kostić, M. Smiljković, J. Petrović et al., "Chemical, nutritive composition and a wide range of bioactive properties of honey mushroom Armillaria mellea (Vahl: Fr.) Kummer," *Food & Function*, vol. 8, no. 9, pp. 3239–3249, 2017.

[20] M. Smiljkovic, M. I. Dias, D. Stojkovic et al., "Characterization of phenolic compounds in tincture of edible Nepeta nuda: development of antimicrobial mouthwash," *Food & Function*, vol. 9, no. 10, pp. 5417–5425, 2018.

[21] P. Dey, D. Parai, M. Banerjee, S. T. Hossain, and S. K. Mukherjee, "Naringin sensitizes the antibiofilm effect of ciprofloxacin and tetracycline against Pseudomonas aeruginosa biofilm," *International Journal of Medical Microbiology*, vol. 310, no. 3, Article ID 151410, 2020.

[22] I. Khan, A. Bahuguna, P. Kumar, V. K. Bajpai, and S. C. Kang, "Antimicrobial potential of carvacrol against uropathogenic Escherichia coli via membrane disruption, depolarization, and reactive oxygen species generation," *Frontiers in Microbiology*, vol. 8, 2017.

[23] M. Smiljkovic, D. Stanisavljevic, D. Stojkovic et al., "Apigenin-7-O-glucoside versus apigenin: insight into the modes of anticandidal and cytotoxic actions," *EXCLI Journal*, vol. 16, pp. 795–807, 2017.

[24] A. S. O. Lemos, L. M. Campos, L. Melo et al., "Antibacterial and antibiofilm activities of psychorubrin, a pyranonaphthoquinone isolated from Mitracarpus frigidus (Rubiaceae)," *Frontiers in Microbiology*, vol. 9, 2018.

[25] D. Stojković, M. I. Dias, D. Drakulić et al., "Methanolic extract of the herb ononis spinosa L. Is an antifungal agent with no cytotoxicity to primary human cells," *Pharmaceuticals (Basel, Switzerland)*, vol. 13, no. 4, pp. 1–13, 2020.

[26] A. Ž Kostić, U. M. Gašić, M. B. Pešić et al., "Phytochemical analysis and total antioxidant capacity of rhizome, aboveground vegetative parts and flower of three Iris species," *Chemistry & Biodiversity*, vol. 16, no. 3, Article ID e1800565, 2019.

[27] B. Hoffmann and K. Herrmann, "Flavonolglykoside des Beifu⁄ (Artemisia vulgaris L.), Estragon (Artemisia dracunculus L.) und Wermut (Artemisia absinthium L.)," *Zeitschrift f⁄r Lebensmittel-Untersuchung und -Forschung*, vol. 174, no. 3, pp. 211–215, 1982.

[28] S. Klick and K. Herrmann, "Bestimmung von Hydroxybenzoes⁄ure-Verbindungen in Gew⁄rzen und weiteren pflanzlichen Lebensmitteln," *Zeitschrift f⁄r Lebensmittel-Untersuchung und -Forschung*, vol. 187, no. 5, pp. 444–450, 1988.

[29] J.-P. Lai, Y. H. Lim, J. Su, H.-M. Shen, and C. N. Ong, "Identification and characterization of major flavonoids and caffeoylquinic acids in three Compositae plants by LC/DAD-APCI/MS," *Journal of Chromatography B*, vol. 848, no. 2, pp. 215–225, 2007.

[30] S. Şahin, Ö. Aybastier, and E. Işik, "Optimisation of ultrasonic-assisted extraction of antioxidant compounds from Artemisia absinthium using response surface methodology," *Food Chemistry*, vol. 141, no. 2, pp. 1361–1368, 2013.

[31] S. Benyahia, F. Benayache, and S. Benayache, "Flavonoids from artemisia arborescens," *Chemistry of Natural Compounds*, vol. 49, no. 6, pp. 1132-1133, 2014.

[32] D. Melguizo-Melguizo, E. Diaz-de-Cerio, R. Quirantes-Piné, J. Švarc-Gajić, and A. Segura-Carretero, "The potential of Artemisia vulgaris leaves as a source of antioxidant phenolic compounds," *Journal of Functional Foods*, vol. 10, pp. 192–200, 2014.

[33] B. Han, Z. Xin, S. Ma et al., "Comprehensive characterization and identification of antioxidants in Folium Artemisiae Argyi using high-resolution tandem mass spectrometry," *Journal of Chromatography B*, vol. 1063, pp. 84–92, 2017.

[34] D. N. Olennikov, N. I. Kashchenko, N. K. Chirikova et al., "Caffeoylquinic acids and flavonoids of fringed sagewort (artemisia frigida willd.): HPLC-DAD-ESI-QQQ-MS profile, HPLC-DAD quantification, in vitro digestion stability, and antioxidant capacity," *Antioxidants (Basel, Switzerland)*, vol. 8, no. 8, 2019.

[35] M. Masoudi and M. Saiedi, "A Review study of Ethnopharmacology, Phytochemistry, and Anti-inflammatory, antioxidant, and anti-microbial effect of Artemisia absinthium," *Der Pharmacia Lettre*, vol. 9, no. 4, pp. 155–162, 2017.

[36] F. Juteau, I. Jerkovic, V. Masotti et al., "Composition and antimicrobial activity of the essential oil ofArtemisia absinthiumfrom Croatia and France," *Planta Medica*, vol. 69, no. 2, pp. 158–161, 2003.

[37] M. D. P. Rodríguez-Torres, L. S. Acosta-Torres, L. A. Díaz-Torres, G. Hernández Padrón, R. García-Contreras, and B. E. Millán-Chiu, "Artemisia absinthium-based silver nanoparticles antifungal evaluation against three Candida species," *Materials Research Express*, vol. 6, no. 8, Article ID 085408, 2019.

[38] A. Fernández-Calienes Valdés, J. Mendiola Martínez, R. Scull Lizama, M. Vermeersch, P. Cos, and L. Maes, "In vitro antimicrobial activity of the Cuban medicinal plants Simarouba glauca DC, Melaleuca leucadendron L and Artemisia absinthium L," *Memórias Do Instituto Oswaldo Cruz*, vol. 103, no. 6, pp. 615–618, 2008.

[39] M. Ivanov, A. Kannan, D. S. Stojković et al., "Flavones, flavonols, and glycosylated derivatives—impact on candida albicans growth and virulence, expression of cdr1 and erg11, cytotoxicity," *Pharmaceuticals*, vol. 14, no. 1, pp. 1–12, 2021.

[40] T. Vila, A. S. Sultan, D. Montelongo-Jauregui, and M. A. Jabra-Rizk, "Oral candidiasis: a disease of opportunity," *Journal of Fungi*, vol. 6, no. 1, 2020.

[41] A. A. Mohamed, X. L. Lu, and F. A. Mounmin, "Diagnosis and treatment of esophageal candidiasis: current updates," *Canadian Journal of Gastroenterology and Hepatology*, vol. 2019, Article ID 3585136, 6 pages, 2019.

[42] G. Liguori, B. Lamas, M. L. Richard et al., "Fungal dysbiosis in mucosa-associated microbiota of Crohn's disease patients," *Journal of Crohn's and Colitis*, vol. 10, no. 3, pp. 296–305, 2016.

[43] D. T. Huong, Y. Zhao, N. T. Nguyet et al., "Candida kruseicolonization in patients with gastrointestinal diseases," *Medical Mycology*, vol. 51, no. 8, pp. 884–887, 2013.

[44] A. Boudjelal, A. Smeriglio, G. Ginestra, M. Denaro, and D. Trombetta, "Phytochemical profile, safety assessment and wound healing activity of artemisia absinthium L," *Plants*, vol. 9, no. 12, pp. 1–14, 2020.

[45] M. F. Khan, H. Tang, J. T. Lyles, R. Pineau, Z.-U.-R. Mashwani, and C. L. Quave, "Antibacterial properties of medicinal plants from Pakistan against multidrug-resistant ESKAPE pathogens," *Frontiers in Pharmacology*, vol. 9, p. 815, 2018.

[46] S. Hasannezhad, A. Mohamadi Sani, and F. Yaghooti, "Chemical composition and antibacterial activity of essential oil and methanolic extract of artemisia absinthium L. Aerial part on typical food-borne pathogens," *Journal of Essential Oil Bearing Plants*, vol. 19, no. 5, pp. 1066–1074, 2016.

[47] A. D. Alanís, F. Calzada, J. A. Cervantes, J. Torres, and G. M. Ceballos, "Antibacterial properties of some plants used in Mexican traditional medicine for the treatment of gastrointestinal disorders," *Journal of Ethnopharmacology*, vol. 100, no. 1–2, pp. 153–157, 2005.

[48] M. M. Pettigrew, J. F. Gent, Y. Kong et al., "Gastrointestinal microbiota disruption and risk of colonization with carbapenem-resistant Pseudomonas aeruginosa in intensive care unit patients," *Clinical Infectious Diseases*, vol. 69, no. 4, pp. 604–613, 2019.

[49] J. M. Rhodes, "The role of Escherichia coli in inflammatory bowel disease," *Gut*, vol. 56, no. 5, pp. 610–612, 2007.

[50] A. Fukui, T. Takagi, Y. Naito et al., "Higher levels of Streptococcus in upper gastrointestinal mucosa associated with symptoms in patients with functional dyspepsia," *Digestion*, vol. 101, no. 1, pp. 38–45, 2020.

[51] J. Huang, C. Qian, H. Xu, and Y. Huang, "Antibacterial activity of Artemisia asiatica essential oil against some common respiratory infection causing bacterial strains and its mechanism of action in Haemophilus influenzae," *Microbial Pathogenesis*, vol. 114, pp. 470–475, 2018.

[52] E. C. von Rosenvinge, G. A. O'May, S. Macfarlane, G. T. Macfarlane, and M. E. Shirtliff, "Microbial biofilms and gastrointestinal diseases," *Pathogens and Disease*, vol. 67, no. 1, pp. 25–38, 2013.

[53] Z. Deng, X. M. Luo, J. Liu, and H. Wang, "Quorum sensing, biofilm, and intestinal mucosal barrier: involvement the role of probiotic," *Frontiers in Cellular and Infection Microbiology*, vol. 10, Article ID 538077, 2020.

[54] A. Rosenbach, D. Dignard, J. V. Pierce, M. Whiteway, and C. A. Kumamoto, "Adaptations of Candida albicans for growth in the mammalian intestinal tract," *Eukaryotic Cell*, vol. 9, no. 7, pp. 1075–1086, 2010.

[55] Y. C. Fiamegos, P. L. Kastritis, V. Exarchou et al., "Antimicrobial and efflux pump inhibitory activity of caffeoylquinic acids from Artemisia absinthium against gram-positive pathogenic bacteria," *PLoS One*, vol. 6, no. 4, Article ID e18127, 2011.

[56] E. A. Moacă, I. Z. Pavel, C. Danciu et al., "Romanian wormwood (artemisia absinthium L.): physicochemical and nutraceutical screening," *Molecules (Basel, Switzerland)*, vol. 24, no. 17, p. 3087, 2019.

[57] J. H. Yang, E. Lee, B. H. Lee et al., ""Ethanolic extracts of artemisia apiacea hance improved atopic dermatitis-like skin lesions in vivo and suppressed TNF-Alpha/IFN-gamma–induced proinflammatory chemokine production in vitro," *Nutrients*, vol. 10, no. 7, 2018.

[58] X. Zhang, Z. Lin, J. Fang et al., "An on-line high-performance liquid chromatography-diode-array detector-electrospray ionization-ion-trap-time-of-flight-mass spectrometry-total antioxidant capacity detection system applying two antioxidant methods for activity evaluation of the edible flowers from Prunus mume," *Journal of Chromatography A*, vol. 1414, pp. 88–102, 2015.

[59] E. Hvattum and D. Ekeberg, "Study of the collision-induced radical cleavage of flavonoid glycosides using negative electrospray ionization tandem quadrupole mass spectrometry," *Journal of Mass Spectrometry: JMS*, vol. 38, no. 1, pp. 43–49, 2003.

[60] D. Zheleva-Dimitrova, G. Zengin, V. Balabanova et al., "Chemical characterization with in vitro biological activities of Gypsophila species," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 155, pp. 56–69, 2018.

[61] M. U. Rashid, M. Alamzeb, S. Ali et al., "A new irregular monoterpene acetate along with eight known compounds with antifungal potential from the aerial parts of Artemisia incisa Pamp (Asteraceae)," *Natural Product Research*, vol. 31, no. 4, pp. 428–435, 2017.

Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2021, Article ID 9929093, 15 pages
https://doi.org/10.1155/2021/9929093

Research Article

Molecular Targets and Mechanisms of *Scutellariae radix-Coptidis rhizoma* Drug Pair for the Treatment of Ulcerative Colitis Based on Network Pharmacology and Molecular Docking

Kai Niu,[1] Qifang Li,[2] Yuan Liu,[1] Yi Qiao,[3] Bingbing Li,[1] Chao Wei,[1] Kunrui Wang,[1] Lu'an Cui,[1] Canlei Zheng,[1] Rong Wang,[1] Li Zhang,[1] Honghua Zhang,[4] Bing Sun,[1] and Bin Yu[1]

[1]*College of Integrated Chinese and Western Medicine, Jining Medical University, Jining 272067, China*
[2]*Department of Traditional Chinese Medicine, Affiliated Hospital of Jining Medical University, Jining 272060, China*
[3]*School of Public Health, Jining Medical University, Jining 272067, China*
[4]*College of Pharmaceutical Sciences, Hangzhou Normal University, Hangzhou 311121, China*

Correspondence should be addressed to Bin Yu; yubinsd@yeah.net

Received 2 April 2021; Accepted 28 May 2021; Published 4 June 2021

Academic Editor: Dejan Stojkovic

This study aims to analyze the targets of the effective active ingredients of *Scutellariae radix-Coptidis rhizoma* drug pair (SCDP) in ulcerative colitis (UC) by network pharmacology and molecular docking and to explore the associated therapeutic mechanism. The effective active ingredients and targets of SCDP were determined from the TCMSP database, and the drug ingredient-target network was constructed using the Cytoscape software. The disease targets related to UC were searched in GeneCards, DisGeNET, OMIM, and DrugBank databases. Then, the drug ingredient and disease targets were intersected to construct a protein-protein interaction network through the STRING database. The Metascape database was used for the Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analyses of the predicted targets of SCDP for UC. The Autodock software was used for molecular docking between the main active ingredient and the core target to evaluate the binding ability. SCDP has 43 effective active ingredients and 134 intersection targets. Core targets included AKT1, TP53, IL-6, VEGFA, CASP3, JUN, TNF, MYC, EGFR, and PTGS2. GO functional enrichment analysis showed that biological process was mainly associated with a cytokine-mediated signaling pathway, response to an inorganic substance, response to a toxic substance, response to lipopolysaccharide, reactive oxygen species metabolic process, positive regulation of cell death, apoptotic signaling pathway, and response to wounding. KEGG enrichment analysis showed main pathway concentrations were related to pathways in cancer, AGE-RAGE signaling pathway in diabetic complications, bladder cancer, IL-17 signaling pathway, apoptosis, p53 signaling pathway, and PI3K-Akt signaling pathway. The drug active ingredient-core target-key pathway network contains 41 nodes and 108 edges, of which quercetin, wogonin, baicalein, acacetin, oroxylin A, and beta-sitosterol are important active ingredients; PTGS2, CASP3, TP53, IL-6, TNF, and AKT1 are important targets; and the pathways involved in UC treatment include pathways in cancer, PI3K-Akt signaling pathway, AGE-RAGE signaling pathway in diabetic, apoptosis, IL-17 signaling pathway and herpes simplex infection. The active ingredient has a good binding capacity to the core target. SCDP key active ingredients are mainly quercetin, wogonin, baicalein, acacetin, oroxylin A, and beta-sitosterol, which function mainly by regulating targets, such as PTGS2, CASP3, TP53, IL-6, TNF, and AKT1, and are associated with multiple signaling pathways as pathways in cancer, PI3K-Akt signaling pathway, apoptosis, IL-17 signaling pathways.

1. Introduction

Ulcerative colitis (UC), a type of inflammatory bowel disease (IBD), is a chronic inflammatory disease of the intestine characterized by abnormal intestinal mucosal structure, changes in intestinal bacterial composition, and systemic biochemical dysfunction. It is an incurable disease with low mortality [1]. The incidence of this disease is 11.60 per 100,000 individuals and 24.5 per 100,000 individuals in China and Hong Kong, respectively, which is lower than the incidence in Western countries but shows an increasing annual trend [2–5]. The main clinical symptoms are abdominal pain, diarrhea, bloody purulent stools, and weight loss accompanied by numerous neutrophils, macrophages, and inflammatory factors infiltrating the intestinal mucosa. The pathogenesis of this disease is not clear, and studies suggest a complex interaction among host genetic factors, immunity, and microbial environmental exposure [6]. In addition to aminosalicylic acid preparations and corticosteroids, immunomodulators, Janus kinase inhibitors, and some biological agents, such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-17A, and IL-12/IL-23p40 antibodies, have been applied in the clinical treatment of UC [7]. However, more and more patients eventually become refractory or intolerant to the side effects or complications of drugs, and the persistence of chronic inflammatory conditions often becomes a risk factor for inducing colorectal cancer [8, 9]. Under the circumstance, traditional Chinese medicines (TCMs) play an important role in the treatment of UC and are regarded as complementary and alternative medicine treatment options. Also, much evidence has shown that TCM therapies, including Chinese herbal medicine, Chinese patent medicine, acupuncture, moxibustion, have potentially positive effects on UC [10, 11].

Scutellariae radix-Coptidis rhizoma drug pair is the main ingredient herb for clearing away heat and dampness. Their compatibility is often used to treat damp-heat syndromes, dysentery, diarrhea, and other diseases [12–14]. Furthermore, they are the main ingredient drugs in the classical prescriptions as Gegen Qinlian decoction, Wumei pill, Banxia Xiexin decoction, and Shaoyao decoction, and others [15–18]. The combination of *Scutellaria baicalensis* and *Coptis chinensis* is mainly responsible for clearing away heat and dampness and has an antidiarrheal and antidysentery efficacy. Furthermore, Hu et al. used a database to analyze the medication regularity of TCM compounds in the treatment of UC. Results showed that *Scutellaria baicalensis* and *Coptis chinensis* were among the top 26 drugs with the highest medication, of which and *Coptis chinensis* was the most frequently used drug [19]. Professor Jingri Xie advocated that *Scutellaria baicalensis*, *Coptis chinensis*, *Phellodendron amurense*, and *Sophora flavescens* can be used together and they are particularly suitable for patients with the damp-heat syndrome, such as abdominal bloat, bitter mouth, nausea, vomiting, and/or yellow tongue coating [20]. Previous work in our laboratory also confirmed that *Scutellaria baicalensis* and *Coptis chinensis* are the drugs which were used more frequently in the treatment of UC and are commonly paired drugs to treat damp-heat intrinsic syndrome [21, 22].

One proposed mechanism of the *Scutellariae radix-Coptidis rhizoma* drug pair (SCDP) in the treatment of UC is that baicalein can reduce the number of apoptotic intestinal epithelial cells due to its ability to reduce the expression of the 78-kDa glucose-regulated protein (GRP78) and caspase-3 [23]. Researchers have found that berberine-nano-structured lipid carriers can block the nuclear translocation of nuclear factor-kappa B (NF-κB), reduce the expression of the proinflammatory cytokines IL-1β, IL-6, matrix metalloproteinase-9, chemokine motif receptor 1 (CX3CR1), cyclooxygenase-2 (COX-2), and telomerase reverse transcriptase, and increase the expression of the tight junction scaffolding protein zonula occludens-1, to play a therapeutic role in UC [24]. Gegen Qinlian decoction, comprising herbs *Scutellariae radix* and *Coptidis rhizoma*, exert therapeutic effects by inhibiting the expression of toll-like receptor 4 (TLR4)/NF-κB pathway and suppressing the inflammatory factors secretion as TNF-α, IL-6, IL-1β, and IL-4 in the colon of UC animal models [15]. In addition, a recent report has revealed that baicalin, which was the main ingredient of *Coptidis rhizoma*, can exert a therapeutic effect on UC through increasing glutathione peroxidase and superoxide dismutase contents and inhibiting oxidative stress response as well as apoptosis [10].

Network pharmacology and molecular docking are new technologies based on systems biology and databased molecular correlation analysis in the exploration of new drugs and prediction of drug targets. Network pharmacology emphasizes multi-ingredient-multitargets-multipathway regulation of the signal pathway. Many studies have confirmed that network pharmacology is significant for determining the target of drugs and screening the active ingredients of drugs [25–27]. In this study, the mechanism of *Scutellariae radix-Coptidis rhizoma* drug pair in the treatment of UC was analyzed by network pharmacology technology and molecular docking approaches to help better guide clinical treatment and mechanism research.

2. Materials and Methods

2.1. SCDP Ingredients Collection and Target Gene Prediction. The Traditional Chinese Medicine Systems Pharmacology (TCMSP) database and analysis platform (https://tcmspw.com/tcmsp.php) was used for the active ingredient screening. Oral bioavailability (OB) ≥30% and drug-likeness (DL) ≥0.18 were used as absorption, distribution, metabolism, and excretion parameters. Target genes were predicted using TCMSP and the Swiss Target Prediction database (http://www.swisstargetprediction.ch/) after identifying the active ingredients. The names of the predicted target genes were standardized from the UniProt database (https://www.uniprot.org/). The SCDP ingredients and target gene network were drawn using the Cytoscape software, and the top 10 active ingredients were identified by their degree values.

2.2. Prediction of UC-Related Pathogenic Genes. GeneCards (https://www.genecards.org/), DisGeNET (https://www.disgenet.org/), OMIM (http://www.omim.org/), and DrugBank (https://www.drugbank.ca/) were

selected to obtain UC-related pathogenic genes. Both databases can be used to find the most cutting-edge disease-related genes. All the databases were used "ulcerative colitis" as the keyword.

2.3. Protein-Protein Interaction (PPI) Analysis and Hub Genes. PPI is particularly important for biological process analyses and is vital for understanding complex mechanisms in a living cell. PPI network mapping was performed on the obtained bioactive ingredients and disease targets using the Search Tool for the Retrieval of Interacting Genes (STRING) database (http://string-db.org/) with the species limited to "*Homo sapiens*" and a confidence score of >0.4. Next, the interaction files were downloaded and imported into Cytoscape to visualize the PPI network and analyze the topological value. Then, based on the MCODE plug-in, the targets with a median value and a median value that was not less than the average value were recognized as the key targets of SCDP for the treatment of UC. And the top 10 ranked proteins were defined as hub targets based on the degree level.

2.4. Enrichment Analysis of Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG). The predicted targets were imported into the Metascape database (http://metascape.org/) for GO and KEGG enrichment analyses by entering the list of intersection target gene names and selecting the species as "*Homo sapiens*" for customized analysis with a cutoff P value of <0.01. According to the results, a network visualization was constructed by the tools in the bioinformatics website (http://www.bioinformatics.com.cn).

2.5. SCDP Drug Ingredient-Target Genes-Pathway Interaction Network. The SCDP active ingredients predicted target genes, and the top 20 key pathways for enrichment analysis were imported into the Cytoscape software to construct the drug ingredient-target genes-pathway interaction network. Topological analysis was performed on the constructed network according to the values of degree centrality, betweenness centrality, and closeness centrality.

2.6. Molecular Docking. Molecular docking is used to interpret the binding area of small molecule ligands and macromolecular receptors through computer simulation and then calculates the physical and chemical parameters for predicting the affinity between the two. Receptor and ligand files were downloaded from PubChem (https://pubchem.ncbi.nlm.nih.gov/) and PDB (https://www.rcsb.org) databases.

And then, molecular docking was performed using the AutoDockTools software. The docking range between the protein and the active ingredient was set using AutoGrid, and molecular docking was performed by running the "Genetic Algorithm" using Docking settings, with binding energy less than 0 indicating a good potential for ligand binding to the receptor.

3. Results

3.1. SCDP Ingredients Collection and Target Gene Prediction. According to the two screening conditions of OB value and DL index, 50 active ingredients of SCDP, including 36 ingredients in *Scutellariae radix* and 14 ingredients in *Coptidis rhizoma,* were obtained from TCMSP. Eight ingredients were eliminated because they showed no predicted targets in either the TCMSP or Swiss Target Prediction databases. Finally, 43 ingredients, including 32 in *Scutellariae radix*, 11 in *Coptidis rhizoma*, and coptisine and epiberberine as two common ingredients, were obtained (Table 1). Then the target genes of 43 active ingredients were collected for target genes prediction in TCMSP and Swiss Target Prediction. After merging the data and deleting duplicates, 223 target genes were finally distinguished.

3.2. Drug Active Ingredient-Target Gene Interaction Network. Drug active ingredient-target gene interaction networks were obtained by importing the active ingredients and predicted genes into Cytoscape 3.8.0. There are 266 nodes and 824 edges (Figure 1). The edges represent the interrelationships between the drug and active ingredients and between the ingredients and targets. Meanwhile, the degree value represents the number of routers connected to each node in the network. The higher the degree value, the more likely the node plays a key role in the network. In this network, the same compound acted on multiple targets. Simultaneously, multiple compounds shared the same target. Furthermore, the top 10 key active ingredients and 19 core targets (degree ≥10) were screened according to the degree value as shown in Tables 2 and 3.

The purple squares represent the drug *Scutellariae radix* and *Coptidis rhizoma*. The green octagons represent the ingredients in *Scutellariae radix*, while green hexagons represent the ingredients of *Coptidis rhizoma*. The red circle represents the common active ingredient of SCDP. The blue diamond represents the predicted target. The area and color transparency of the node represent its value. The darker the area and color, the more important the node. SCDP: *Scutellariae radix-Coptidis rhizoma* drug pair.

3.3. Prediction of UC-Related Pathogenic Genes. The disease genes related to UC were collected from GeneCards, DisGeNET, OMIM, and DrugBank, with "*ulcerative colitis*" as the keyword. Taking 1.795 and 4.51 as the median "Relevance score," 1111 relevant targets were screened in GeneCards. Meanwhile, 1458, 552, and 65 pathogenic genes were obtained from the DisGeNET, OMIM, and DrugBank databases, respectively. The UniProt database was used to standardize target names, and finally a total of 2386 UC-related pathogenic target genes were obtained after collating and removing duplicates.

3.4. Construction of the PPI Network and Core Target Screening. A total of 134 intersection targets were obtained by screening drug ingredient targets and disease targets

Table 1: Ingredients in *Scutellariae radix-Coptidis rhizoma* drug pair.

Drug source	Molecule ID	Molecule name	OB (%)	DL
Scutellariae radix	MOL001689	Acacetin	34.97	0.24
	MOL000173	Wogonin	30.68	0.23
	MOL000228	(2*R*)-7-Hydroxy-5-methoxy-2-phenylchroman-4-one	55.23	0.2
	MOL002714	Baicalein	33.52	0.21
	MOL002909	5,7,2,5-Tetrahydroxy-8,6-dimethoxyflavone	33.82	0.45
	MOL002910	Carthamidin	41.15	0.24
	MOL002913	Dihydrobaicalin_qt	40.04	0.21
	MOL002914	Eriodyctiol (flavanone)	41.35	0.24
	MOL002915	Salvigenin	49.07	0.33
	MOL002917	5,2′,6′-Trihydroxy-7,8-dimethoxyflavone	45.05	0.33
	MOL002925	5,7,2′,6′-Tetrahydroxyflavone	37.01	0.24
	MOL002927	Skullcapflavone II	69.51	0.44
	MOL002928	Oroxylin A	41.37	0.23
	MOL002932	Panicolin	76.26	0.29
	MOL002933	5,7,4′-Trihydroxy-8-methoxyflavone	36.56	0.27
	MOL002934	Neobaicalein	104.34	0.44
	MOL002937	Dihydrooroxylin	66.06	0.23
	MOL000358	Beta-sitosterol	36.91	0.75
	MOL000359	Sitosterol	36.91	0.75
	MOL000525	Norwogonin	39.4	0.21
	MOL000552	5,2′-Dihydroxy-6,7,8-trimethoxyflavone	31.71	0.35
	MOL000073	Ent-epicatechin	48.96	0.24
	MOL000449	Stigmasterol	43.83	0.76
	MOL001458	Coptisine	30.67	0.86
	MOL001490	Bis[(2*S*)-2-ethylhexyl] benzene-1,2-dicarboxylate	43.59	0.35
	MOL002879	Diop	43.59	0.39
	MOL002897	Epiberberine	43.09	0.78
	MOL008206	Moslosooflavone	44.09	0.25
	MOL010415	11,13-Eicosadienoic acid, methyl ester	39.28	0.23
	MOL012245	5,7,4′-Trihydroxy-6-methoxyflavanone	36.63	0.27
	MOL012246	5,7,4′-Trihydroxy-8-methoxyflavanone	74.24	0.26
	MOL012266	Rivularin	37.94	0.37
Coptidis rhizoma	MOL001454	Berberine	36.86	0.78
	MOL002894	Berberrubine	35.74	0.73
	MOL002897	Epiberberine	43.09	0.78
	MOL002903	(*R*)-Canadine	55.37	0.77
	MOL002904	Berlambine	36.68	0.82
	MOL002907	Corchoroside A_qt	104.95	0.78
	MOL000622	Magnograndiolide	63.71	0.19
	MOL000785	Palmatine	64.6	0.65
	MOL000098	Quercetin	46.43	0.28
	MOL001458	Coptisine	30.67	0.86
	MOL002668	Worenine	45.83	0.87

OB: oral bioavailability; DL: drug-likeness.

using the Wayne diagram. The PPI network was construed by the STRING databases of the 134 targets, as shown in Figure 2, with 134 nodes, 2612 edges, an average node degree of 39, and a PPI enrichment $p < 1.0e - 16$.

The obtained TSV format files of the PPI network were imported into the Cytoscape software for visualization analysis. As the interactions between proteins in the PPI networks are reciprocal, it is usually classified as an undirected graph. Some regions with high density were present in the PPI complex network, and they are called a community or module. The networks within modules are potential subnetworks of the PPI network, with a high density of connections in the subnetworks and few connections in other regions. Therefore, modules are considered to be biologically significant sets. The sets have two meanings: (1) protein complex, (i.e., multiple proteins combine to form a complex, which then plays a biological role); (2) functional module (e.g., proteins located in the same pathway interact more closely). The MCODE plug-in was then applied to analyze the interactions using a molecular complex detection algorithm, and 3 module clusters were obtained (Figure 3). According to the degree values, the top 10 core targets were identified as AKT1, TP53, IL-6, VEGFA, CASP3, JUN, TNF, MYC, EGFR, and PTGS2 (Figure 3), whose full names are provided in Table 4.

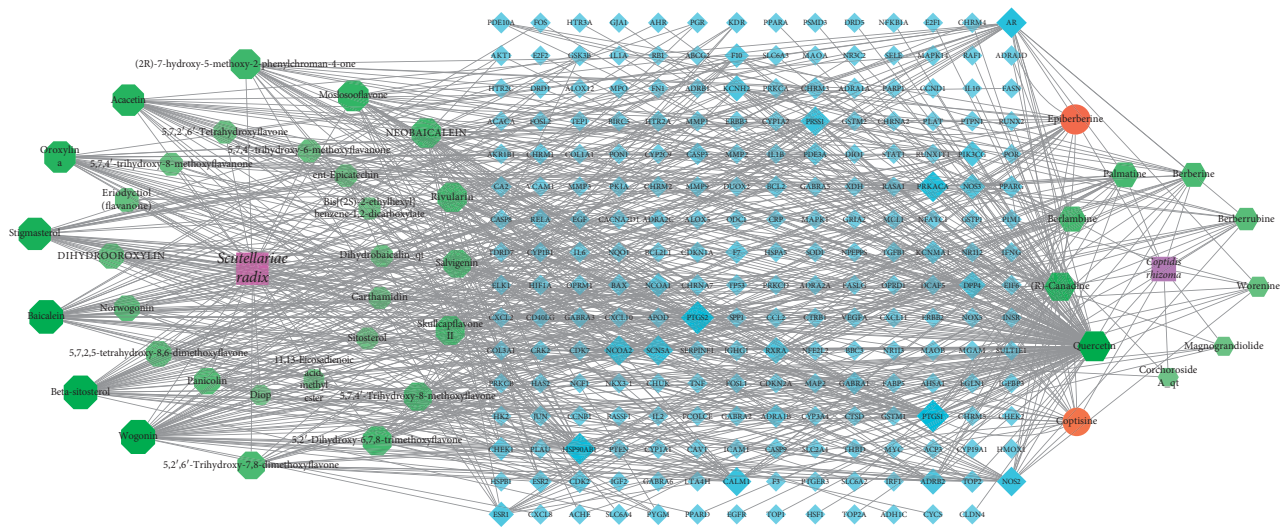


Figure 1: Drug ingredient-target interaction network. The purple squares represent the drug Scutellariae radix and Coptidis rhizoma. The green octagons represent the ingredients in Scutellariae radix, while green hexagons represent the ingredients of Coptidis rhizoma. The red circle represents the common active ingredient of SCDP. The blue diamond represents the predicted target. The area and color transparency of the node represent its value. The darker the area and color, the more important the node. SCDP, Scutellariae radix-Coptidis rhizoma drug pair.

Table 2: Top 10 key active ingredients in *Scutellariae radix-Coptidis rhizoma* drug pair.

Rank	Molecule ID	Name	Degree ≥24
1	MOL000098	Quercetin	151
2	MOL000173	Wogonin	46
3	MOL000358	Beta-sitosterol	38
4	MOL002714	Baicalein	38
5	MOL000449	Stigmasterol	32
6	MOL002903	(R)-Canadine	32
6	MOL002928	Oroxylin A	27
8	MOL001689	Acacetin	27
9	MOL008206	Moslosooflavone	25
10	MOL002897	Epiberberine	24

Table 3: Core targets of active ingredients in the *Scutellariae radix-Coptidis rhizoma* drug pair.

Rank	Name	Degree ≥ 10	Closeness centrality	Neighbourhood connectivity
1	PTGS2	37	0.5248	22.3429
2	PTGS1	33	0.5166	22.9375
3	HSP90AB1	30	0.5009	23.4667
4	PRKACA	26	0.5009	24.5200
5	AR	25	0.4514	25.5652
6	SCN5A	24	0.4775	26.5652
7	NCOA2	24	0.4809	24.7391
8	PRSS1	23	0.4484	27.5238
9	NOS2	23	0.3228	19.6190
10	CALM1	22	0.3529	21.5909
11	DPP4	17	0.4368	29.4706
12	RXRA	16	0.4530	31.4667
13	ADRB2	15	0.4625	32.8000
14	KCNH2	15	0.4366	30.9231
15	PIK3CG	14	0.4515	32.4286
16	NOS3	14	0.3973	29.8182
17	ESR1	13	0.3078	18.1818
18	F10	11	0.4213	32.2727
19	NCOA1	10	0.3236	24.0000

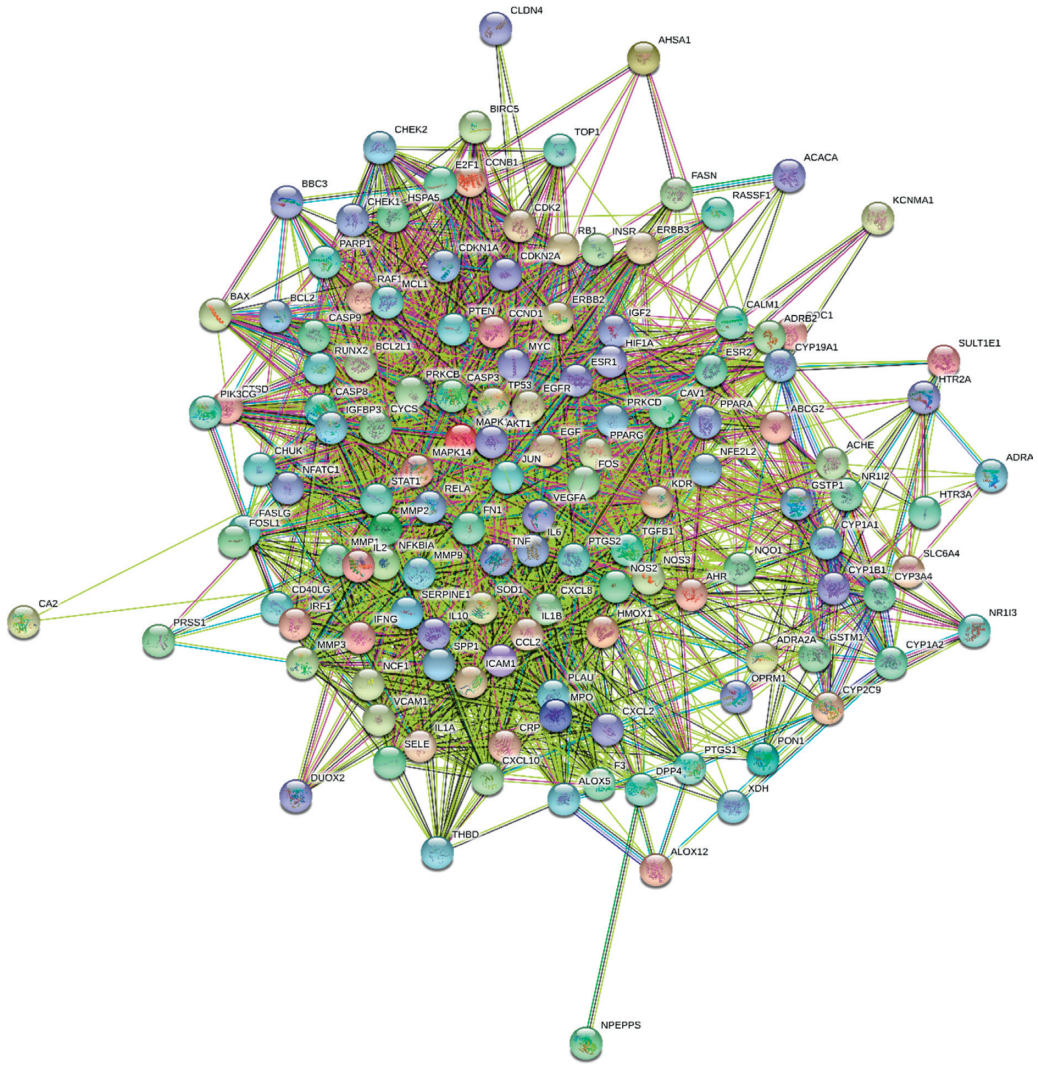


Figure 2: Common targets in the PPI network of SCDP for the treatment of ulcerative colitis. The PPI network includes 134 nodes, 2612 edges, and an average node degree of 39. PPI: protein-protein interaction; SCDP: *Scutellariae radix-Coptidis rhizoma* drug pair.

3.5. GO and KEGG Enrichment Analyses. Metascape was used for GO and KEGG analyses of the 134 hub genes to obtain enriched ontology clusters. As a result, we obtained 2304 GO terms, including 2073 biological processes (BPs), 79 cellular components (CCs), and 152 molecular functions (MFs). Figure 4 shows that BPs were mainly related to cytokine-mediated signaling pathway, response to inorganic substances, response to toxic substances, response to lipopolysaccharides (LPS), reactive oxygen species (ROS) metabolic processes, positive regulation of cell death, apoptotic signaling pathway, and response to wounding. The CCs were mainly related to membrane raft and transcription factor complex. Moreover, the MFs were mainly related to protein kinase binding, transcription factor binding, cytokine receptor binding, and protein homodimerization activity (Figure 4).

KEGG pathway enrichment analysis screening resulted in 164 signaling pathways. Visual analysis of the top 20 pathways showed that the main signaling pathways of SCDP in the treatment of UC were concentrated in pathways in cancer, the advanced glycation end products and the receptor for AGEs (AGE-RAGE) signaling pathway in diabetic complications, bladder cancer, IL-17 signaling pathway, apoptosis, p53 signaling pathway, and phosphoinositide 3-kinase (PI3K)-Akt signaling pathway (Figure 5).

3.6. Drug Ingredient-Target-Pathway Interaction Network Construction. The Cytoscape software was used to construct an active ingredient-core target-key pathway network, which contained 41 nodes and 108 edges (Figure 6). According to the degree values (betweenness centralities and closeness centralities in the drug ingredient-target-pathway network), the main active ingredients of SCDP for UC treatment were determined to be quercetin, wogonin, baicalein, acacetin, oroxylin A, and beta-sitosterol, with the main targets being PTGS2, CASP3, TP53, IL-6, TNF, and AKT1 (Tables 5 and 6).

3.7. Molecular Docking. The molecular docking results indicate binding energies of less than −2 kcal/mol after docking, suggesting the formation of stable bonds between

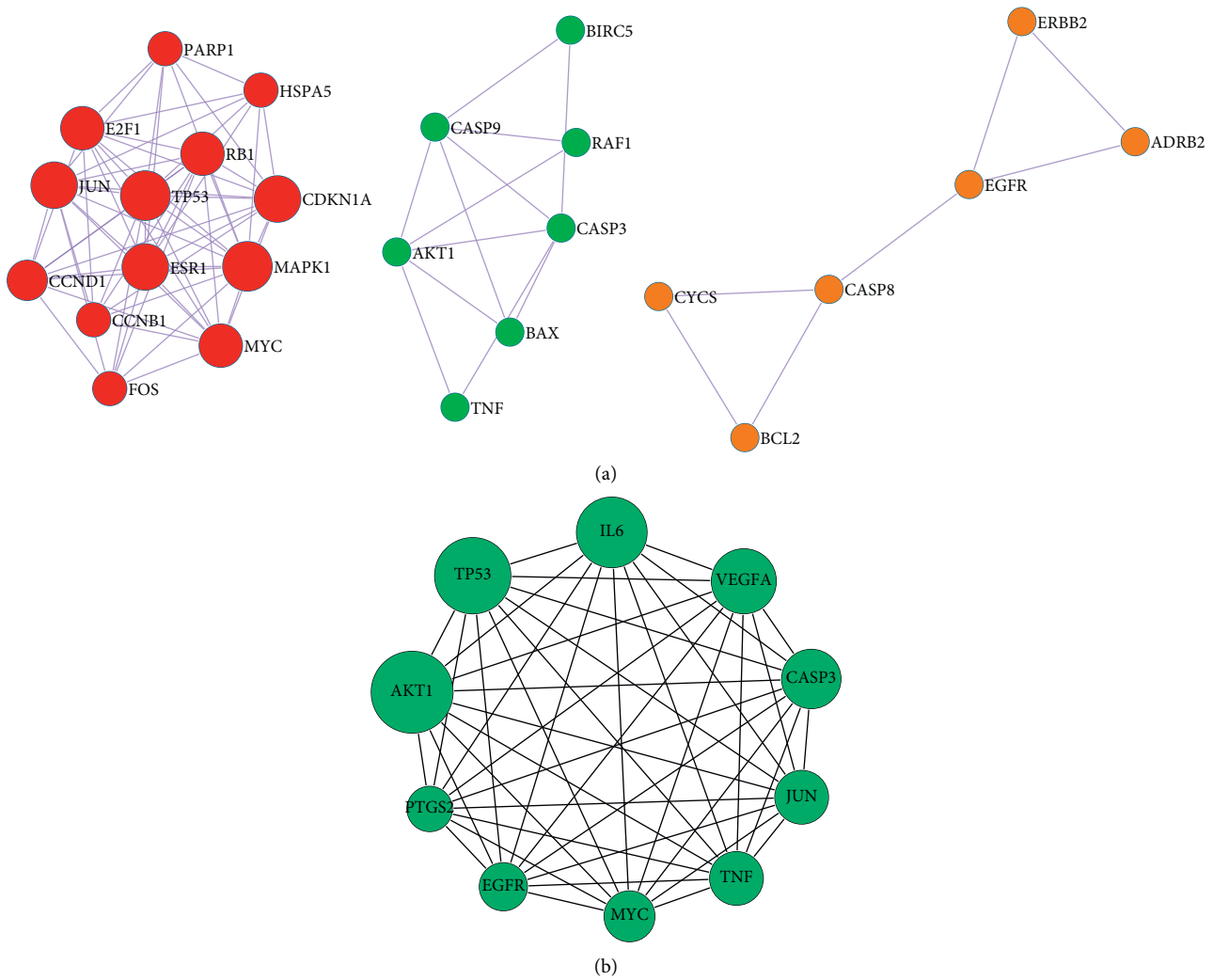


Figure 3: The core target PPI network module clusters (a) and core protein network diagram of SCDP for the treatment of ulcerative colitis (b). PPI: protein-protein interaction; SCDP: *Scutellariae radix-Coptidis rhizoma* drug pair.

Table 4: The main targets of *Scutellariae radix-Coptidis rhizoma* drug pair.

Rank	Gene symbol	Gene name	Degree
1	AKT1	RAC-alpha serine/threonine-protein kinase	108
2	TP53	Cellular tumor antigen p53	98
3	IL-6	Interleukin-6	94
4	VEGFA	Vascular endothelial growth factor A	93
5	CASP3	Caspase-3	88
6	JUN	Transcription factor AP-1	86
6	TNF	Tumor necrosis factor	86
8	MYC	Myc proto-oncogene protein	85
9	EGFR	Epidermal growth factor receptor	84
10	PTGS2	Prostaglandin G/H synthase 2	83

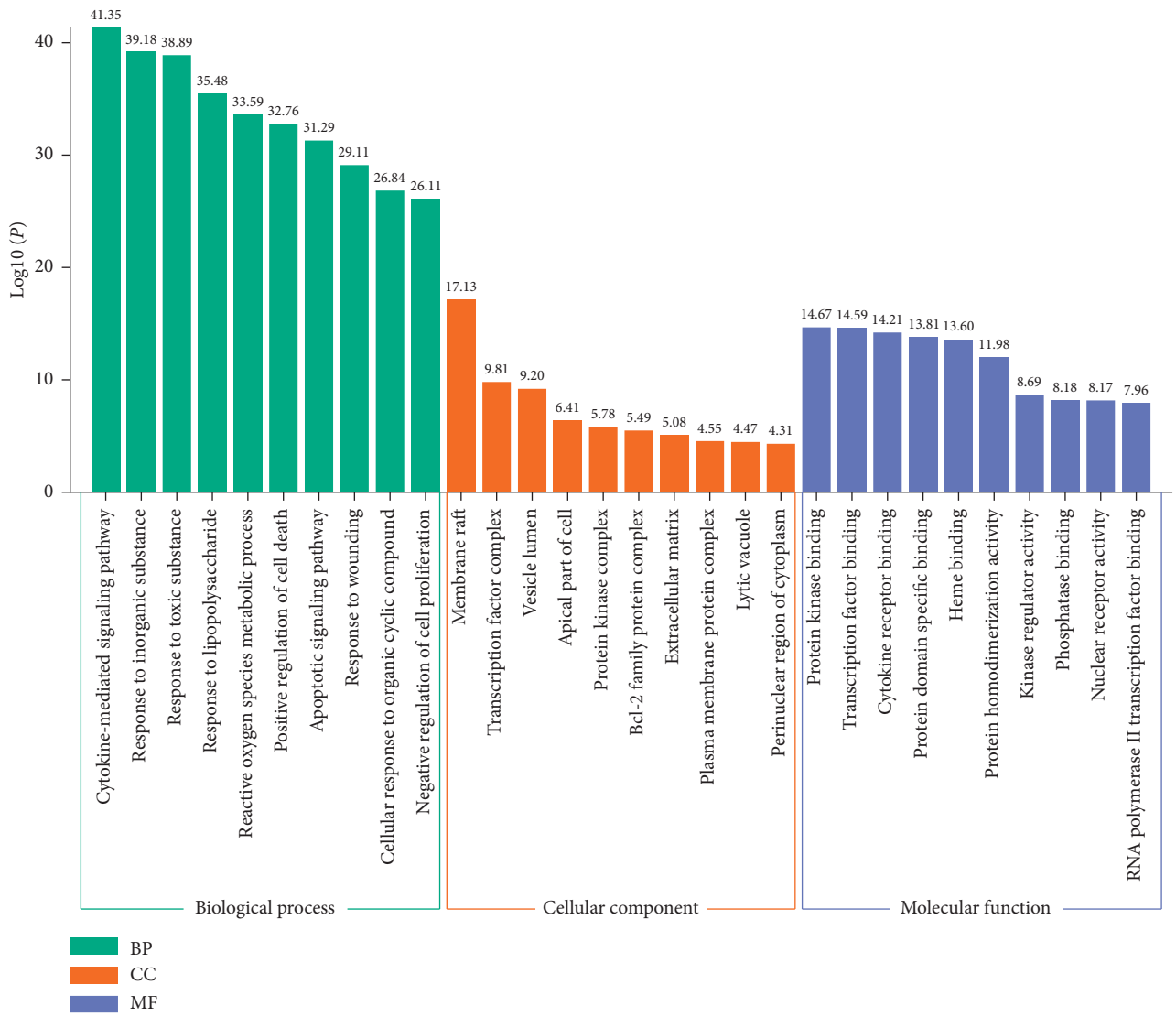


Figure 4: GO terms of candidate targets of SCDP against ulcerative colitis. The top 10 GO functional categories were selected. GO: gene ontology; SCDP: *Scutellariae radix-Coptidis rhizoma* drug pair; BP: biological process; CC: cellular component; MF: molecular function.

the active ingredients and predicted target proteins (Table 7). The PyMOL software was used to visualize the docking results of key active ingredients and core targets, as shown in Figure 7.

4. Discussion

UC is a type of irritable bowel syndrome (IBS), and the main pathogenesis of UC is spleen deficiency as the original cause and damp-heat with stasis toxin as the superficial factors in TCM theory [28–30]. Based on the theory of TCM, UC consists of several types of syndromes, the most common of which (34.8%) being damp-heat accumulation syndrome [4, 31]. Therefore, the main treatment approach is to enhance the spleen Qi and eliminate damp-heat toxin. Damp-heat and stasis toxin are important factors in the recurrence of this disease [32].

SCDP is a commonly used drug pair for eliminating damp-heat toxin. These two herbs are also constituents of many classical formulas, such as decoctions Gegen Qinlian, Huangqin, Shaoyao, Baitouweng, Huanglian Jiedu, Banxia Xiexin, and WuMei pill, for the treatment of diarrhea as a symptom of UC, by TCMs [27].

The damp-heat insidious pathogen has been proposed as the mechanism responsible for UC recurrence, necessitating that damp-heat toxin is cleared for UC treatment. In turn, Cao et al. advocated the use of *Scutellariae radix*, *Coptidis rhizoma*, *Cortex Phellodendri*, *Sophora flavescens*, *pulsatilla*, *ash bark*, among others, in combination with dampness-dissipating drugs, such as *Pogostemon cablin* and *eupatorium*, while Y. Zhang et al. suggested the use of *Coptidis rhizoma*, *Scutellariae radix*, *Cortex Phellodendri, Sophora flavescens,* and *pulsatilla* as the main ingredients in the formulation [27, 33, 34]. To further clarify the internal mechanism of SCDP for UC treatment, we applied network pharmacology to analyze its active ingredients, targets, and pathways, thus providing ideas for future research and drug development.

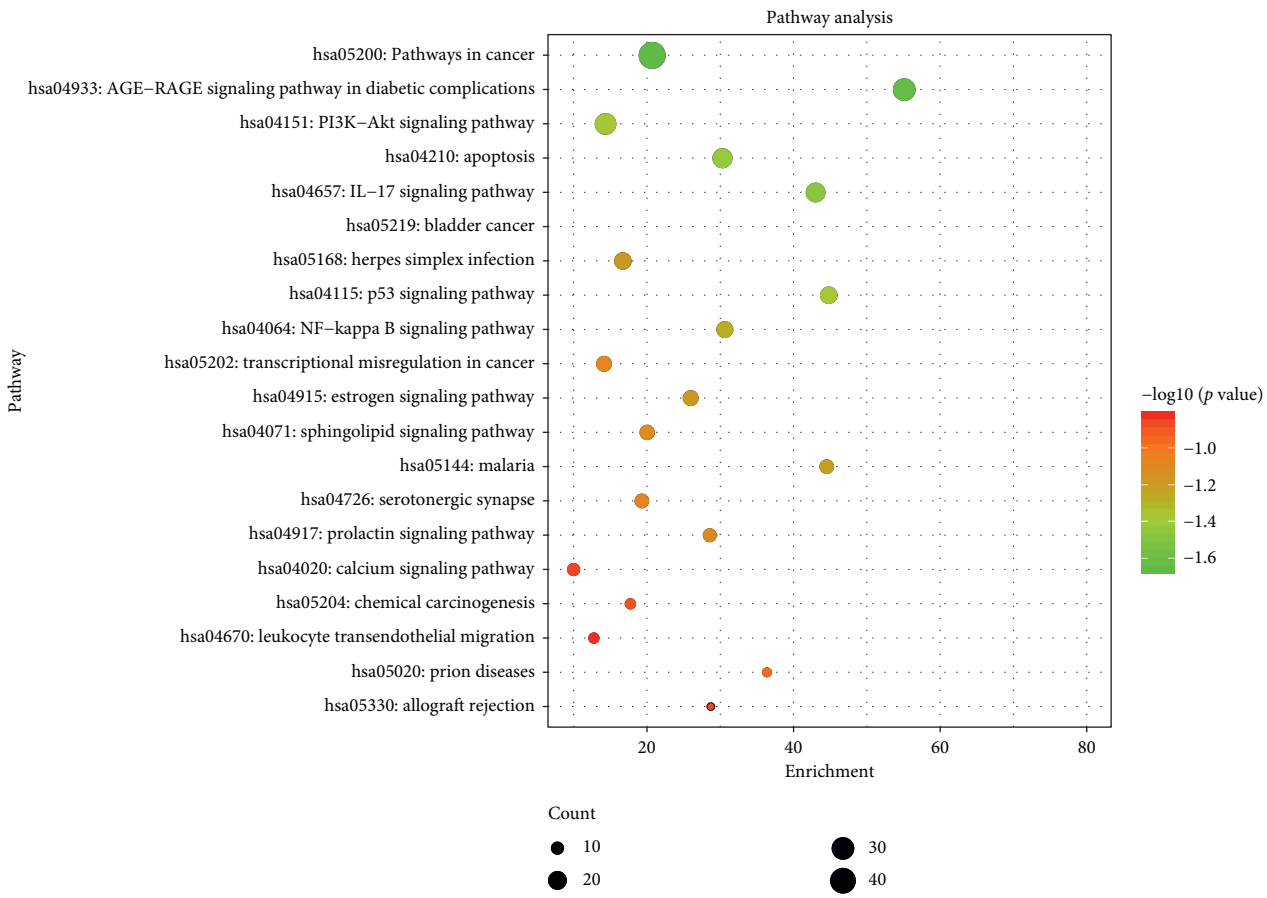


Figure 5: KEGG pathway enrichment of top 20 pathways of SCDP in the treatment of ulcerative colitis. The size of the spot represents the number of genes, and the color represents enrichment of -log10 (p value). KEGG: Kyoto Encyclopedia of Genes and Genomes; SCDP: *Scutellariae radix-Coptidis rhizoma* drug pair.

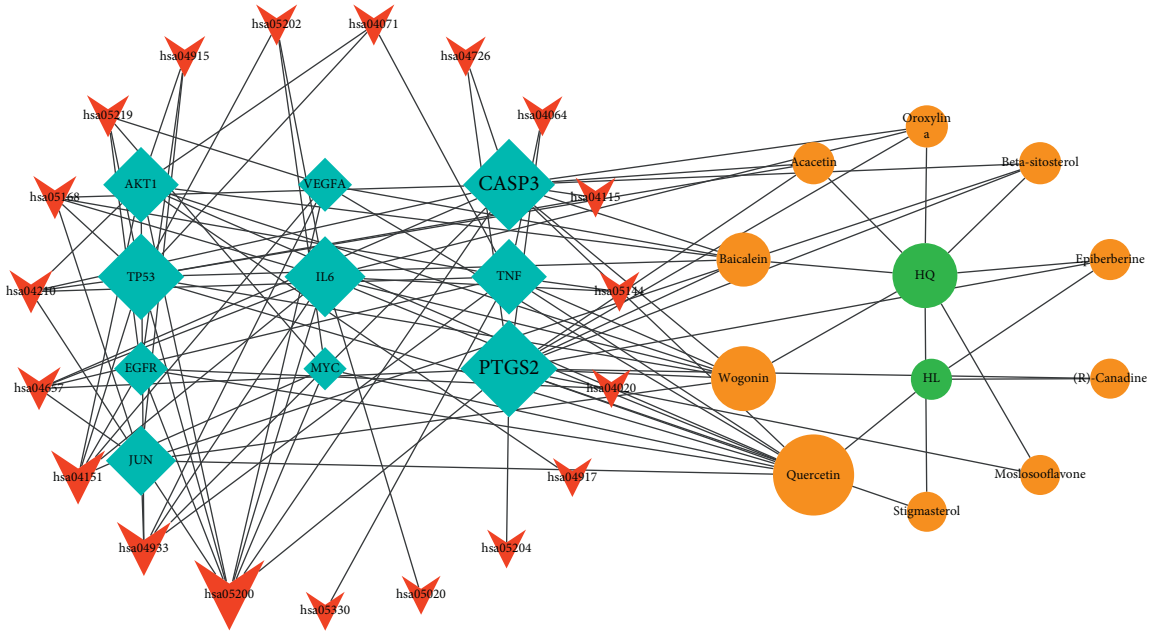


Figure 6: Key ingredient-protein target-pathway network for SCDP in the treatment of ulcerative colitis. Green circles represent the *Scutellariae radix* as "HQ" and *Coptidis rhizoma* as "HL." Orange circle represents key active ingredients, while blue diamond represents key protein targets. The red V-shape represents key pathways from KEGG enrichment analysis. The area and color transparency of the node represent its degree; the darker the area and color, the more important the node. SCDP: *Scutellariae radix-Coptidis rhizoma* drug pair.

Table 5: Key ingredients obtained from ingredient-target-pathway network.

Molecule ID	Name	Degree ≥ 4	Betweenness centrality	Closeness centrality
MOL000098	Quercetin	11	0.1762	0.5714
MOL000173	Wogonin	8	0.0789	0.5128
MOL002714	Baicalein	6	0.0405	0.4545
MOL001689	Acacetin	4	0.0145	0.4167
MOL002928	Oroxylin A	4	0.0171	0.4167
MOL000358	Beta-sitosterol	4	0.0114	0.3922

Table 6: Core targets obtained from ingredient-target-pathway network.

Name	Degree ≥10	Betweenness centrality	Closeness centrality
PTGS2	15	0.2576	0.5063
CASP3	13	0.1119	0.4819
TP53	12	0.1144	0.4706
IL-6	11	0.1265	0.4598
TNF	10	0.1098	0.4494
AKT1	10	0.1007	0.4494

Table 7: Binding energy between key ingredients and target proteins.

Key ingredients	Binding energy (kcal/mol)					
	PTGS2	CASP3	TP53	IL-6	TNF	AKT1
Quercetin	−2.25	−1.94	−1.74	−1.60	−1.73	−2.90
Wogonin	−2.88	−2.57	−3.22	−1.72	−3.10	−2.44
Baicalein	−4.78	−2.33	−2.4	−2.16	−3.38	−2.53
Acacetin	−4.66	−2.59	−2.74	−2.46	−2.78	−2.46
Oroxylin A	−2.95	−2.54	−3.09	−2.11	−2.94	−2.51
Beta-sitosterol	−2.34	−2.23	−1.95	−1.49	−2.80	−3.16

The results show that the key active ingredients are quercetin, wogonin, baicalein, acacetin, oroxylin A, and beta-sitosterol. Previous research discovered that quercetin inhibits the expression of LPS-induced inflammatory genes, mainly by reducing the levels of TNF-α and lipocalin-2 mRNA and enhancing the expression of the Slip protein to protect the UC mucosa [35]. It has been found that quercetin exerts a therapeutic effect in IBD by regulating the structure of the intestinal flora, inhibiting the expression of proinflammatory factors, such as IL-17, TNF-α, IL-6, and increasing the expression of IL-10 [36].

Wogonin has strong anti-inflammatory, antitumor, antiviral and antiallergic properties. It can inhibit the activity of Treg cells, regulate the differentiation of Th17 cells, and inhibit the transcription of the extracellular signal-regulated kinase (ERK), signal transducer and activator of transcription 3 (STAT3), and hypoxia-inducible factor-1α to regulate inflammatory responses [37]. One study found that 25-mg/mL wogonin can reduce the LPS-induced inflammatory response of the TLR4-MyD88-mediated NF-κB pathway in Caco-2 cells, suggesting its protective effect on the intestinal mucosal barrier [38].

Baicalein is the main ingredient of *Coptidis rhizoma*. Studies have suggested its anti-inflammatory effect and discovered that it could improve the inflammatory state of UC and the control drugs [39–41]. The effect of baicalein is similar to that of the reference drug sulfasalazine and comparable to wogonin [42, 43]. In addition, baicalein reduces the levels of the inflammatory mediators IL-33 and NF-κB p65 and increases the level of IκBα to resists the pathological changes of dextran sulfate sodium (DSS)-induced UC [44].

Acacetin (5,7-dihydroxy-4′-methoxyflavone) is an O-methylated flavone naturally present in plants like chrysanthemum and safflower, as well as in Calamintha and Linaria species. Acacetin possesses antiperoxidative, anti-inflammatory, antiplasmodial, and anticancer activities. In addition, acacetin strongly inhibits the expression of proinflammatory cytokines, inducible nitric oxide synthase (iNOS), and COX-2 in LPS-induced RAW 264.7 cells [45]. Research has shown that acacetin treatment could inhibit sepsis-induced acute lung injury and reduced iNOS and COX-2 expression.

Oroxylin A and baicalein are produced as major metabolites of baicalin by the action of bacteria in the large intestine cavity. In addition, both baicalin and baicalein are converted into four new metabolites: wogonin, wogonoside, oroxylin A, and oroxin A [46]. Some studies have shown that oroxylin A is involved in the induction of autophagy and apoptosis, potentially via its regulation of p62-mediated caspase-8, which promotes the transcription of NF-κB downstream target genes and inhibits the transcription of downstream target genes of Nrf2 [47–49].

Beta-sitosterol was found to markedly reduce weight loss, colonic length shortening, and microscopic changes in DSS-induced colitis and to reduce TNF-α, IL-6, and IL-1β levels in the intestinal tissues of experimental colitis mice in a concentration-dependent manner [50].

The core targets of SCDP in UC treatment are PTGS2 (COX2), CASP3, TP53, IL-6, TNF, and AKT1, which are associated with the TNF signaling pathway and NF-κB and oxidative stress pathways. An important target of anti-inflammatory drugs, especially nonsteroidal anti-inflammatory drugs, is prostaglandin endoperoxide synthase or COX. There are two subtypes of COX. COX1 is constitutively expressed and is responsible for regulating normal physiological processes. COX2 is induced under inflammatory conditions and mainly regulates the inflammatory process. It has been suggested that the expression of COX2 is induced in the colon of IBD patients but also in the inflamed tissues of IL-10-deficient IBD mouse models, and reduced COX2 is considered a major target for IBD treatment [46].

A previous study in UC rats revealed that baicalin could up-regulate B-cell lymphoma 2 (Bcl-2) protein expression, lower Bax (also known as Bcl-2-like protein 4) protein

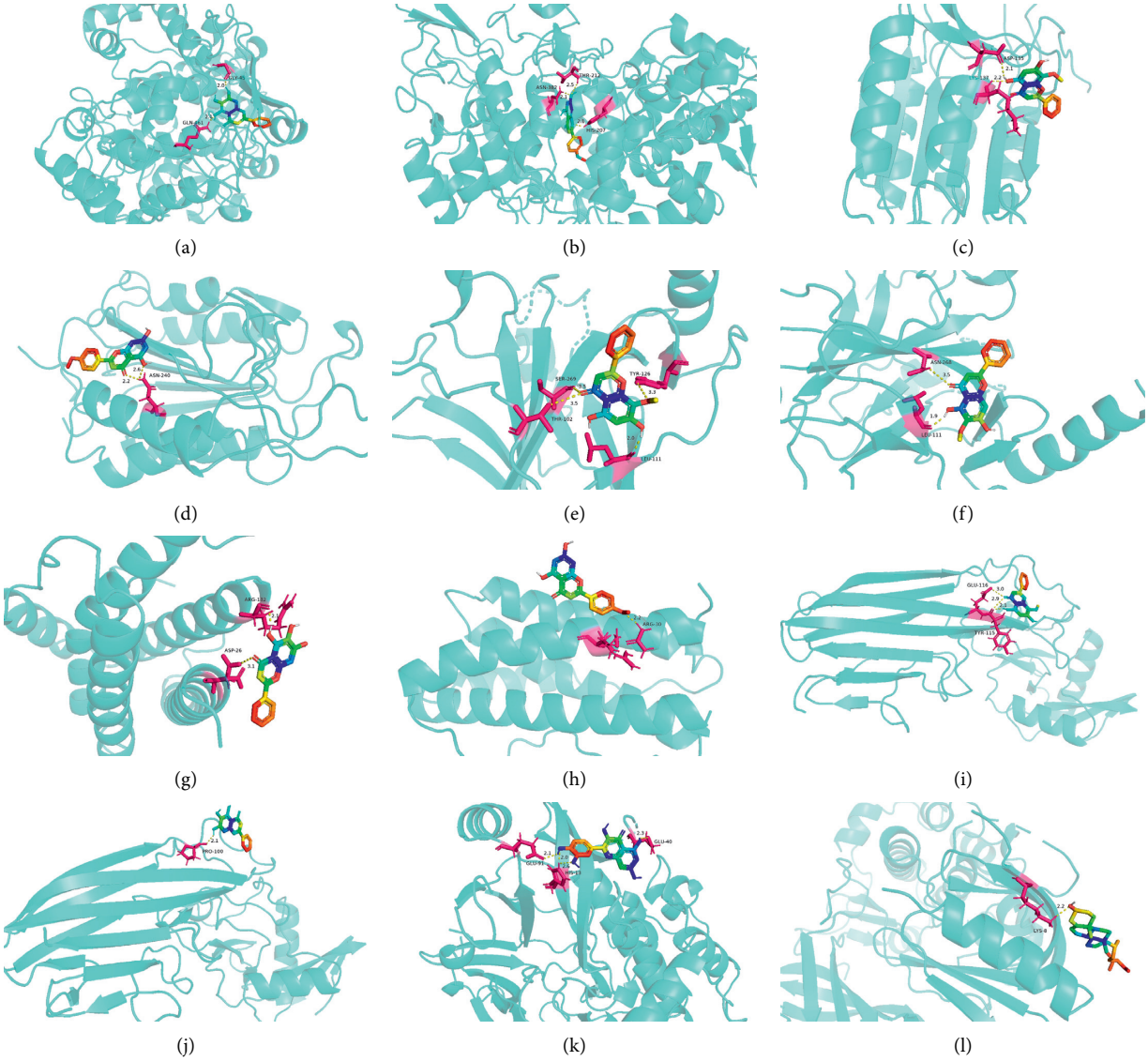


Figure 7: Docking results of the key ingredient and six hubs target proteins. (a) Baicalein and PTGS2; (b) acacetin and PTGS2; (c) wogonin and CASP3; (d) acacetin and CASP3; (e) wogonin and TP53; (f) oroxylin A and TP53; (g) baicalein and IL-6; (h) acacetin and IL-6; (i) wogonin and TNF; (j) baicalein and TNF; (k) quercetin and AKT1; (l) beta-sitosterol and AKT1.

expression, induce marked activation of the caspase-cascade system, and exhibit concentration-dependent down-regulation of Fas and Fas Ligand (FasL) protein expressions to exert therapeutic effects in UC [10]. AKT1 is a key mediator of the PI3K-Akt signaling pathway and is involved in cellular function regulation in various tumors, including gastric cancer, glioma, lung cancer, and esophageal squamous cell carcinoma [51, 52].

Berberine has been found to play a regulatory role in macrophage M1 polarization in DSS-induced colitis through the AKT1-suppressor of cytokine signaling-1 (SOCS1)-NF-κB signaling pathway [53]. IL-6 gene expression is strongly associated with IBD progression [54]. JUN is a proto-oncogene that plays a key role in inflammation, and can be activated by many inflammatory factors directly or indirectly. The activation of JUN further modulates the expression and regulation of relevant inflammatory factors, which, in turn, participate in the regulation of the inflammatory response [55]. Being a proinflammatory regulator, TNF promotes the release of proinflammatory factors and plays an important role in the development and progression of UC. In addition, together with interferons, it alters the barrier function of intestinal epithelial cells, enhances the permeability of the intestinal mucosa and vascular wall, and disrupts the integrity of the intestinal mucosa, leading to ulcer formation [56, 57].

GO analysis and KEGG enrichment results showed that the mechanism of SCDP in the treatment of UC was mainly related to the following pathways: pathways in cancer, PI3K-Akt signaling pathway, the AGE-RAGE signaling pathway in diabetic complications, apoptosis, IL-17 signaling pathway, and herpes simplex infection.

According to Rogler, up to 30% of the patients with chronic active UC or Crohn's disease (CD) are likely to get

colorectal cancer after 35 years since the onset of the disease [58]. Chronic inflammation, the increased turnover of epithelial cells, and ROS production are thought to contribute to the development of dysplastic lesions, which may then transform into colorectal cancer [59]. Meanwhile, chronic UC status is also a major risk factor in the development of colitis-associated cancer [59].

IL-17, mainly secreted by Th17 cells, is involved in inflammatory response, and its levels have been confirmed to correlate with the extent of UC disease. Acting as a proinflammatory factor, IL-23 promotes IL-17 production and forms the IL-23/17 axis to amplify the inflammatory response [60, 61]. IL-17A induces the aggregation of neutrophils on target cells, resulting in the secretion of inflammatory cytokines (IL-6, TNF-α, and IL-1B), chemokines (CXC), granulocyte colony-stimulating factor, and granulocyte-macrophage colony-stimulating factor. Meanwhile, IL-17A is also involved in the regulation of mucosal barrier function and amplification of the complete inflammatory response through the production of antimicrobial peptides, such as β-defensins, S100 β-defensins, and S100 proteins [62]. The strongest evidence comes from the genome-wide association studies that have identified IBD susceptibility single nucleotide polymorphisms in many genes involved in the IL-23/IL-17 axis [63, 64]. Other susceptibility genes in Crohn's disease and UC also appear to be involved in the IL-23/IL-17 axis. For example, STAT3, janus kinase 2 (JAK2) and tyrosine kinase 2 (TYK2) participate in IL-23 signaling, and IL-12B encodes the common subunit of IL-12 and IL-23 [61].

The mutation of the p53 gene is an important genetic alteration involved in the early stages of UC-associated colorectal cancer. The overexpression of p53 protein in the colorectal crypt of UC patients, usually in the absence of atypical hyperplasia observation of atypical hyperplasia, is used by pathologists to define the state between regenerative changes and intraepithelial neoplasia. It has been used as a biomarker in predicting the risk of progression into a malignant tumor. High-frequency mutations of p53 were reported in severe chronic UC patients with undiagnosed cancer [65]. The expression of p53 in UC patients showed a negative correlation with IL-6, which is mainly involved in the development of cancer-induced by chronic inflammation. IL-6 is the main activator of STAT3, and its stimulation products increase the transcription of target genes, as well as cell proliferation/survival. Downregulation of p53 expression is probably the most important step in the transformation of inflamed tissues and cells. Particularly, inflammatory cells that stimulate proliferation and inhibit apoptosis of cells, cytokines, chemokines, and growth factors produce ROS and reactive nitrogen species that cause oxidative DNA damage. The damage may not be adequately repaired because the p53 function is downregulated by IL-6 [66].

In addition, TCMs may exert relevant apoptotic inhibitory effects through the upregulation of p53 and Bcl-2 expression, which is accompanied by the downregulation of TNF-α and IL-1 and the upregulation of IL-6 and IL-10. The PI3K-AKT signaling pathway is involved in the regulation and release of proinflammatory cytokines, including TNF-α and other cytokines closely related to the inflammatory response of UC [67], and plays an important role in the development and progression of UC. TCMs inhibit transforming growth factor-β (TGF-β) expression and lead to a decrease in PI3K/AKT activation, thereby alleviating the symptoms of UC by regulating TGF-β expression [68]. AGE and RAGE are involved in and mediate various signaling pathways of oxidative stress, induce ROS production, and activate NF-κB, leading to inflammatory responses, cell apoptosis, and microvascular diseases [69]. RAGE polymorphisms and increased RAGE levels have been found in IBD patients, and the involvement of AGE/RAGE in inflammation correlates with its activation of NF-κB and its response to oxidative stress [70].

The results of molecular docking showed that quercetin, wogonin, baicalein, acacetin, oroxylin A, and beta-sitosterol, the main active ingredients of SCDP, had a good affinity to the core target genes *PTGS2*, *CASP3*, *TP53*, *IL-6*, *TNF*, and *AKT1*, suggesting that the key active ingredients of SCDP play a therapeutic role in UC by mainly intervening with the key signaling targets, such as the inflammatory response and oxidative stress. In summary, the potential biological mechanism of SCDP in the treatment of UC involves multiple ingredients, targets, and pathways, and the therapeutic effects occur through several pathways as pathways in cancer, PI3K-Akt signaling pathway, apoptosis, and IL-17 signaling pathway.

The presented study provides a scientific basis for the subsequent development and utilization of SCDP. Even so, network pharmacology has only analyzed the main active ingredients and targets of drugs, and further validation of their predicted target pathways is needed experimentally. The conclusions of this research also contribute to the research and development of drugs and further elaboration of mechanisms.

Data Availability

All data are fully available without restriction, and all relevant data are included within the paper.

Disclosure

The funders had no role in study design, in the collection, analysis, and interpretation of data, in the writing of the report, and in the decision to submit the article for publication.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

Authors' Contributions

Kai Niu and Qifang Li contributed equally to this work. B. Y. conceived and designed the study. Q. L., K. N., and K. W. developed the methodology. Q. L., L. C., C. W., Y. L., and B. L. performed the data acquisition. C. Z., R. W., H. Z., C.

W., L. Z., B. S., and B. L. performed data analysis and interpretation. Y. Q. and B. Y. drafted, reviewed, and revised the manuscript. All authors read and approved the final manuscript.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 81603509), the Natural Science Foundation of Shandong Province (No. ZR2014HQ051), and the College Students' Innovation and Entrepreneurship Training Program of Jining Medical University (No. cx2020067).

References

[1] I. Khan, N. Ullah, L. Zha et al., "Alteration of gut microbiota in inflammatory bowel disease (IBD): cause or consequence? IBD treatment targeting the Gut microbiome," *Pathogens*, vol. 8, no. 3, 2019.

[2] S. C. Ng, W. K. Leung, H. Y. Shi et al., "Epidemiology of inflammatory bowel disease from 1981 to 2014: results from a territory-wide population-based registry in Hong Kong," *Inflammatory Bowel Diseases*, vol. 22, no. 8, pp. 1954–1960, 2016.

[3] S. C. Ng, C. N. Bernstein, M. H. Vatn et al., "Geographical variability and environmental risk factors in inflammatory bowel disease," *Gut*, vol. 62, no. 4, pp. 630–649, 2013.

[4] M. Chen, Y. Ding, and Z. Tong, "Efficacy and safety of Sophora flavescens (kushen) based traditional Chinese medicine in the treatment of ulcerative colitis: clinical evidence and potential mechanisms," *Frontiers in Pharmacology*, vol. 11, 2020.

[5] S. C. Ng, H. Y. Shi, N. Hamidi et al., "Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: a systematic review of population-based studies," *The Lancet*, vol. 390, no. 10114, pp. 2769–2778, 2017.

[6] D. R. Plichta, D. B. Graham, S. Subramanian, and R. J. Xavier, "Therapeutic opportunities in inflammatory bowel disease: mechanistic dissection of host-microbiome relationships," *Cell*, vol. 178, no. 5, pp. 1041–1056, 2019.

[7] M. F. Neurath, "Cytokines in inflammatory bowel disease," *Nature Reviews Immunology*, vol. 14, no. 5, pp. 329–342, 2014.

[8] Y. N. Wang, J. Li, W. Y. Zheng et al., "Clinical characteristics of ulcerative colitis-related colorectal cancer in Chinese patients," *Journal of Digestive Diseases*, vol. 18, no. 12, pp. 684–690, 2017.

[9] M. Duijvestein, R. Battat, N. Vande Casteele et al., "Novel therapies and treatment strategies for patients with inflammatory bowel disease," *Curr Treat Options Gastroenterol*, vol. 16, no. 1, pp. 129–146, 2018.

[10] J. Yao, X. Cao, R. Zhang et al., "Protective effect of baicalin against experimental colitis via suppression of oxidant stress and apoptosis," *Pharmacognosy Magazine*, vol. 12, no. 47, pp. 225–234, 2016.

[11] H. W. Lee, J. H. Jun, K.-J. Kil et al., "Herbal medicine (Danggui Shaoyao San) for treating primary dysmenorrhea: a systematic review and meta-analysis of randomized controlled trials," *Maturitas*, vol. 85, pp. 19–26, 2016.

[12] Z. Lu, W. Xiong, S. Xiao et al., "Huanglian Jiedu decoction ameliorates DSS-induced colitis in mice via the JAK2/STAT3 signalling pathway," *Chinese Medicine*, vol. 15, p. 45, 2020.

[13] X. Ke, Y. Huang, L. Li et al., "Berberine attenuates arterial plaque formation in atherosclerotic rats with damp-heat syndrome via regulating autophagy," *Drug Design, Development and Therapy*, vol. 14, pp. 2449–2460, 2020.

[14] X. Xie, X. Ma, S. Zeng et al., "Mechanisms of berberine for the treatment of atherosclerosis based on network pharmacology," *Evidence-based Complementary and Alternative Medicine: ECAM*, vol. 2020, Article ID 3568756, 11 pages, 2020.

[15] R. Li, Y. Chen, M. Shi et al., "Gegen Qinlian decoction alleviates experimental colitis via suppressing TLR4/NF-κB signaling and enhancing antioxidant effect," *Phytomedicine*, vol. 23, no. 10, pp. 1012–1020, 2016.

[16] Y. Wan, L. Xu, Z. Liu et al., "Utilising network pharmacology to explore the underlying mechanism of Wumei Pill in treating pancreatic neoplasms," *BMC Complementary and Alternative Medicine*, vol. 19, no. 1, p. 158, 2019.

[17] Q. Ji, Y. Yang, X. Song, X. Han, and W. Wang, "Banxia Xiexin Decoction in the treatment of chronic atrophic gastritis," *Medicine*, vol. 99, no. 42, Article ID e22110, 2020.

[18] N. Zhu, J. Hou, G. Ma, and J. Liu, "Network pharmacology identifies the mechanisms of action of Shaoyao gancao decoction in the treatment of osteoarthritis," *Medical Science Monitor*, vol. 25, pp. 6051–6073, 2019.

[19] Z. Hu, Y. Zhang, W. Zheng, H. Wang, and X. Sun, "Analysis of the medication rule and mechanism of patent-prescription of Chinese medicine for ulcerative colitis based on patent database," *Chinese Journal of Hospital Pharmacy*, vol. 40, no. 17, pp. 1811–1815, 2021.

[20] W. Shan, J. Xie, and M. Li, "Xie Jingri's experience in the treatment of ulcerative colitis with drug-pairs," *Journal of Changchun University of Chinese Medicine*, vol. 36, no. 3, pp. 451–453, 2019.

[21] H. Li, Y. Xie, Y. Zhu et al., "Literature study on medication regularity and prescription-formulating principle of Chinese medicine for treatment of ulcerative colitis," *Journal of Guangzhou University of Traditional Chinese Medicine*, vol. 37, no. 1, pp. 176–181, 2020, in Chinese.

[22] H. Li, Y. Xie, Q. Li et al., "Analysis of prescription rules in treating ulcerative colitis with liver-spleen disorder syndrome based on traditional Chinese medicine inheritance support system," *China Medicine*, vol. 15, no. 10, pp. 1580–1584, 2020, in Chinese.

[23] S. Yan, L. Yingchao, W. Zhangliu et al., "Effect of berberine from Coptis chinensis on apoptosis of intestinal epithelial cells in a mouse model of ulcerative colitis: role of endoplasmic reticulum stress," *Evidence-based Complementary and Alternative Medicine: ECAM*, vol. 2020, Article ID 3784671, 7 pages, 2020.

[24] J. Deng, Z. Wu, Z. Zhao et al., "Berberine-loaded nanostructured lipid carriers enhance the treatment of ulcerative colitis," *International Journal of Nanomedicine*, vol. 15, pp. 3937–3951, 2020.

[25] F. Cheng, I. A. Kovács, and A.-L. Barabási, "Network-based prediction of drug combinations," *Nature Communications*, vol. 10, no. 1, p. 1197, 2019.

[26] Q. Tao, J. Du, X. Li et al., "Network pharmacology and molecular docking analysis on molecular targets and mechanisms of Huashi Baidu formula in the treatment of COVID-19," *Drug Development and Industrial Pharmacy*, vol. 46, no. 8, pp. 1345–1353, 2020.

[27] Z. Shen, Q. Zhou, Y. Ni, W. He, H. Shen, and L. Zhu, "Traditional Chinese medicine for mild-to-moderate ulcerative colitis," *Medicine*, vol. 98, no. 33, Article ID e16881, 2019.

[28] A. Imdad, M. R. Nicholson, E. E. Tanner-Smith et al., "Fecal transplantation for treatment of inflammatory bowel disease,"

The Cochrane Database of Systematic Reviews, vol. 11, Article ID CD012774, 2018.

[29] Z.-H. Shen, C.-X. Zhu, Y.-S. Quan et al., "Relationship between intestinal microbiota and ulcerative colitis: mechanisms and clinical application of probiotics and fecal microbiota transplantation," *World Journal of Gastroenterology*, vol. 24, no. 1, pp. 5–14, 2018.

[30] M. Wei, H. Li, Q. Li et al., "Based on network pharmacology to explore the molecular targets and mechanisms of gegen Qinlian decoction for the treatment of ulcerative colitis," *BioMed Research International*, vol. 2020, p. 5217405, 2020.

[31] B. Ye, H. Shen, Y. Lu, and Y.-Q. Wang, "Clinical observations on 100 cases of ulcerative colitis treated with the method of clearing away heat, expelling dampness, promoting blood circulation and healing ulcer," *Journal of Traditional Chinese Medicine*, vol. 30, no. 2, pp. 98–102, 2010.

[32] B. Liu, X. Piao, W. Niu et al., "Kuijieyuan decoction improved intestinal barrier injury of ulcerative colitis by affecting TLR4-dependent PI3K/AKT/NF-κB oxidative and inflammatory signaling and gut microbiota," *Frontiers in Pharmacology*, vol. 11, p. 1036, 2020.

[33] Y. Zhang, X. Li, X. Xu, and N. Yang, "Mechanisms of paeonia lactiflora in treatment of ulcerative colitis: a network pharmacological study," *Medical Science Monitor*, vol. 25, pp. 7574–7580, 2019.

[34] S.-Y. Cao, S.-J. Ye, W.-W. Wang, B. Wang, T. Zhang, and Y.-Q. Pu, "Progress in active compounds effective on ulcerative colitis from Chinese medicines," *Chinese Journal of Natural Medicines*, vol. 17, no. 2, pp. 81–102, 2019.

[35] M. Dicarlo, G. Teti, G. Verna et al., "Quercetin exposure suppresses the inflammatory pathway in intestinal organoids from Winnie mice," *International Journal of Molecular Sciences*, vol. 20, no. 22, 2019.

[36] R. Lin, M. Piao, and Y. Song, "Dietary quercetin increases colonic microbial diversity and attenuates colitis severity in Citrobacter rodentium-infected mice," *Frontiers in Microbiology*, vol. 10, p. 1092, 2019.

[37] L. Fan, D. Qiu, G. Huang et al., "Wogonin suppresses IL-10 production in B cells via STAT3 and ERK signaling pathway," *Journal of Immunology Research*, vol. 2020, Article ID 3032425, 12 pages, 2020.

[38] W. Wang, T. Xia, and X. Yu, "Wogonin suppresses inflammatory response and maintains intestinal barrier function via TLR4-MyD88-TAK1-mediated NF-κB pathway in vitro," *Inflammation Research*, vol. 64, no. 6, pp. 423–431, 2015.

[39] W. Lee, S.-K. Ku, and J.-S. Bae, "Anti-inflammatory effects of baicalin, baicalein, and wogonin in vitro and in vivo," *Inflammation*, vol. 38, no. 1, pp. 110–125, 2015.

[40] C. Liu, Y. Li, Y. Chen et al., "Baicalein restores the balance of Th17/treg cells via aryl hydrocarbon receptor to attenuate colitis," *Mediators of Inflammation*, vol. 2020, Article ID 5918587, 19 pages, 2020.

[41] B. Dinda, S. Dinda, S. DasSharma, R. Banik, A. Chakraborty, and M. Dinda, "Therapeutic potentials of baicalin and its aglycone, baicalein against inflammatory disorders," *European Journal of Medicinal Chemistry*, vol. 131, pp. 68–80, 2017.

[42] A. Salaritabar, B. Darvishi, F. Hadjiakhoondi et al., "Therapeutic potential of flavonoids in inflammatory bowel disease: a comprehensive review," *World Journal of Gastroenterology*, vol. 23, no. 28, pp. 5097–5114, 2017.

[43] X. Zhong, Y.-J. Surh, S.-G. Do et al., "Baicalein inhibits dextran sulfate sodium-induced mouse colitis," *Journal of Cancer Prevention*, vol. 24, no. 2, pp. 129–138, 2019.

[44] C.-L. Zhang, S. Zhang, W.-X. He et al., "Baicalin may alleviate inflammatory infiltration in dextran sodium sulfate-induced chronic ulcerative colitis via inhibiting IL-33 expression," *Life Sciences*, vol. 186, pp. 125–132, 2017.

[45] M.-H. Pan, C.-S. Lai, Y.-J. Wang, and C.-T. Ho, "Acacetin suppressed LPS-induced up-expression of iNOS and COX-2 in murine macrophages and TPA-induced tumor promotion in mice," *Biochemical Pharmacology*, vol. 72, no. 10, pp. 1293–1303, 2006.

[46] S. Liang, X. Deng, L. Lei et al., "The comparative study of the therapeutic effects and mechanism of baicalin, baicalein, and their combination on ulcerative colitis rat," *Frontiers in Pharmacology*, vol. 10, p. 1466, 2019.

[47] Y. Zhao, Q. Zhu, X. Bu et al., "Triggering apoptosis by oroxylin A through caspase-8 activation and p62/SQSTM1 proteolysis," *Redox Biology*, vol. 29, Article ID 101392, 2020.

[48] S. Tm and K. Mk, "Molecular cross-talk between the NRF2/KEAP1 signaling pathway, autophagy, and apoptosis," *Free Radical Biology & Medicine*, vol. 50, no. 9, 2011.

[49] H. Huang, X. Zhang, and J. Li, "Protective effect of oroxylin A against lipopolysaccharide and/or D-galactosamine-induced acute liver injury in mice," *Journal of Surgical Research*, vol. 195, no. 2, pp. 522–528, 2015.

[50] K. Ding, Y. Y. Tan, Y. Ding et al., "β-Sitosterol improves experimental colitis in mice with a target against pathogenic bacteria," *Journal of Cellular Biochemistry*, vol. 120, no. 4, pp. 5687–5694, 2019.

[51] T. Que, Y. Song, Z. Liu et al., "Decreased miRNA-637 is an unfavorable prognosis marker and promotes glioma cell growth, migration and invasion via direct targeting Akt1," *Oncogene*, vol. 34, no. 38, pp. 4952–4963, 2015.

[52] N. Zhang and X. Chen, "Potential role of O-GlcNAcylation and involvement of PI3K/Akt1 pathway in the expression of oncogenic phenotypes of gastric cancer cellsin vitro," *Biotechnology and Applied Biochemistry*, vol. 63, no. 6, pp. 841–851, 2016.

[53] Y. Liu, X. Liu, W. Hua et al., "Berberine inhibits macrophage M1 polarization via AKT1/SOCS1/NF-κB signaling pathway to protect against DSS-induced colitis," *International Immunopharmacology*, vol. 57, pp. 121–131, 2018.

[54] T. T. Pizarro, S. A. De La Rue, and F. Cominelli, "Role of interleukin 6 in a murine model of Crohn's ileitis: are cytokine/anticytokine strategies the future for IBD therapies?" *Gut*, vol. 55, no. 9, pp. 1226-1227, 2006.

[55] Y. S. Kim, Y. Ahn, M. H. Hong et al., "Rosuvastatin suppresses the inflammatory responses through inhibition of c-Jun N-terminal kinase and nuclear factor-κb in endothelial cells," *Journal of Cardiovascular Pharmacology*, vol. 49, no. 6, pp. 376–383, 2007.

[56] K. Goh and S.-D. Xiao, "Inflammatory bowel disease: a survey of the epidemiology in Asia," *Journal of Digestive Diseases*, vol. 10, no. 1, pp. 1–6, 2009.

[57] Y. Ishiguro, "Mucosal proinflammatory cytokine production correlates with endoscopic activity of ulcerative colitis," *Journal of Gastroenterology*, vol. 34, no. 1, pp. 66–74, 1999.

[58] G. Rogler, "Chronic ulcerative colitis and colorectal cancer," *Cancer Letters*, vol. 345, no. 2, pp. 235–241, 2014.

[59] E. N. D. Low, N. M. Mokhtar, Z. Wong, and R. A. Raja Ali, "Colonic mucosal transcriptomic changes in patients with long-duration ulcerative colitis revealed colitis-associated cancer pathways," *Journal of Crohn's and Colitis*, vol. 13, no. 6, pp. 755–763, 2019.

[60] B. Verstockt, G. Van Assche, S. Vermeire, and M. Ferrante, "Biological therapy targeting the IL-23/IL-17 axis in

inflammatory bowel disease," *Expert Opinion on Biological Therapy*, vol. 17, no. 1, pp. 31–47, 2017.

[61] A. Geremia and D. P. Jewell, "The IL-23/IL-17 pathway in inflammatory bowel disease," *Expert Review of Gastroenterology & Hepatology*, vol. 6, no. 2, pp. 223–237, 2012.

[62] S. Xu and X. Cao, "Interleukin-17 and its expanding biological functions," *Cellular & Molecular Immunology*, vol. 7, no. 3, pp. 164–174, 2010.

[63] M. F. Neurath, "IL-23 in inflammatory bowel diseases and colon cancer," *Cytokine & Growth Factor Reviews*, vol. 45, pp. 1–8, 2019.

[64] E. Bianchi and L. Rogge, "The IL-23/IL-17 pathway in human chronic inflammatory diseases-new insight from genetics and targeted therapies," *Genes & Immunity*, vol. 20, no. 5, pp. 415–425, 2019.

[65] K. Kobayashi, H. Tomita, M. Shimizu et al., "p53 expression as a diagnostic biomarker in ulcerative colitis-associated cancer," *International Journal of Molecular Sciences*, vol. 18, no. 6, 2017.

[66] H. Su, Q. Kang, H. Wang et al., "Changes in expression of p53 and inflammatory factors in patients with ulcerative colitis," *Experimental and Therapeutic Medicine*, vol. 17, no. 4, pp. 2451–2456, 2019.

[67] X. L. Huang, J. Xu, X. H. Zhang et al., "PI3K/Akt signaling pathway is involved in the pathogenesis of ulcerative colitis," *Inflammation Research*, vol. 60, no. 8, pp. 727–734, 2011.

[68] X. Wang, D. Li, Y. Zhang, S. Wu, and F. Tang, "Costus root granules improve ulcerative colitis through regulation of TGF-β mediation of the PI3K/AKT signaling pathway," *Experimental and Therapeutic Medicine*, vol. 15, no. 5, pp. 4477–4484, 2018.

[69] F. Kei, Y. Sho-ichi, and O. Seiya, "Role of AGEs-RAGE system in cardiovascular disease," *Current Pharmaceutical Design*, vol. 20, no. 14, pp. 2395–2402, 2014.

[70] F. A. Moura, M. O. F. Goulart, S. B. G. Campos, and A. S. da Paz Martins, "The close interplay of nitro-oxidative stress, advanced glycation end products and inflammation in inflammatory bowel diseases," *Current Medicinal Chemistry*, vol. 27, no. 13, pp. 2059–2076, 2020.

Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2021, Article ID 9985325, 18 pages
https://doi.org/10.1155/2021/9985325

Research Article

Chemical Composition and Antimicrobial Activity of Two Sri Lankan Lichens, *Parmotrema rampoddense*, and *Parmotrema tinctorum* against Methicillin-Sensitive and Methicillin-Resistant *Staphylococcus aureus*

Polwatta Samaraweera Arachchige Ishara Shiromi,[1] Ruwani Punyakanthi Hewawasam,[2] Rankoth Gedara Udeni Jayalal,[1] Hasanga Rathnayake,[2] Weerasinghe Mudiyanselage Dilip Gaya Bandara Wijayaratne,[3] and Dakshika Wanniarachchi[4]

[1]*Department of Natural Resources, Faculty of Applied Sciences, Sabaragamuwa University of Sri Lanka, Belihuloya, Sri Lanka*
[2]*Department of Biochemistry, Faculty of Medicine, University of Ruhuna, Galle, Sri Lanka*
[3]*Department of Microbiology, Faculty of Medicine, University of Ruhuna, Galle, Sri Lanka*
[4]*Instrument Center, Faculty of Applied Sciences, University of Sri Jayewardenepura, Nugegoda, Sri Lanka*

Correspondence should be addressed to Ruwani Punyakanthi Hewawasam; ruwaniph@yahoo.com

Received 16 March 2021; Revised 30 April 2021; Accepted 22 May 2021; Published 2 June 2021

Academic Editor: Gokhan Zengin

Introduction. Medicinal utility of lichens is ascribed to the presence of various secondary metabolites of low molecular weight and they have been used in traditional medicine including Ayurveda in the treatment of wounds and skin disorders. Despite the urgent need to effectively address the antibiotic resistance worldwide, the discovery of new antibacterial drugs has declined in the recent past. This emphasizes the increasing importance of investigating and developing new classes of antibiotics that can withstand antibiotic resistance. *Aims of the study*. The present study was conducted to investigate the chemical composition and the antibacterial activity of hexane, ethanol, and aqueous extracts of *Parmotrema rampoddense* and *Parmotrema tinctorum*, two lichens collected from Belihuloya, Sri Lanka, against Gram-negative and Gram-positive bacteria including twenty clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA). *Materials and methods*. Phytochemical analysis, thin layer chromatography (TLC), and Gas Chromatography Mass Spectrometry (GC-MS) were performed to determine the chemical composition of the two lichens. Hexane, ethanol, and aqueous extracts of both lichens were tested against clinical isolate of Gram-negative and Gram-positive bacteria including twenty clinical isolates of MRSA. Bacterial susceptibility was tested using a disc diffusion assay. Minimum inhibitory concentration (MIC) was determined by a broth microdilution method. Vancomycin was used as the positive control. *Results*. Alectorialic acid, atranorin, atraric acid, orcinol, and O-orsellinaldehyde were among the secondary metabolites identified by the TLC and GC-MS analysis. None of the lichen extracts were active against Gram-negative bacteria but both lichens showed a concentration-dependent activity against methicillin-sensitive *Staphylococcus aureus* (MSSA) and MRSA. Ethanol extract of *P. rampoddense* showed the highest activity against MSSA with the MIC, 0.0192 mg/ml, but all MRSA isolates investigated showed MIC between 0.096 and 2.4 mg/ml for the same extract. *Conclusion*. Both lichens, *P. rampoddense* and *P. tinctorum*, represent potentially important sources of future antimicrobial drugs. Further investigation on the ethanol extract of *P. rampoddense* will enable us to determine the most active phytoconstituents responsible for the activity, their mechanism of action against bacterial pathogens, and also their cytotoxicity against normal cells.

1. Introduction

The recurrence of high-profile, multidrug-resistant (MDR) pathogens is a growing concern as global mortality rates from bacterial infections continue to increase worldwide [1]. *Staphylococcus aureus* is the primary causative agent of community acquired skin and soft tissue infections, and it is also an important cause of hospital-associated invasive infections including bacteremia, pneumonia, sepsis, and endocarditis. Despite the urgent need to effectively address antibiotic resistance, the discovery of new antibacterial drugs has declined in recent years. This is largely due to the resistance developed by pathogens long before many drugs come to the market. Furthermore, microbial resistance has severely impacted a drug's long-term potential to return a profit [2]. These issues emphasize the increasing importance of investigating and developing new classes of antibiotics that can withstand pathogen resistance or express a new mode of action, thereby increasing the potential lifetime of the antibacterial agents [3].

Sri Lanka is considered as a biodiversity hotspot in the world due to its diverse and luxuriant ecosystems [4]. Approximately 30% of Sri Lanka is covered by forest and the island is home to around 3000 angiosperm species and one quarter of it are endemic [5]. Also, Sri Lanka harbors a highly diverse lichen flora, approximately 1200 of which have already been identified. It is expected that 2000 or more lichen species could be found on the island nation due to the minimal knowledge of Sri Lankan lichen species [6]. Lichens are widely used in folk medicine in many countries, such as India, Sri Lanka, and the Ayurveda as well as Unani systems of medicine describe their use in the treatment of a multitude of diseases [7]. They are symbiotic systems consisting of a fungus and either an algae or a cyanobacterium. They represent one of the more promising potential sources of low molecular weight secondary compounds and more than 1000 different secondary compounds have already been reported from lichens and their cultured mycobionts [8].

Metabolites isolated from lichens exert a plethora of biological actions including antibiotic, analgesic, antimycotic, antiviral, anti-inflammatory, and cytotoxic activities [9, 10]. The activity of lichen derived compounds has also been proven against some MDR pathogenic bacteria including vancomycin-resistant enterococci and methicillin-resistant *Staphylococcus aureus* (MRSA) [11, 12]. Atranorin, salazinic acid, lecanoric acid, and usnic acid are well-known active constituents isolated from lichens and are reported to possess strong biological activities such as antibacterial, antifungal, antitumor, and antioxidant activities. Usnic acid, the most investigated metabolite with proven antibiotic activity, has been used in the development of pharmaceuticals and cosmetics. Recently, derivatives of usnic acid were synthesized which enhanced its antimicrobial activity [13]. According to literature, approximately 8% of the terrestrial ecosystem comprises lichens; thus, around 20,000 lichens are distributed throughout the world. However, their biological activities and characterization of active constituents remain unexplored to a significant extent [14].

This is the first detailed report of the chemical composition and the comparison of antimicrobial activity of aqueous, hexane, and ethanol extracts of *Parmotrema rampoddense* and *Parmotrema tinctorum* against CLSI (Clinical & Laboratory Standards Institute) standard strain of methicillin-sensitive *Staphylococcus aureus*, ATCC 25923 (MSSA), and clinical isolates of MRSA. *P rampoddense*, widely known as the "long-whiskered ruffle lichen," is a species of the family Parmeliaceae (Figure 1(a)). It is widely distributed in tropical regions and grows on the bark of oak and palm trees. It has a largely expanded thallus and the thallus attaches to the substratum loosely. The upper surface of the thallus is mineral gray or whitish gray. The lower surface is black and wrinkled. The upper surface is rugose, smooth, and soredia are present. The lobes of the thallus are ciliated. This species has a corticolous habitat [15].

P. tinctorum (Figure 1(b)) has a thallus which is loosely attached to the substratum. The thallus is membranaceous to leathery. Irregular lobes are present in the thallus. Margins are either entire or scalloped. The upper surface has a gray color of a frosted look from a powdery coating (glaucous). The thallus is smooth, and marginal regions are folded longitudinally. The lower surface is black and wrinkled. This species can be easily distinguished from the other Parmeliaceae family species due to the loosely and largely arranged thallus, which is broad, ecilliated, and laminally isidiate lobes [15].

Both lichens have been used by the traditional ayurvedic medical practitioners in Sri Lanka in the treatment of wounds and various infectious diseases. Therefore, the present study was conducted to investigate the chemical composition of *P. rampoddense* and *P. tinctorum* collected from Belihuloya, Sri Lanka, by Thin layer chromatography (TLC) and Gas Chromatography-Mass Spectrophotometry (GC-MS) analysis and the antibacterial activity of hexane, ethanol, and aqueous extracts of each lichen against Gram-negative and Gram-positive bacteria including twenty clinical isolates of MRSA.

2. Materials and Methods

2.1. Collection of Lichen Species. Lichen samples of *P. rampoddense* (6°45′37″ °N 80°47′24″ °E) and *P. tinctorum* (6°45′38″ °N 80°47′23″ °E) were collected from the Nonpareil Estate, Belihuloya, Sri Lanka with permission of related authorities. Voucher specimens were deposited at the lichen herbarium of the Department of Natural Resources, Faculty of Applied Sciences, Sabaragamuwa University of Sri Lanka (Specimen No SUSLNR20 and SUSLNR21).

2.2. Identification of Lichens. The lichen samples were identified using stereo and light microscopes. Specifically, a dissecting microscope (LABOMED CZM6) was used to identify morphological characteristics of the thallus and the reproductive structures, while a compound microscope (Bio Blue 110–240v/50–60 Hz) was used to study the anatomy of thalli and fruiting bodies. Spot test reactions were carried out on thalli under the dissecting microscope.

(a) (b)

Figure 1: Lichens collected from Belihuloya, Sri Lanka. (a) *P. rampoddense* and (b) *P. tinctorum*, (scale bars: 1 cm).

2.3. Extraction of Lichen Metabolites. Each lichen thalli was washed thoroughly to remove debris in tap water and distilled water, oven dried at 40°C, and coarsely powdered. A cold extraction procedure was followed for the preparation of hexane and ethanol extracts. From each powdered lichen, 10 g was mixed with 100 ml of the solvent. The contents were placed in a mechanical shaker for 72 h at 25°C. Then, the extracts were filtered through Whattman No 1 filter paper, and the extract was concentrated at 40°C using a rotary evaporator (Hahnvapor, Hahnshin Scientific Co, HS-2005V-N). The concentrated extract was transferred to a preweighed sterile glass container and allowed to dry under a stream of air. The resulting crude extracts were dissolved in a minimum amount of 10% dimethyl sulfoxide (DMSO) separately, and the working stock solution was obtained by redissolving in 10% DMSO to yield a final concentration of 300 mg/ml for each extract. Each extract was stored at 4°C in sterile airtight containers in the dark for further studies.

The aqueous extract was prepared using 2.5 g of dried, coarsely powdered lichen refluxed in 30 ml of distilled water for 3 h. The resulting crude extract was dried at 40°C and was dissolved in a minimum amount of 10% DMSO, and the final concentration of the extract was adjusted to 300 mg/ml. The extract was stored at 4°C in sterile airtight containers in the dark for further studies.

2.4. Identification of Secondary Metabolites of Lichen Extracts

2.4.1. Phytochemical Analysis of Lichen Extracts. A qualitative phytochemical analysis was carried out to identify the major classes of phytochemicals (tannins, alkaloids, phenolic compound, cyanogenic glycosides, cardiac glycosides, anthracene glycosides, reducing sugars, saponins, and flavonoids) present in various aqueous and alcoholic extracts of the two lichens by the methods described by Trease and Evans [16] and Sofowora [17]. Precautions were taken to remove the interference from chlorophyll before the commencement of the experiment.

2.4.2. Identification of Lichen Metabolites by Thin-Layer Chromatography (TLC). TLC fingerprint was obtained using the solvent system C (toluene: acetic acid = 170 : 30), which showed a fine separation with a maximum number of components. Two control samples including *Parmelia caperata* and known metabolites isolated from lichens were used as controls. Bands generated by constituents that could not be detected in the visible region were visualized under UV light at 254 nm and 366 nm. TLC plate was also sprayed with 10 % H_2SO_4 and subjected to heating at 110–120°C for 10 min before the observation for color changes and measurement of Rf value of the spots observed. The relative Rf (Retention factor) values for the chemicals extracted from both species were compared with the published literature to identify the specific compounds in each extract [18]. The experiment was performed in duplicate.

2.4.3. GC-MS Analysis. GC–MS analyses were performed on an Agilent 7890 A/5975C-GC/MSD inert detector operating in EI mode with capillary-column chromatography HP-5 ms (30 m × 250 μm × 0.25 μm). The autosampler was used to inject 1 μl of the sample with a 10 : 1 split ratio. The temperature of the injection port was 250°C. Temperature programming was conducted with the starting column

temperature 70°C, which was held for 4 min, and then increased to 270°C at a rate of 10°C/min, held for 20 min. Helium was used as a carrier gas at a flow rate of 1.0 ml/min. The ionic source temperature was 230°C, the quadruple temperature was 150°C, and the scanning quality range was set at 50–550 amu. The percentage composition of the extracts was computed from the GC peak areas. Qualitative analysis on the identification of compounds was carried out using the NIST standard spectral library (https://www.nist.gov/). Nonisothermal retention indices were calculated using the definition of Van den Dool and Kratz [19].

$$\mathrm{RI} = 100n + \frac{100\left(t_x - t_n\right)}{\left(t_{n+1} - t_n\right)}, \quad (1)$$

where t_n and t_{n+1} are retention times of the reference n-alkane hydrocarbons eluting immediately before and after the chemical compound "X." The retention time of the compound "X" is given in t. Here, standard n-alkane series containing C_8–C_{20} hydrocarbons (40 mg/L, Sigma-Aldrich)) in hexane was used for calculating the retention indices.

2.5. Determination of the Antimicrobial Activity of Lichen Extracts

2.5.1. Microbial Strains. Both lichens were first screened against CLSI (Clinical and Laboratory Standards Institute) control strains of three Gram-negative bacteria, *Escherichila coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), and *Klebsiella pneumoniae* (ATCC 700603), and a Gram-positive bacterium, *S. aureus* (ATCC 25923). Standard bacterial cultures were obtained from the Department of Microbiology, Faculty of Medicine, University of Ruhuna, Sri Lanka.

Since all lichen extracts showed antimicrobial activity only against *S. aureus* CLSI control strain, 20 MRSA strains (SA 1–20) were used for the second phase of the study along with MSSA as the reference organism. The MRSA strains used in this study were isolated from pus samples obtained for culture and sensitivity testing from patients having skin and soft tissue infections among patients admitted to the Teaching Hospital, Karapitiya, Sri Lanka. Organisms were subcultured for 24 h on blood agar and MacConkey agar, and were confirmed by Gram stain, catalase test, slide and tube coagulase tests, and the zone diameter of cefoxitin (30 μg) on Mueller–Hinton agar at the Department of Microbiology, Faculty of Medicine, University of Ruhuna, Sri Lanka.

2.5.2. Determination of Antibiotic Resistance Profile. Antibacterial activity was determined against CLSI control strain (ATCC 25923) and 20 MRSA strains separately using the disc diffusion assay as previously optimized by us and described by Rathnayake et al. [20]. The crude extract (300 mg/ml), 10-fold, and 100-fold dilutions of each lichen extract, extracted in ethanol, hexane, and water were prepared in 10% DMSO. Bacterial cell suspensions were adjusted to a density of 1×10^8 CFU/ml inoculum using 0.5 McFarland turbidity standards. Each standardized inoculum was distributed evenly on the surface of dried sterile Mueller–Hinton agar plates. Sterile blank filter paper discs (Whattman No. 1, diameter = 6 mm) were impregnated with 10 μlof each lichen extract and were placed on the seeded Mueller–Hinton agar plates. Cefotaxime (30 μg/disk) was used as the positive control against CLSI control strains, vancomycin (30 μg/disc) disk was used as the positive control against MRSA, and 10% DMSO-soaked filter paper disk was used as the negative control in both sets of experiments. The plates were incubated aerobically at 35 ± 2°C for 18–24 h. At the end of the incubation period, the antimicrobial spectrum of lichen extracts was determined for each bacterial species by the measurement of zone size around each disc. Diameters of the zones of inhibition were measured using a Vernier caliper and compared with the controls. Each test was carried out in triplicate, and the average of the diameters was calculated.

2.5.3. Determination of Activity Index (AI) and Relative Percentage Inhibition (RPI). Activity index (AI) for each lichen extract was calculated using the following formula [21]:

$$\mathrm{AI} = \frac{\text{Inhibition zone diameter of the sample}}{\text{Inhibition zone of the standard}}. \quad (2)$$

The relative percentage index was calculated against the positive control using the following formula [21]:

$$\mathrm{RPI} = \frac{100\left(X - Y\right)}{\left(Z - Y\right)}, \quad (3)$$

where X = total area of inhibition of the lichen extract, Y = total area of inhibition of the solvent, and Z = total area of inhibition of the standard drug. The total area of the inhibition was calculated using the formula,

$$\mathrm{Area} = \pi r^2, \quad (4)$$

where r = radius of zone of inhibition.

2.5.4. Determination of Minimum Inhibitory Concentration (MIC). Broth micro-dilution method described in Rathnayake et al. was used to determine the MIC of lichen extracts that gave a positive result for the disk diffusion assay against CLSI control strains and 20 MRSA strains separately [20]. Serial 5-fold dilutions of the lichen extracts were prepared in the 10% DMSO, yielding serial dilutions of the crude lichen extract (300 mg/ml). Bacterial inoculum was prepared in Mueller–Hinton broth, and the turbidity was adjusted to approximately 0.5 McFarland turbidity standards. For the MIC assay, 96-well microtiter plates were used, and 150 μL of plant extract was added to each well of the microplate. Bacterial suspension (50 μL) was added to each well except the negative controls. Vancomycin (MIC ≤ 2 μg/ml) was used as a positive control. DMSO (10%) and plant extracts without bacterial suspension were used as the negative controls. Microtiter plates were incubated at 35 ± 2°C for 24 h. Antimicrobial activity was assessed by the measurement of absorbance at 630 nm using a microplate

reader. The lowest concentration (highest dilution) of the extract that prevented visible bacterial growth depending on the absorbance was regarded as the MIC. The assay was done in triplicate for each extract separately, and average absorbance values were used to determine the MIC.

2.6. Ethical Approval. Ethical approval for the study was obtained from the Ethical Review Committee, Faculty of Medicine, University of Ruhuna, Sri Lanka.

2.7. Statistical Analysis. The mean and the standard error of the mean of all replicates were calculated and data were analysed using one-way ANOVA to compare the mean between groups. $p < 0.05$ was considered as statistically significant.

3. Results

In the present study, the chemical composition and antimicrobial activity of three different extracts of two Sri Lankan lichens, *P. rampoddense* and *P. tinctorum* were compared against methicillin-sensitive and methicillin-resistant *S. aureus.*

3.1. Identification of Secondary Metabolites of Lichen Extracts. Qualitative analysis of phytoconstituents revealed that both lichens contain saponins, flavonoids, and polyphenols. Also, anthracene glycosides were present in *P. tinctorum* but not in *P. rampoddense*. Proteins, alkaloids, tannins, reducing sugars, cyanogenic glycosides, and cardenolide glycosides were absent in both lichen extracts.

The thin layer chromatograms developed with the medium polar to polar solvent system gave the best separation of compounds in the two lichen extracts indicating that these extracts contain relatively polar phytoconstituents (Figure 2). TLC data indicated the presence of alectorialic acid (h) at Rf. Value of 0.25, atranorin (c) at Rf value of 0.71, and unknown compounds (d, e, f, and g) at Rf values of 0.57, 0.5, 0.42, and 0.45, respectively, in *P. rampoddense* (Pr).

Similarly *P. tinctorum* (Pt) showed the presence of atranorin (i) at Rf value of 0.71, lecanoric acid at Rf value of 0.21(k), and unknown compound (j) at Rf values of 0.49. The control 1 (C_1) of *Parmelia caperata* showed the presence of usnic acid (a) at Rf value of 0.64, and caperatic acid (b) at Rf value of 0.24. Furthermore, control 2 (C_2) consisted of chloro-atranorin (At) at Rf values of 0.76 (l) and stictic acid (m) at Rf value of 0.11.

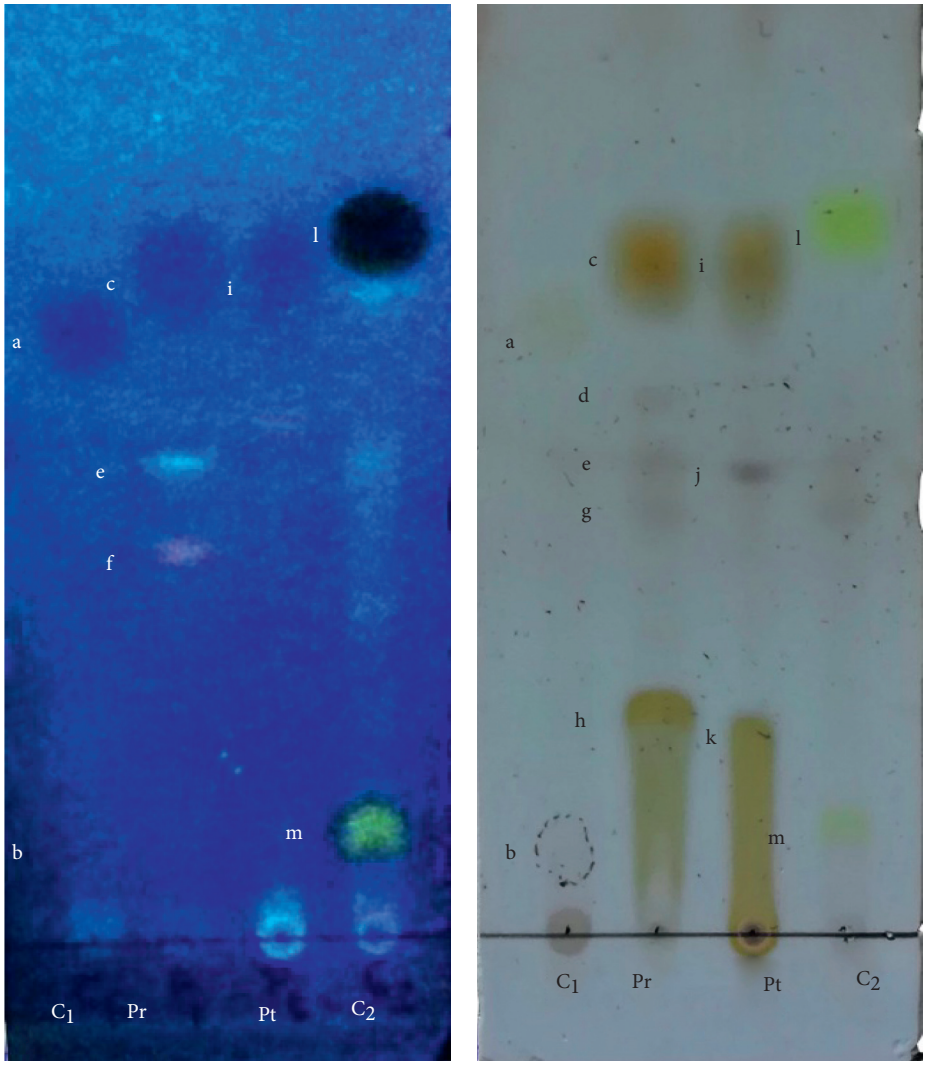
3.1.1. GC-MS Spectrophotometry

GC-MS Analysis of P. rampoddense. GC-MS analysis was carried out on hexane, chloroform, and acetonitrile extracts of *P. rampoddense* whole lichen and 13 major chemical constituents were detected. The active principles with their retention time (RT), Retention Index, molecule name, and percentage of the chemical in the crude extract are presented in Table 1. The molecular structures of major constituents identified from *P. rampoddense* are shown in Figure 3. The chromatograms obtained for hexane, acetonitrile, and chloroform extracts of P. rampoddense showed the presence of 1,4 benzene,2,6-dimethyl, also known as 2,6-dimethylhydroquinone (13.33 min), and benzoic acid, 2,4-dihydroxy-3,6-dimethyl-, methyl ester, also known as atraric acid (16.89 min), in all three solvents. Apart from the above compounds, the GC-MS profiles of hexane extract showed the presence of heptacosane (24.122 min), Nonadecane, 9-methyl (24.97 min), benzeneacetic acid, 2-tetradecyl ester (25.96 min), and octacosane (27.1 min) as the main constituents (Figure 4(a)). Similarly, the GC-MS profile of acetonitrile extract showed the presence of 3-methoy-5-propylphenol (14.34 min) 1,11-dodecadiene (16.40 min), ethyl 2-hydroxy-4-methoxy-6-propylbenzoate (17.89 min), and octadecanoic acid, ethyl ester (19.75 min) (Figure 4(b)). Furthermore, eicosane (14.32 min), tridecane, 3-methyl (7.54 min), octacosane (24.97 min), and tetracosane (25.96 min) were identified in the GC-MS chromatogram of the chloroform extract of *P. rampoddense* (Figure 4(c)). Other constituents were represented by very low percentages.

GC-MS Analysis of P. tinctorum. GC-MS analysis was carried out on hexane, chloroform, and acetonitrile extracts of *P. tinctorum* whole lichen, and 12 major chemical constituents were detected. The active principles with their retention time (RT), molecule name, and percentage of the chemical in the crude extract are presented in Table 2. The molecular structures of major constituents identified from *P. tinctorum* are shown in Figure 5. The chromatograms of hexane, acetonitrile, and chloroform extracts of *P. tinctorum* showed the presence of 2,6-dimethylhydroquinone (13.33 min), atraric acid (16.9 min), and 9-octadecanoic acid, methyl ester (20.7 min) as major constituents present in all three extracts. Pentadecanoic acid, 14-methyl, methyl ester (19.07 min), and octadecanoic acid (20.98 min) (Figure 6(a)) were identified in the GC-MS chromatogram of the hexane extract of *P. tinctorum*. Similarly, the GC-MS profile of acetonitrile extract also showed the presence of orcinol (12.65 min), o-orsellenaldehyde (15.03 min), 7-hexadecanoic acid, methyl ester (19.06 min) (Figure 6(b)). In addition to the above major compounds, the GC-MS profiles of chloroform extract showed 3,5-dihydroxytoluene (orcinol) (12.65 min), benzaldehyde, 2,4-dihydroxy-6-methyl (o-orsellenaldehyde) (15.03 min) with high percentages (Figure 6(c)). Other constituents were represented by very low percentages.

3.2. Determination of Antimicrobial Activity of Lichen Extracts

3.2.1. Antimicrobial Profile of Lichen Extracts Using Agar Disc Diffusion Assay. Ethanol, hexane, and aqueous extracts of *P. tinctorum* and *P. rampoddense* were first subjected to an agar disc diffusion assay to identify the presence of potential antibacterial compounds against both Gram-positive and Gram-negative bacteria. Crude extract and its 10-fold and 100-fold dilutions were prepared, and the inhibitory activity of each extract was quantified by the measurement of the



(a) (b)

Figure 2: Thin-layer chromatograms developed with the solvent system C (toluene 170: acetic acid 30) visualized under UV light at 366 nm (a) and heated with 10% H_2SO_4 (b).

Table 1: Chemical constituents identified in abundance in the GC-MS analysis of *P. rampoddense*.

	Name of the compound	Hexane			Acetonitrile			Chloroform		
		RT (min)	RI	% area	RT (min)	RI	% area	RT (min)	RI	% area
1	Limonene	—	—	—	7.01	1035	1.145	—	—	—
2	Benzene, 1,3-diethyl	7.421	1055	0.271	—	—	—	—	—	—
3	Tridecane,3-methyl	7.543	1061	0.902	—	—	—	7.544	1061	4.431
4	Dodecane,1-ido	11.365	1284	0.523	—	—	—	—	—	—
5	1,4-Benzenediol,2,6-dimethyl	13.337	1425	0.299	13.338	1425	1.794	13.337	1425	3.234
6	Eicosane	14.326	1501	0.999	—		—	14.323	1501	9.651
7	3-Methoxy-5-propylphenol	—	—	—	14.349	1503	0.12	—	—	—
8	1,11-Dodecadiene	—	—	—	16.401	1676	2.147	—	—	—
9	Heptacosane	16.856	1717	0.307	—		—	16.856	1717	2.654
10	Atraric acid	16.899	1721	0.308	16.898	1721	2.193	16.897	1721	7.622
11	Ethyl 2-hydroxy-4-methoxy-6-propylbenzoate	—	—	—	17.899	1908	50.357	—	—	—
12	Palmitic acid	19.404	1982	0.676	—		—	—	—	—
13	Octadecanoic acid, ethyl ester	—	—	—	19.752	1997	2.594	—	—	—
14	Octacosane	21.638		0.323				24.971		4.160
15	Tetradecane	22.499		1.111				—		—

Table 1: Continued.

	Name of the compound	Hexane			Acetonitrile			Chloroform		
		RT (min)	RI	% area	RT (min)	RI	% area	RT (min)	RI	% area
16	Tetracosane	23.325		2.751				25.962		4.432
17	Heptacosane	24.122		6.123				24.121		3.69
18	Nonadecane,9-methyl	24.974		9.799						
19	Benzeneacetic acid, 2-tetradecyl ester	25.964		12.110						
20	Eicosane, 7-hexyl	26.680		0.462						
21	Octacosane	27.145		13.877						
22	Squalene	27.637		1.804						
23	Heptacosane	28.580		10.263						
24	Benzeneacetic acid, 2-tetradecyl ester	30.351		7.912						
25	Octacosane	35.305		3.602						

RT-Retention time (min.), RI–Nonisothermal retention index calculated using Van Den Dool and Kratz method using C_8–C_{20} alkane series. % Area is expressed as percentage of the peak area to the total peak area.

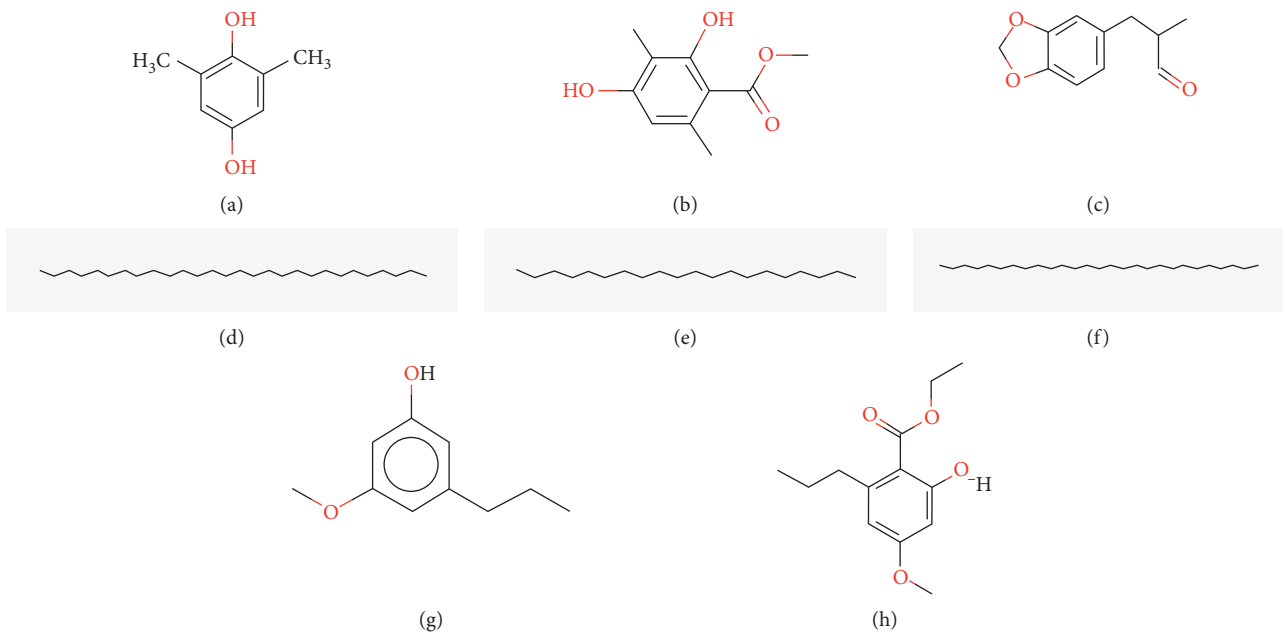
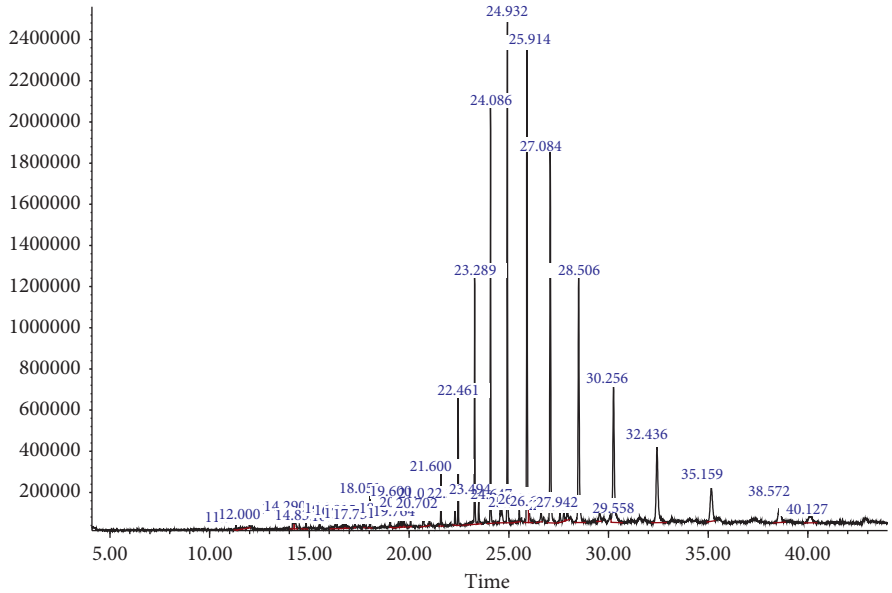


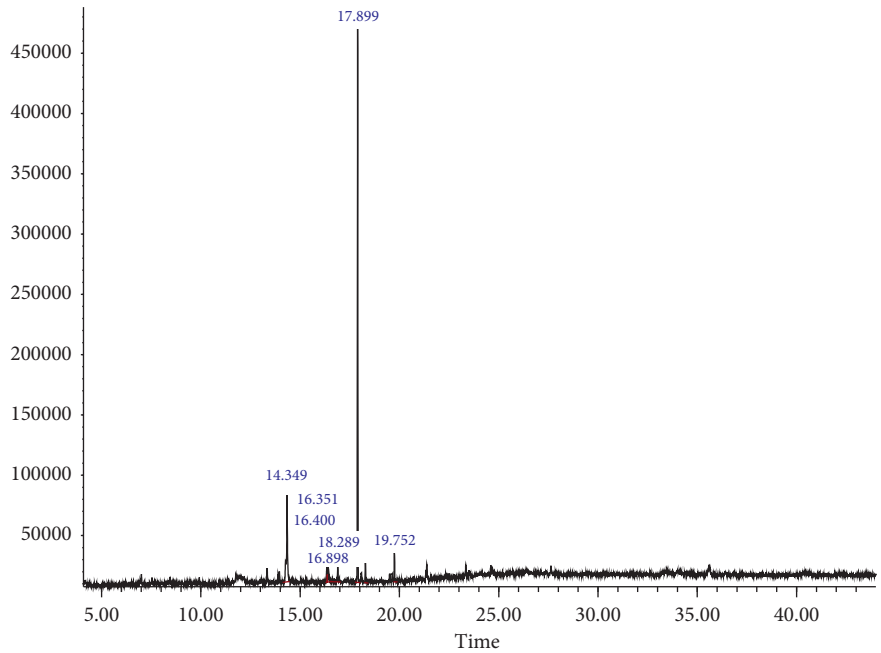
Figure 3: Compounds present in *P. rampoddense*. (a) 1,4-Benzenediol, 2,6-dimethyl. (b) Benzoic acid, 2,4-dihydroxy-3,6-dimethyl-, methyl ester (atraric acid). (c) 1,3-benzodioxole-5-propanal,.alpha.-methyl (helional). (d) Octacosane ($C_{28}H_{58}$). (e) Eicosane ($C_{20}H_{42}$). (f) Heptacosane ($C_{27}H_{56}$). (g) Phenol, 3-methoxy-5-propyl. (h) Ethyl 2-hydroxy-4-methoxy-6-propylbenzoate (Ethyl divaricatinate).

diameter of the zone of inhibition. Measurable inhibitory zones were observed only against the Gram-positive bacterium, MSSA. None of the lichen extracts showed any activity against the Gram-negative bacteria, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*. *P. tinctorum* gave inhibitory zones only for the ethanol and the hexane extracts but *P. rampoddense* showed inhibitory zones for all three extracts (Table 3, Figure 7). Compared to *P. tinctorum*, which showed an inhibitory zone of 9.4 mm only at 300 mg/ml, *P. rampoddense* showed larger inhibitory zones (14.4, 15.3, 16.5 mm at 300, 30, and 3 mg/ml, respectively) in the presence of all three concentrations of the ethanol extract. However, the inhibitory zones given by the hexane (7.8, 6.5 mm at 300 and 30 mg/ml, respectively) and the aqueous extracts (8.1, 7.9 mm at 300 and 30 mg/ml, respectively) of *P. rampoddense* were comparatively lower (Table 3, Figure 7).

The same trend was observed against twenty clinical isolates of MRSA. Relatively larger inhibitory zones between 7.9 and 11.4 mm were observed from the undiluted crude ethanol extract of *P. rampoddense*, while ten-fold and hundred-fold dilutions of the ethanol extract gave inhibitory zones between 8.5–12.4 mm and 6.1–8.2 mm, respectively. However, diameters of inhibitory zones of water and hexane extracts were lower ranging from 6.1–7.4 mm and 6.1–8.0 mm, respectively (Table 4, Figure 8). Hexane and aqueous extracts of *P. rampoddense* gave measurable inhibitory zones only at 300 mg/ml. Consistent with the results obtained against MSSA, relatively lower inhibitory zones were observed in the presence of ethanol and hexane extracts of *P. tinctorum*, but the aqueous extract was inactive against MRSA. All results were compared against the standard drug, Vancomycin (30 μg/disc), which showed inhibitory zones of 20.8 mm and 13.53 mm, respectively, against MSSA and MRSA.

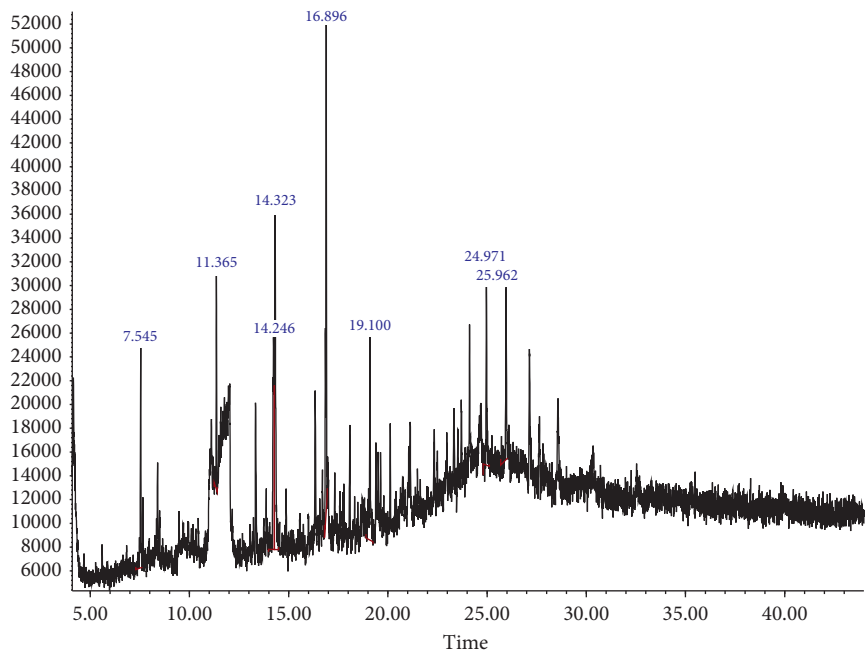


(a)



(b)

Figure 4: Continued.



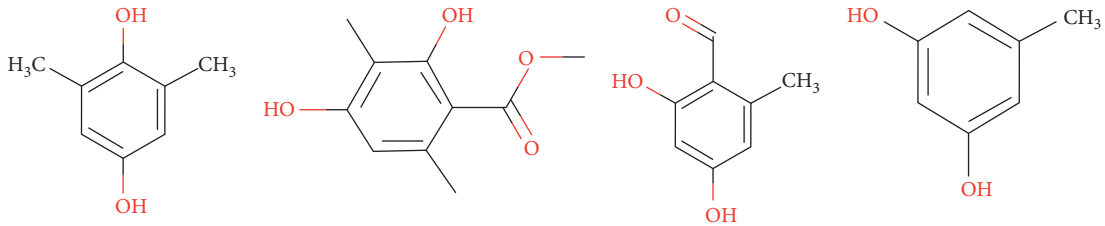
(c)

Figure 4: GC-MS chromatograms of crude hexane (a), acetonitrile (b), and chloroform (c) extracts of *P. rampoddense*.

Table 2: Chemical constituents identified in abundance in the GC-MS analysis of *P.tinctorum*.

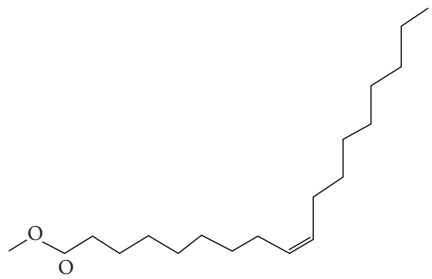
	Name of the compound	Hexane			Acetonitrile			Chloroform		
		RT (min)	RI	% area	RT (min)	RI	% area	RT (min)	RI	% area
1	Benzaldehyde,2,4,-dimethyl	—	—	—	10.409	1223	0.265	10.409	1223	0.73
2	Hexadecane,2,6,10,14-tetramethyl	—	—	—	—	—	—	11.337	1283	0.228
3	3,5-Dihydroxytoluene	—	—	—	12.65	1374	30.086	12.654	1375	2.572
4	1,4-Benzenediol, 2,6-dimethyl	13.342	1425	1.915	13.335	1425	2.939	13.339	1425	4.084
5	Chloroatranol (3-chloro-2,6-dihydroxy-4-methyl benzaldehyde)	—	—	—	—	—	—	14.44	1511	0.633
6	Benzaldehyde,2,4-dihydroxy-6-methyl	—	—	—	15.039	1560	2.758	15.035	1560	11.671
7	Atraric acid	16.888	1720	6.723	16.888	1720	16.422	16.901	1721	52.174
8	7-Hexadecanoic acid, methyl ester	—		—	19.069	1966	0.685	18.879	1956	0.478
9	Pentadecanoic acid,14-methyl,methyl ester	19.07	1966	1.436	—	—	—	19.073	1966	1.616
10	*n*-Hexadecanoic acid	—	—	—	—	—	—	19.392	1982	1.402
11	9-Octadecanoic acid, methyl ester	20.761	—	1.932	20.76	-	0.696	20.763	—	2.058
12	Octadecanoic acid	20.92	—	0.702	—	—	—	20.983	—	0.285

RT, retention time (min.), RI, –nonisothermal retention index calculated using Van Den Dool and Kratz method using C_8–C_{20} alkane series. %, area is expressed as percentage of the peak area to the total peak area.



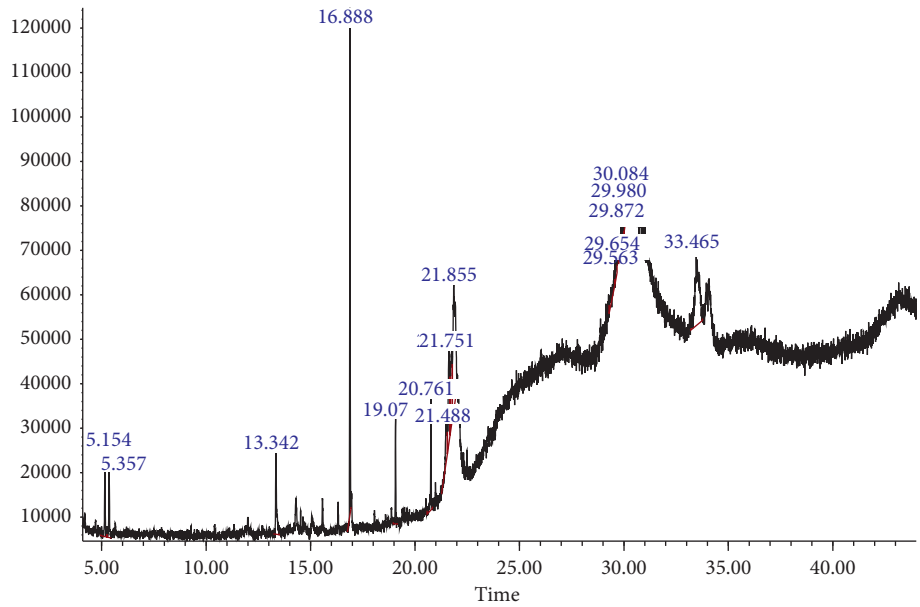
(a) (b) (c) (d)

Figure 5: Continued.

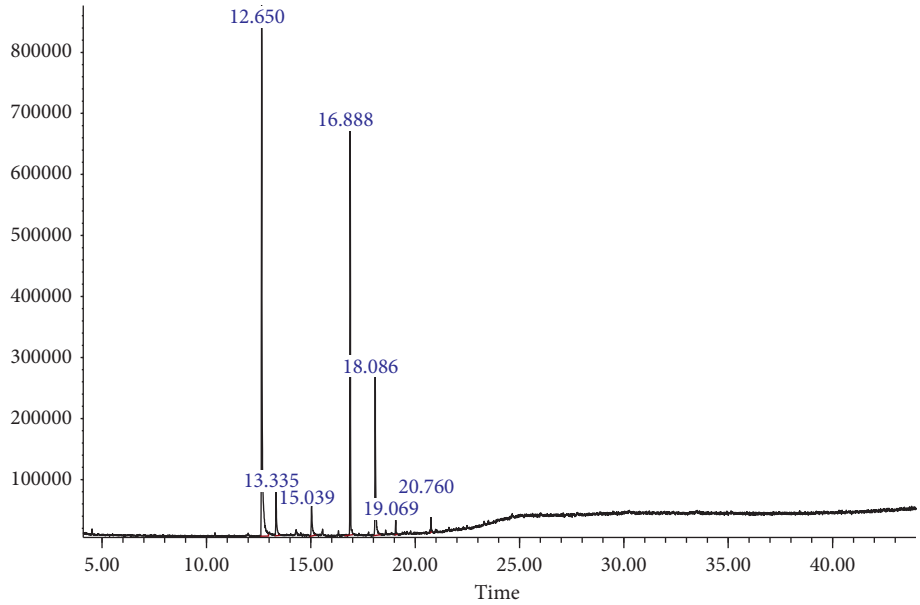


(e)

Figure 5: Compounds present in *P. tinctorum*. (a) 1,4-Benzenediol,2,6-dimethyl. (b) Benzoic acid, 2,4-dihydroxy-3,6-dimethyl-, methyl ester (atraric acid). (c) Benzaldehyde,2,4-dihydroxy-6-methyl (O-orsellinaldehyde). (d) 3,5-dihydroxytoluene (orcinol). (e) 9-octadecanoic acid, methyl ester.

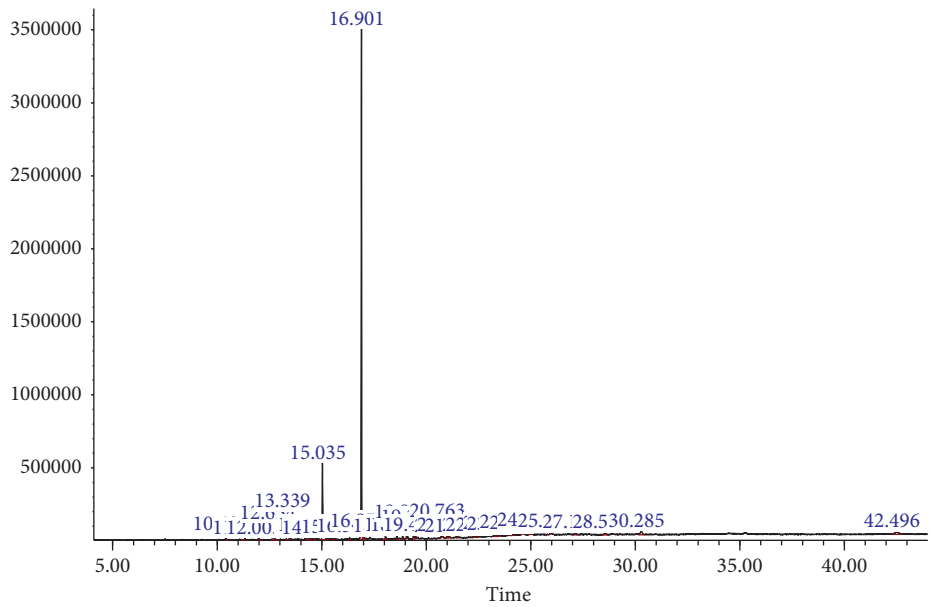


(a)



(b)

Figure 6: Continued.



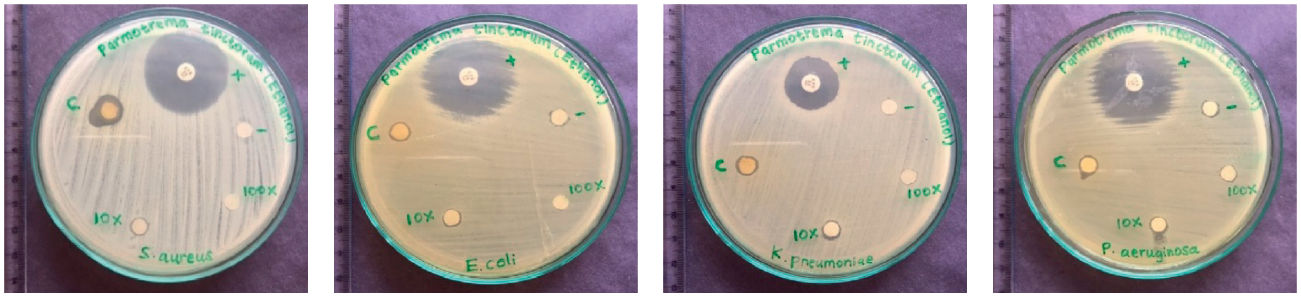
(c)

Figure 6: GC-MS chromatograms of crude hexane (a), acetonitrile (b), and chloroform (c) extracts of *P. tinctorum*.

Table 3: Comparison of zone diameters of the disc diffusion assay performed on *P.tinctorum* and *P. rampoddense* against MSSA (ATCC 25923) and 20 clinical isolates of MRSA.

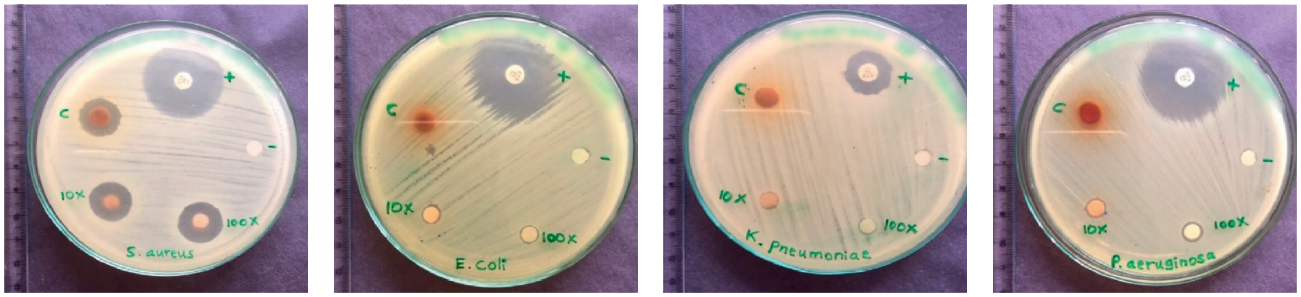
Extract	Concentration (mg/ml)	Diameters of the inhibitory zones (mm)			
		MSSA (ATCC 25923)*		MRSA#	
		P. tinctorum	*P.rampoddense*	*P. tinctorum*	*P. rampoddense*
Ethanol	300	9.4 ± 0.1	14.4 ± 0.8	7.13 (6.2–9.6), $n = 17$	9.61 (7.9–11.4), $n = 20$
	30	—	15.3 ± 0.6	7.11 (6.1–11.1), $n = 16$	10.38 (8.5–12.4), $n = 20$
	3	—	16.5 ± 0.4	—	7.03 (6.1–8.2), $n = 15$
Hexane	300	7.7 ± 0.2	7.8 ± 0.1	7.24 (6.3–9.1), $n = 18$	6.76 (6.1–7.4), $n = 18$
	30	7.5 ± 0.1	6.5 ± 0.1	6.78 (6.4–7.1), $n = 5$	—
	3	6.8 ± 0.3	—	—	—
Aqueous	300	—	8.1 ± 0.2		7.28 (6.1–8.0), $n = 13$
	30	—	7.9 ± 0.1	—	—
	3	—	—	—	—
Positive control	30 μg/disk	20.8 ± 0.4	13.53 ± 0.23		
Negative control	10%	—	—		

*Data are expressed as mean ± SEM. #Data are expressed as mean (Min-Max) of the 20 clinical isolates of MRSA. n denotes the number of MRSA isolates which gave a measurable diameter. Vancomycin was used as the positive control. 10% DMSO was used as the negative control. – denotes a measurable diameter was not observed.



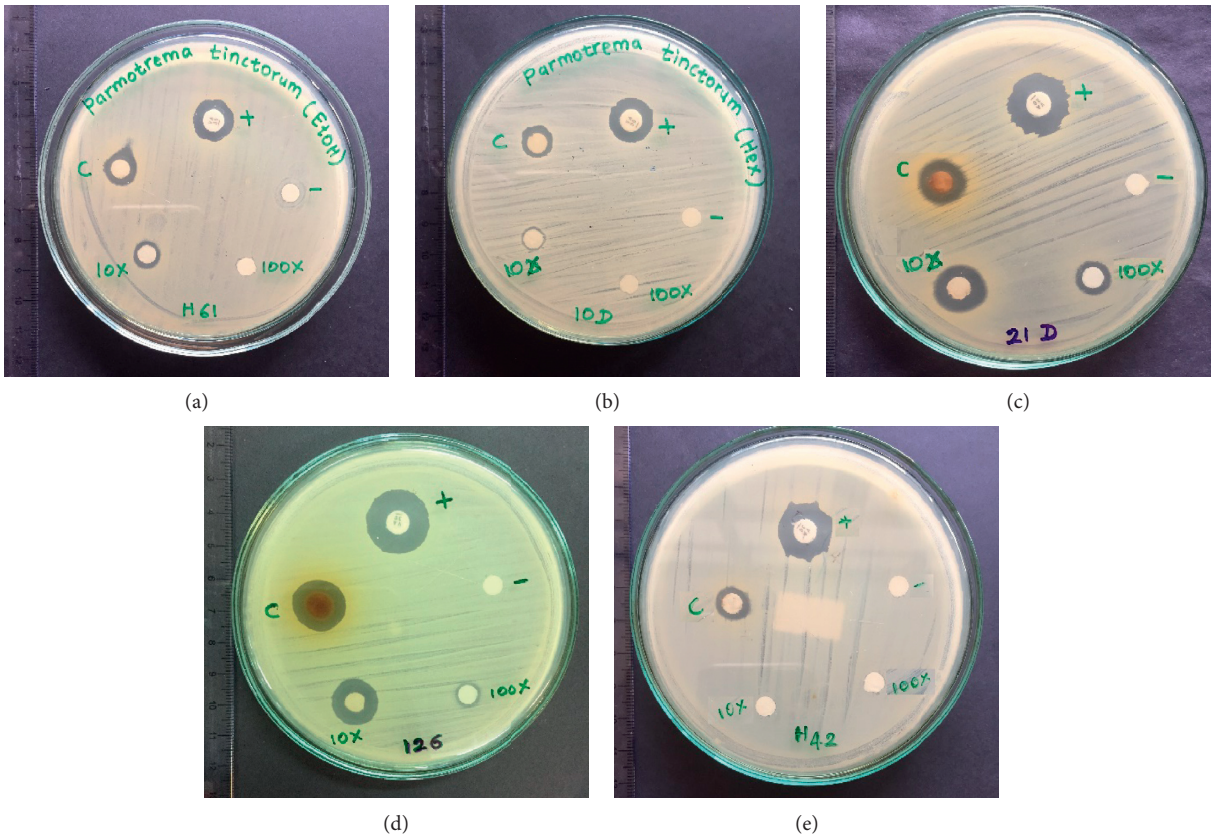
(a) (b) (c) (d)

Figure 7: Continued.



(e) (f) (g) (h)

Figure 7: Inhibitory zones obtained for *P. tinctorum* and *P. rampoddense* tested against Gram-positive and Gram-negative bacteria. Top row shows the inhibitory zones of crude ethanol extracts of *P. tinctorum* against (a) *S aureus*, (b) *E coli*, (c) *K pneumonia*, and (d) *P aeruginosa,* and botom row shows the inhibitory zones of crude ethanol extracts of *P.rampoddense* against (e) *S aureus,* (f) *E coli,* (g) *K pneumonia*, and (h) *P aeruginosa*; (+): positive control, cefotaxime (30 μg/disk); (−): negative control, 10% DMSO; (c) crude extract; 10*x*: 10-fold dilution of crude extract; 100*x*: 100-fold dilution of the crude extract.



(a) (b) (c)

(d) (e)

Figure 8: Inhibitory zones of crude extracts of *P. tinctorum:* (a) ethanol extract; (b) hexane extract. Crude extracts of *P. rampoddense*: (c) ethanol extract, (d) hexane extract, and (e) aqueous extract, against different strains of methycillin resistant *Staphylococcus aureus*. The lichen extracts that gave positive results for the disc diffusion assay for some MRSA strains are shown here. (+): positive control, vancomycin (30 μg/disc); (-): negative control, 10% DMSO; (c) crude extract; 10*x*: 10-fold dilution of crude extract; 100*x*: 100-fold dilution of the crude extract.

3.2.2. Activity Index (AI) and Relative Percentage Inhibition (RPI) of Lichen Extracts. Antibacterial activity of the extracts was compared with the antibacterial activity of the standard drug (positive control), Vancomycin. Ethanol extract of *P. rampoddense* exhibited the maximum AI (Figure 9) as well as the RPI (Figure 10) against MSSA (AI of 0.69, RPI of 44.46%) and MRSA (AI of 0.68, RPI of 37.38) compared to the hexane and aqueous extracts. AI and RPI calculated for *P. tinctorum* were relatively lower in all three extracts compared to *P. rampoddense.*

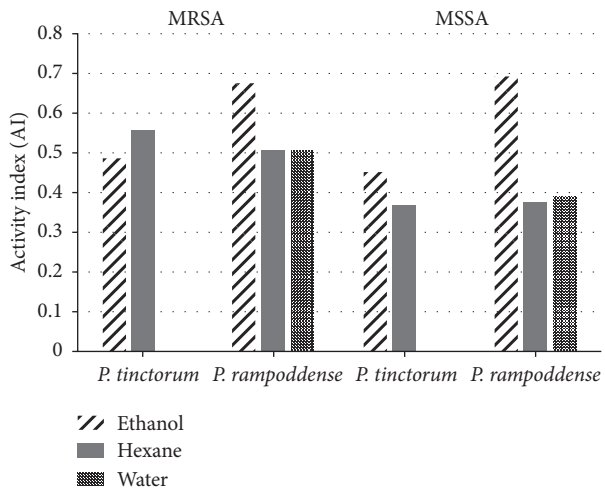


Figure 9: Activity index calculated for the activity of different extracts of *P. tinctorum* and *P. rampoddense* against MRSA and MSSA.

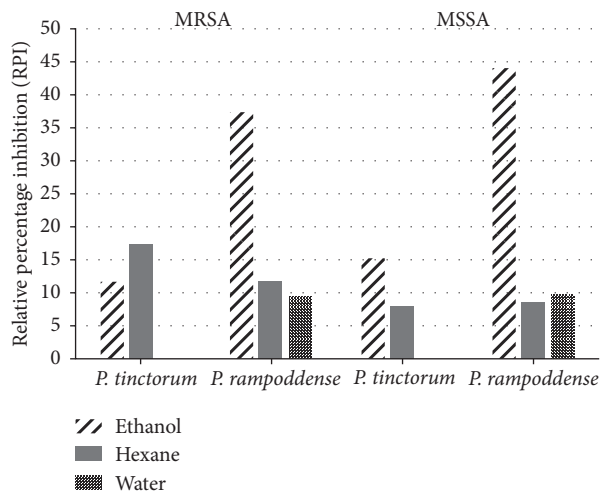


Figure 10: Relative percentage index calculated for the activity of different extracts of *P. tinctorum* and *P. rampoddense* against MRSA and MSSA.

3.2.3. Minimum Inhibitory Concentration of Lichen Extracts. As expected, results of the broth microdilution assay confirmed that MSSA (ATCC 25923) was more susceptible against ethanol extract of *P. rampoddense* with the lowest MIC of 0.0192 mg/ml than the MRSA, which showed a MIC of 0.096 mg/ml for 25% of clinical isolates (Table 5). MIC ranges obtained for the ethanol and hexane extracts of *P. tinctorum* were 2.4–12 mg/ml, whereas MIC ranges obtained for the hexane, ethanol, and aqueous extracts of *P. rampoddense* were 12–60, 0.096–2.4, and 2.4–60 mg/ml, respectively. Out of the 20 MRSA isolates used in the study, 60% and 90% were inhibited by the presence of 2.4 mg/ml of ethanol and hexane extracts of *P. tinctorum*, respectively. However, hexane extract of *P. rampoddense* was less effective in the inhibition of MRSA isolates in which 75% of the isolates were inhibited at 60 mg/ml and 25% of the isolates were inhibited at 12 mg/ml. Comparatively, the aqueous extract of *P. rampoddense* showed better activity in which 25%, 55%, and 20% of the MRSA isolates were inhibited by 60, 12, and 2.4 mg/ml of the lichen extract, respectively. The best activity was shown by the ethanol extract of *P. rampoddense* where 25% of the MRSA isolates were inhibited by 0.096 mg/ml of the extract, whereas 25% and 50% of the isolates were inhibited by 0.48 mg/ml and 2.4 mg/ml of the same extract, respectively.

4. Discussion

Resistant microbial populations emerged in a higher magnitude in the recent past due to inappropriate and irrational use of antibiotics. Among the mechanisms suggested, alteration of target sites, enzymatic degradation, and active efflux of drugs are some of the strategies used by the pathogenic bacteria to develop inherent resistance developed against antibiotics. Unfortunately, Food and Drug Administration (FDA) has approved only two antibacterial agents for use in humans from 2008 to 2016, compared to the 16 drugs approved from 1983 to 1987 [22]. Therefore, novel bioactive preparations from lichens may offer a plethora of interesting possibilities to combat drug resistance.

Relatively, a limited number of studies have thus far explored the biological activities of lichens worldwide [23]. Antibacterial activities of lichens were first reported in 1944 by Burkholder et al. [24] and since then, several studies have emerged on their protective effect against both Gram-negative and Gram-positive bacteria [24]. Results from previous studies have suggested that it is much difficult for bacteria to develop resistance against multiple and chemically complex phytochemicals present in plant extracts including lichens [25]. It is believed that secondary metabolites of plants were optimized during evolution so that they can interfere with molecular targets in herbivores and microbes and act as defense mechanisms [26].

This study provides data based on the first broad-scale screening of antimicrobial activity of two lichens collected from Belihuloya, Sri Lanka, against Gram-negative and Gram-positive bacteria including twenty clinically isolated MRSA. In the preliminary study we conducted, it was reported that lichens extracted to three different solvents of varying polarities were not active against any of the Gram-negative bacteria, *Escherichia coli, Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*. Our results are comparable to the results reported previously by other authors [27–30]. This can be ascribed to the sensitivity and differences in the permeability of the cell wall of bacteria. The cell wall of Gram-positive bacteria is made up of peptidoglycans and teichoic acids, whereas the cell wall of Gram-negative bacteria are made up of peptidoglycans, lipopolysaccharides, and lipoproteins, which offers more resistance than the Gram-positive bacteria [31].

We determined the antibacterial activity of *P. tinctorum* and *P. rampoddense* against the standard strain of *S. aureus* (ATCC 25923), and 20 clinically isolated strains of MRSA (MRSA 1–20). These bacteria were used as test microorganisms due to their clinical significance, which imposes serious threats against individuals. According to the results

Table 4: Zone diameters of the disc diffusion assay performed on *P. tinctorum* and *P. rampoddense* against 20 clinical isolates of MRSA.

Organism	*P. tinctorum*				*P. rampoddense*					
	Ethanol		Hexane		Ethanol		Hexane		Aqueous	
	(+) control	Crude	(+) control	Crude	(+) control	Crude	(+) control	Crude	(+) control	Crude
1	11.9	5.2	10.9	6.9	14.6	9.9	13.7	7	13.9	7.4
2	14	9.6	14.3	7.3	14.9	11.4	13.5	6.7	13.9	0
3	16.5	6.2	15.9	7.2	14.9	10.6	12.9	6.5	12.4	5.3
4	17.3	7.9	14.7	7.1	13.3	8.9	13	7.2	13	8
5	12.8	10	11.1	7	13.9	9.1	13	7.3	13.2	6
6	12.4	6.6	11	5.7	12.9	10.2	13.2	5.5	12.7	7.3
7	14.8	5.4	10.8	6.3	14.2	9	12.5	6	14.3	8
8	13.5	6.8	11.4	7	14.4	8.2	13.3	6.1	13.4	7.3
9	15	6.1	14	6.9	16.6	10	13.5	6.2	14.4	7
10	13.4	7.1	11.2	7.1	15.7	8.5	13.1	7.3	12.5	6
11	14.9	7.4	14.5	6.4	14.4	9.4	13	7.2	14.6	7.2
12	13.4	6.2	15	8.7	16	10.6	13.7	7.4	14.3	7
13	16.5	8.1	11.4	7.5	11.8	9.8	13.9	6.8	12.9	5.6
14	16	6.3	12.6	9.1	14.7	10	13	6.9	13.4	6.7
15	10.8	6.2	9.6	7	13.8	7.9	12.5	5.8	13.8	5.5
16	12.1	7.4	11.7	7.2	12.6	9.8	12.4	6	13.3	7
17	15.2	5.2	16.8	7	15.6	11.1	13.1	6.4	12.9	5.8
18	13	6.5	14	7.4	13.8	9.4	13	6.5	13.6	6.1
19	13.1	6.6	11.7	6	12.8	8.9	13.2	7	13.4	7.8
20	16.4	6.2	11.8	7.3	13	9.4	13.3	7.2	13.4	7.8
Average	14.15	6.85	12.72	7.10	14.19	9.60	13.14	6.65	13.46	6.44

Vancomycin was used as the positive control. 10% DMSO was used as the negative control and they did not show any measurable zones. All crude extracts were at 300 mg/ml. Some ethanol and hexane extracts of *P. tinctorum* and *P. rampoddense* gave measurable zones at 30 and 3 mg/ml concentrations but they are not shown here.

obtained, MSSA was more sensitive than MRSA against both *P. tinctorum* and *P. rampoddense*. Our results against *S. aureus* are comparable to several other studies reported previously by multiple authors [32–34]. We also used crude aqueous, ethanol, and hexane extracts of all lichens for the study. Crude extracts are often used in traditional systems of medicine, such as Ayurveda, commonly practiced in Sri Lanka, due to their belief in the synergistic effect of a multitude of phytoconstituents, which may give rise to the observed bioactivity. It is also believed that the separation of these compounds may alter the activity of individual compounds, which may be below the sensitivity threshold [35].

According to the results, we found that all hexane and ethanol extracts of the tested lichen species, except the aqueous extracts, were active against both methicillin-sensitive and methicillin-resistant *S. aureus* at 300 mg/ml but some were even active at lower concentrations such as 30 and 3 mg/ml (Tables 3 and 5). Although both hexane and ethanol extracts demonstrated activity against all the MRSA strains, the ethanol extract was more active than the hexane extract (Table 4). Ethanol extract of *P. rampoddense* showed the highest activity index (Figure 9) and relative percentage index (Figure 10) and also the lowest MIC values of 0.096 mg/ml (Table 4) against five isolates of methicillin-resistant *S. aureus*. Hence, it can be concluded that the polarity of the solvent used during the extraction process determines which phytoconstituents were extracted that has a direct effect on the antimicrobial potential of the lichens under study.

Different types of secondary metabolites, such as depsides, depsidones, hydroxybenzoic acid derivatives, anthraquinones, dibenzofurans, and aliphatic acids, are produced by lichens and are deposited in the upper cortex or fruiting bodies on the thallus in liquid or crystal form [31]. The antimicrobial activity could be correlated with the secondary metabolites of *P. rampoddense*, which is composed of alectorialic acid and atranorin as the main substances as supported by our TLC results (Figure 2). A significant amount of Atraric acid detected by GC-MS is derived from the well-known lichen compound atranorin [36]. Furthermore, the hexane extract of the lichen indicated significant quantities of n-alkanes and methyl esters of fatty acids similar to *Xanthoria parietina* species [37]. It is a known fact that relatively unstable lichen substances are degraded in the lichen itself or *in vitro* in their extracts at room temperature and Zakeri et al. [38] have shown that alectorialic acid is a relatively unstable substance which can be degraded to other compounds at room temperature. Therefore, degradation products have been reported in the GC-MS analysis. Lichen acids, atranorin, and lecanoric acid were detected in the TLC profile of *P. tinctorum* and the GC-MS analysis confirmed the presence of atraric acid, orcinol, and O-orsellinaldehyde among the major constituents. Previous studies have also detected O-orsellinaldehyde and orcinol as bioactive derivatives obtained from lecanoric acid [38–40]. Similar antimicrobial activities were reported by Gomes et al. for the lecanoric acid isolated from the lichen, *P. tinctorum* [41]. Alectorialic acid and atranorin were among the several metabolites with

Table 5: Minimum inhibitory concentration (MIC) of different lichen extracts determined by broth microdilution assay against MSSA (ATCC 25923) and 20 clinical isolates of MRSA.

Bacterial strain	Minimum inhibitory concentration (MIC) (mg/ml)				
	Hexane extract		Ethanol extract		Aqueous extract
	P. tinctorum	*P. rampoddense*	*P. tinctorum*	*P. rampoddense*	*P. rampoddense*
MSSA	2.4	12	2.4	0.0192	2.4
MRSA 1	12	12	2.4	2.4	60
MRSA 2	12	60	12	0.096	60
MRSA 3	2.4	60	2.4	0.096	12
MRSA 4	2.4	60	2.4	0.48	60
MRSA 5	2.4	12	12	2.4	12
MRSA 6	2.4	60	12	2.4	12
MRSA 7	2.4	60	12	0.096	12
MRSA 8	2.4	60	2.4	2.4	12
MRSA 9	2.4	60	2.4	2.4	12
MRSA 10	2.4	12	12	0.48	12
MRSA 11	2.4	60	2.4	2.4	12
MRSA 12	2.4	60	2.4	0.48	2.4
MRSA 13	2.4	12	2.4	2.4	2.4
MRSA 14	2.4	60	2.4	0.096	12
MRSA 15	2.4	60	2.4	2.4	12
MRSA 16	2.4	60	12	0.096	2.4
MRSA 17	2.4	12	12	2.4	60
MRSA 18	2.4	60	2.4	0.48	60
MRSA 19	2.4	60	12	2.4	2.4
MRSA 20	2.4	60	2.4	0.48	12

antimicrobial activities identified by Pilzk et al. in the lichens, *Ramalina* and *Usnea* [31]. According to Jayaprakasha and Rao, phenolic constituents, including methyl orsenillate, orsenillic acid, atranorin and lecanoric acid, were extracted from the lichen *Parmotrema stuppeum* [31]. Atranorin, usnic acid, norstictic acid, protoacetraric acid, atranol, lecanoric acid, stictic acid, and divericatic acids extracted from lichens, *Melanelia subaurifera* and *Melanelia fuliginosa*, were also reported to possess antibacterial activities [42]. In another study, Rankovic et al. also reported that atranorin exhibited antimicrobial activities against *S. aureus, B. subtilis*, and *E coli* [43]

Rajan et al. who determined the antimicrobial activity of acetone extracts of *P. rampoddense* and *P. tinctorum* against *S. aureus* (ATCC 29213) also detected the presence of alectorialic acid and atranorin in both species [44]. Although they investigated the antimicrobial effect of both acetone and methanol extracts, none of the methanol extracts were effective against *S. aureus* (ATCC 29213). Interestingly, in our study, ethanol extracts of both lichens, which also have a higher polarity comparable to methanol, showed the highest antibacterial activity even at 3 mg/ml (*P. rampoddense).* Rajan et al. also revealed that the MIC of acetone extracts of both lichens were 0.5 mg/ml against *S. aureus* (ATCC 29213) [44]. We revealed that *P. rampoddense* ethanol extract at 0.0192 mg/ml was effective against the CLSI control strain of *S aureus* (ATCC 25923). The same lichen extract was also effective against all MRSA strains with MIC between 0.096 and 2.4 mg/ml. Variation in the results reported for *P. rampoddense* and *P. tinctorum* between the two research groups may be due to a combination of factors, including the extraction of different lichen species, type of extract and its concentration, the solvent used for extraction, and the specific bacterial strain [45]. The differences in results between the two studies could also be due to certain adaptations and modifications that could take place for the survival of the species in different climatic conditions and geographical locations. Specific factors that influence the antimicrobial properties of lichen extracts warrant further investigations.

Since we have identified several chemical constituents already proven to be having antimicrobial properties in the two lichen species investigated, it can be assumed that the lichen crude extracts were active against the test organisms due to the probable mechanisms of those secondary metabolites. It is also proven that multiple components in a crude extract act at different sites, thereby contributing to the overall activity of the extract. The lichen extracts may exert their anti-microbial activity by altering the key events in the pathogenesis and not just by killing the bacteria itself [46]. Such key processes may include the inhibition of cell wall synthesis, inhibition of protein synthesis, alteration of the cell membrane integrity, inhibition of nucleic acid synthesis, and anti-metabolite activity [47]. The antibiotic activity could also be correlated with the mechanism of action of various lichen acids, which could inhibit oxidative phosphorylation. As such events happen, oxygen consumption, electron transport chain, and other mitochondrial functions may also be inhibited, leading to cell death [48]. Therefore, it is imperative to investigate the mechanism of action of active constituents of *P. rampoddense* and *P. tinctorum* and optimize their activity to develop them as potential antimicrobial agents in the future.

5. Conclusion

Our study provides evidence that the two lichens, *P. rampoddense* and *P. tinctorum*, collected from Belihuloya, Sri Lanka, represent potentially important source of future antimicrobial drugs. Our research provides evidence that all extracts showed a significant inhibitory activity but ethanol extract of *P. rampoddense* is more effective than *P. tinctorum* at MIC 0.0192 mg/ml against methicillin-sensitive *Staphylococcus aureus* and at the minimum MIC of 0.096 mg/ml against methicillin-resistant *Staphylococcus aureus*. Among the chemical constituents identified, alectorialic acid, lecanoric acid, atranorin, atraric acid, orcinol, and O-orsellinaldehyde may be responsible for the antimicrobial activity of the two lichens used in the study. These two lichen species merit further investigation to determine their mechanism of action against bacterial pathogens and also their levels of cytotoxicity against normal cells.

Abbreviations

MRSA:	Methicillin-resistant *Staphylococcus aureus*
MSSA:	Methicillin-sensitive *Staphylococcus aureus*
CLSI:	Clinical & laboratory standards institute
P. rampoddense:	*Parmotrema rampoddense*
P. tinctorum:	*Parmotrema tinctorum*
TLC:	Thin later chromatography
GC-MS:	Gas chromatography mass spectrophotometry
ATCC:	American type culture collection
DMSO:	Dimethyl sulfoxide
AI:	Activity index
RPI:	Relative percentage inhibition
MIC:	Minimum inhibitory concentration
Rf:	Retention factor.

Data Availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interests.

Authors' Contributions

PSAIS was involved in data collection, data processing, and analysis. RPH, RGUJ, HR, and WMDGBW were involved in the conceptualization of the study, supervision, and guidance in conducting experiments. RPH drafted and revised the manuscript. RGUJ was involved in the identification and characterization of the lichens. DW was involved in the GC-MS analysis. All the authors reviewed and approved the final manuscript submitted.

Acknowledgments

Technical and other laboratory support was provided by the technical officers and nonacademic staff members at the Departments of Biochemistry and Microbiology, Faculty of Medicine, University of Ruhuna, Sri Lanka. The authors are grateful to the Department of Natural Resources, Faculty of Applied Sciences, Sabaragamuwa University of Sri Lanka and Instrument Center, Faculty of Applied Sciences, University of Sri Jayewardenepura, Nugegoda, Sri Lanka. This study was supported by the Faculty Research Grant of the Faculty of Medicine, University of Ruhuna, Sri Lanka.

References

[1] H. W. Boucher, G. H. Talbot, J. S. Bradley et al., "Bad bugs, no drugs: No ESKAPE! an update from the Infectious Diseases Society of America," *Clinical Infectious Diseases*, vol. 48, no. 1, pp. 1–12, 2009.

[2] M. Leeb, "Antibiotics: a shot in the arm," *Nature*, vol. 431, no. 89, pp. 2-3, 2004.

[3] T. Kokubun, W. K. Shiu, and S. Gibbons, "Inhibitory activities of lichen derived compounds against methicillin and multidrug-resistant *Staphylococcus aureus*," *Planta Medica*, vol. 73, no. 17, pp. 6–9, 2007.

[4] R. M. Dharmadasa, G. C. Akalanka, P. R. M. Muthukumarana, and R. G. S. Wijesekara, "Ethnopharmacological survey on medicinal plants used in snakebite treatments in Western and Sabaragamuwa provinces in Sri Lanka," *Journal of Ethnopharmacology*, vol. 179, pp. 110–127, 2016.

[5] S. Wijesundara, H. S. Kathriarachchi, S. W. Ranasinghe, and G. Hapuarachchi, "The national red list 2012 of Sri Lanka," in *Conservation Status of the Fauna and Flora*, D. K. Weerakoon and S. Wijesundara, Eds., pp. 340–345, Ministry of Environment, Colombo, Sri Lanka, 2012.

[6] G. Weerakoon, P. A. Wolseley, O. Arachchige, M. Eugenia da Silva Cáceres, U. Jayalal, and A. Aptroot, "Eight new lichen species and 88 new records from Sri Lanka," *The Bryologist*, vol. 119, no. 2, pp. 131–142, 2016.

[7] K. S. Vinayaka, S. Shetty, and Y. L. Krishnamurthy, "Utilization of lichens in the central Western Ghats area of Karnataka," *British Lichenological Society Bulletin*, vol. 109, pp. 56–62, 2011.

[8] M. J. Calcott, D. F. Ackerley, A. Knight, R. A. Keyzers, and J. G. Owen, "Secondary metabolism in the lichen symbiosis," *Chemical Society Reviews*, vol. 47, no. 5, pp. 1730–1760, 2018.

[9] V. Shukla, G. P. Joshi, and M. S. M. Rawat, "Lichens as a potential natural source of bioactive compounds: a review," *Phytochemistry Reviews*, vol. 9, no. 2, pp. 303–314, 2010.

[10] K. Molnár and E. Farkas, "Current results on biological activities of lichen secondary metabolites: a review," *Zeitschrift für Naturforschung C*, vol. 65, no. 3-4, pp. 157–173, 2010.

[11] S. Huneck, "The significance of lichens and their metabolites," *Naturwissenschaften*, vol. 86, no. 12, pp. 559–570, 1999.

[12] J. Boustie, S. Tomasi, and M. Grube, "Bioactive lichen metabolites: alpine habitats as an untapped source," *Phytochemistry Reviews*, vol. 10, no. 3, pp. 287–307, 2011.

[13] K. Victor, L. Boris, G. Athina et al., "Design, synthesis and antimicrobial activity of usnic acid derivatives," *MedChemComm*, vol. 9, no. 5, pp. 870–882, 2018.

[14] N. Toma, L. Ghetea, R. Nitu, and D. I. Corol, "Progress and perspectives in the biotechnology of lichens," *Romanian Biotechnological Letters*, vol. 6, pp. 1–15, 2001.

[15] P. K. Divakar and D. K. Upreti, *Parmelioid Lichens in India (A Revisionary Study)*, M/s Bishen Singh Mahendra Pal Singh (BSMPS), Dehradun, India, 2005.

[16] G. E. Trease and W. C. Evans, *A Textbook of Pharmacognosy*, Bailliere Tindall Ltd., London, UK, 1989.

[17] A. Sofowora, *Medicinal Plants and Traditional Medicine in Africa*, Spectrum Books Ltd., Ibadan, Nigeria, 1993.

[18] A. Orange, P. W. James, and F. J. White, *Microchemical Methods for the Identification of Lichens*, British Lichen Society, London, UK, 2010.

[19] H. van Den Dool and P. Dec Kratz, "A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography," *Journal of Chromatography A*, vol. 11, pp. 463–471, 1963.

[20] H. Rathnayake, M. H. N. De Zoysa, R. P. Hewawasam, and W. M. D. G. B. Wijerathne, "Comparison of *in vitro* antibacterial activity of *Epaltes divaricata* and *Vetiveria zizanioides* against Methicillin resistant *Staphylococcus aureus*," *Scientifica*, vol. 2020, Article ID 8239053, 8 pages, 2020.

[21] D. Dharajiya, P. Patel, M. Patel, and N. Moitra, "*In vitro* antimicrobial activity and qualitative phytochemical analysis of *Withania somnifera* (L.) *dunal* extracts," *International Journal of Pharmaceutical Sciences Review and Research*, vol. 27, no. 60, pp. 349–354, 2014.

[22] P. D. Gupta and T. J. Birdi, "Development of botanicals to combat antibiotic resistance," *Journal of Ayurveda and Integrative Medicine*, vol. 8, no. 4, pp. 266–275, 2017.

[23] G. Shrestha, J. Raphael, S. D. Leavitt, and L. L. St Clair, "In vitroevaluation of the antibacterial activity of extracts from 34 species of North American lichens," *Pharmaceutical Biology*, vol. 52, no. 10, pp. 1262–1266, 2014.

[24] P. R. Burkholder, A. W. Evans, I. Mcveigh, and H. K. Thornton, "Antibiotic activity of lichens," *Proceedings of the National Academy of Sciences*, vol. 30, no. 9, pp. 250–255, 1944.

[25] P. D. Gupta, P. G. Daswani, and T. J. Birdi, "Approaches in fostering quality parameters for medicinal botanicals in the Indian context," *Indian Journal of Pharmacology*, vol. 46, pp. 363–71, Article ID 363e71, 2014.

[26] M. Wink, M. L. Ashour, and M. Z. El-Readi, "Secondary metabolites from plants inhibiting ABC transporters and reversing resistance of cancer cells and microbes to cytotoxic and antimicrobial agents," *Frontiers in Microbiology*, vol. 3, Article ID 1e15, 2012.

[27] I. Francolini, P. Norris, A. Piozzi, G. Donelli, and P. Stoodley, "Usnic acid, a natural antimicrobial agent able to inhibit bacterial biofilm formation on polymer surfaces," *Antimicrobial Agents and Chemotherapy*, vol. 48, no. 11, pp. 4360–4365, 2004.

[28] M. Melgarejo, O. Sterner, J. V. Castro, and P. Mollinedo, "More investigations in potent activity and relationship structure of the lichen antibiotic (+)-usnic acid and its derivate dibenzoylusnic acid," *Revista Boliviana de Quimica*, vol. 25, no. 2, pp. 4–9, 2008.

[29] B. Paudel, H. D. Bhattarai, D. P. Pandey, J. S. Hur, S. G. Hong et al., "Antioxidant, antibacterial activity and brine shrimp toxicity test of some Mountainous lichens from Nepal," *Biological Research*, vol. 45, no. 3, pp. 87–91, 2012.

[30] M. Yilmaz, T. Tay, M. Kivanç, H. Türk, and A. O. Türk, "The antimicrobial activity of extracts of the lichen *Hypogymnia tubulosa* and its 3-hydroxyphysodic acid constituent," *Zeitschrift Fur Naturforschung. C, Journal of Biosciences*, vol. 60, no. 3, pp. 35–38, 2005.

[31] Z. Pilzk, A. J. Mycol, L. P. Timbreza, J. L. Reyes, C. Flores et al., "Antibacterial activities of the lichen *Ramalina* and *Usnea* collected from Mt. Banoi , Batangas and Dahilayan, Bukidnon, against multi-drug resistant (MDR) bacteria," *Austrian Journal of Mycolology*, vol. 26, pp. 27–42, 2017.

[32] M. Gulluce, A. Aslan, M. Sokmen et al., "Screening the antioxidant and antimicrobial properties of the lichens *Parmelia saxatilis, Platismatia glauca, Ramalina pollinaria, Ramalina polymorpha*, and *Umbilicaria nylanderiana*," *Phytomedicine*, vol. 13, no. 5, pp. 15–21, 2006.

[33] P. Srivastava, D. K. Upreti, T. N. Dhole, A. K. Srivastava, and M. T. Nayak, "Antimicrobial property of extracts of Indian lichen against human pathogenic bacteria," *Interdisciplinary Perspectives on Infectious Diseases*, vol. 2013, Article ID 709348, 6 pages, 2013.

[34] B. Paudel, H. D. Bhattarai, J. S. Lee, S. G. Hong, H. W. Shin, and J. H. Yim, "Antibacterial potential of antarctic lichens against human pathogenic Gram-positive bacteria," *Phytotherapy Research*, vol. 22, no. 9, pp. 1269–1271, 2008.

[35] S. Mohotti, S. Rajendran, T. Muhammad, A. A. Strömstedt, A. Adhikari et al., "Screening for bioactive secondary metabolites in Sri Lankan medicinal plants by micro fractionation and targeted isolation of antimicrobial flavonoids from *Derris scandens*," *Journal of Ethnopharmacology*, vol. 10, no. 246, pp. 112–158, 2020.

[36] G. Bourgeois, C. Suire, N. Vivas, and C. Vitry, "Atraric acid, a marker for epiphytic lichens in the wood used in cooperage: identification and quantification by GC/MS/(MS)," *Analusis*, vol. 27, no. 3, pp. 281–283, 1999.

[37] A. Torres, I. Dor, J. Rotem, M. Srebnik, and V. M. Dembitsky, "Characterization of surface n-alkanes and fatty acids of the epiphytic lichen Xanthoria parietina, its photobiont a green alga Trebouxia sp., and its mycobiont, from the Jerusalem hills," *European Journal of Biochemistry*, vol. 270, no. 10, pp. 2120–2125, 2003.

[38] Z. Zakeri, J. A. Elix, and V. Otte, "Degradation of alectorialic acid in the lichen genus *Usnea*," *The Lichenologist*, vol. 49, no. 5, pp. 539–543, 2017.

[39] A. T. Gomes, N. K. Honda, F. M. Roese, R. M. Muzzi, and M. R. Marques, "Bioactive derivatives obtained from lecanoric acid, a constituent of the lichen *Parmotrema tinctorum* (Nyl.) Hale (*Parmeliaceae*)," *Revista Brasileira de Farmacognosia*, vol. 12, pp. 74-75, 2002.

[40] N. T. Tuan, P. D. Nguyen, V. H. Mai, T. X. T. Dai, T. D. Luu et al., "Chemical constituents of the lichen *Parmotrema tinctorum* and their antifungal activity," *Chemistry of Natural Compounds*, vol. 56, pp. 1–3, 2020.

[41] A. T. Gomes, A. J. Smania, C. Seidel, E. F. A. Smania, N. K. Honda et al., "Antibacterial activity of orsellinates," *Brazilian Journal of Microbiology*, vol. 34, pp. 194–196, 2003.

[42] P. Z. Vasudeo and C. Lew, "Biopharmaceutical potential of lichens," *Pharmaceutical Biology*, vol. 50, pp. 778–798, 2011.

[43] B. Rankovic, M. Kosanic, N. Manojlovic, A. Rancic, and T. Stanojkovic, "Chemical composition of *Hypogymnia physodes* lichen and biological activities of some of its major metabolites," *Medicinal Chemistry Research*, vol. 23, pp. 408–416, 2014.

[44] V. P. Rajan, S. Gunasekaran, S. Ramanathan, V. Murugaiyah, M. Samsudin, and L. B. Din, "Biological activities of four *Parmotrema* species of Malaysian origin and their chemical constituents," *Journal of Applied Pharmaceutical Science*, vol. 6, pp. 36–43, 2016.

[45] P. Prateeksha, R. Palya, V. Bajpai et al., "The genus Usnea: a potent phytomedicine with multifarious ethnobotany, phytochemistry and pharmacology," *RSC Advances*, vol. 6, no. 26, pp. 21672–21696, 2016.

[46] S. Brijesh, P. G. Daswani, P. Tetali, N. H. Antia, and T. J. Birdi, "Studies on the antidiarrhoeal activity of *Aegle marmelos* unripe fruit," *BMC Complementary and Alternative Medicine*, vol. 9, no. 47, 2009.

[47] M. Kosanić and B. Ranković, "Lichen secondary metabolites as potential antibiotic agents," in *Lichen Secondary Metabolites Bioactive Properties and Pharmaceutical Potential*, B. Ranković, Ed., pp. 81–104, Springer International Publishing, Berlin, Germany, 2015.

[48] M. Hauck and S.-R. Jürgens, "Usnic acid controls the acidity tolerance of lichens," *Environmental Pollution*, vol. 156, no. 1, pp. 115–122, 2008.