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High-performance Liquid Chromatography Analysis and Antimicrobial Activities of Libyan *Cistus salviifolius* Extract

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: This research is focused on the in vitro evaluation of *Cistus salviifolius* L. antimicrobial properties and the determination of the contents of phenols and flavonoids.
Study Design: This research is analytical study aimed to illustrate the antimicrobial properties and to analyze the methanolic extract contents of aerial parts of *Cistus salviifolius* L. by high-performance liquid chromatography techniques.
Duration: The study was performed within six months in the Faculty of Pharmacy, Benghazy University.
Methodology: Antimicrobial properties was tested against twelve organisms using Kirby-Bauer disk diffusion sensitivity test and the determination of the contents of phenols and flavonoids was evaluated by running high-performance liquid chromatography techniques.
Results: The findings indicated that catechin is the most abundant flavonoid in *C. salviifolius*, while gallic acid was the major phenol in the methanolic extract of the plant. The results also revealed

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that the methanol extracts had a significant antimicrobial potential particularly against *Bacillus subtilis* and *Escherichia coli* with MIC (0.98 and 0.49) µg/ml respectively, furthermore the extract was effective against *Aspergillus fumigatus* with MIC 0.98 µg/ml.
Conclusion: *C. salviifolius* was highly rich with flavonoids and phenols and has a significant antimicrobial effect.

Keywords: C. salviifolius; antimicrobial; HPLC; flavonoids; phenols.

1. INTRODUCTION

For decades, medicinal herbs have been regularly considered as a valuable source for the screening of bioactive compounds to treat various medical conditions [1]. Most of the modern health care industries are mainly focused on these plants [2]. The World Health Organization (WHO) has conducted an investigation reporting that 20,000 kinds of medicinal florae around the world are being used in both traditional medicines and pharmaceutical preparations, but only 1.4% of the consumed plants have well-recognized active constituents [3]. Cistus is an evergreen genus of flowering shrubs that belongs to the Cistaceae, which comprises 8 genera and 180 species [4-6]. This family is known with the common name Rock-Rose [7], Cistaceae reveals the largest diversity in the floristic area of the Mediterranean [6]. Cistus covers about 30 indigenous species of this region [4], among which is *Cistus salviifolius* L. that is native to the Libyan flora. The shrub grows to 60 cm with ovate-elliptic leaves and white flowers, the flowering season begins in March and lasts to May [7, 8]. For centuries the value of Cistus herbs was documented, the leaves of numerous species contain a brown aromatic sticky resin called labdanum. This type of resin has been routinely used for the management of coughs, colds, rheumatism, diarrhea, and menstrual problems, it is also employed in the production of perfumes [9]. In addition, the extract of *C. salviifolius* leaves is also used as a substitute for tea [5] and has been utilized as a traditional cure for gout [10] and ulcers [11]. In Morocco, the anti-microbial properties of the herb have also been confirmed against *Mycobacterium smegmatis* and *Mycobacterium aurum* [12].

Patients in developing countries have restricted access to modern synthetic drugs due to the relatively high cost [12]. Moreover, the emergence of antibiotic-resistant bacteria has dramatically increased. Generally, many microorganisms have the genetic capabilities to evolve and transmit drug resistance [13]. Several virulent multi-drug resistant bacteria have been extensively documented as a common finding [14,15]. Thus, antimicrobial agents of plant origin have become a promising alternative [16, 17]. Recently, various researches have been conducted to test the *in-vitro* antimicrobial properties of different herbal extracts [18,19], which may involve stems, flowers, leaves, or roots [19,20].

Advanced work needs to be accomplished to assess the antimicrobial activities of plant materials of interest against the target microorganisms. Most of the studies regarding the antibacterial potential of herbs belonging to the Cistus genus have been carried out in the Middle Eastern and Mediterranean countries, with a large contribution from Morocco, [21] Spain, [22,23] Portugal, [20] France, [24] Greek, [25-27] and Turkey [28,29]. There are some available investigations concerning the antimicrobial properties of *Cistus salviifolius* L, which revealed a powerful effect against some clinically isolated bacteria such as *Pseudomonas aeruginosa* and *Escherichia coli* [30].

This research aims to characterize the antimicrobial properties and to determine the contents of essential active compounds, polyphenols, and flavonoids by running high-performance liquid chromatography techniques to analyze the methanolic extract of aerial parts of *Cistus salviifolius* L.

2. MATERIALS AND METHODS

2.1 Plant Preparation

Cistus salviifolius L. aerial parts were collected from the Botraba region; around one hundred kilometers east of Benghazi/Libya. A sample of the plant was kept in plastic bags and sent to the Department of Pharmacognosy (Faculty of Pharmacy, University of Benghazi) for identification. The aerial parts of the plant were left to dry in the open air. The dried herb was grounded using a blender and kept to be used for

extraction, chromatographic screening, and antimicrobial studies.

2.2 Extraction of the Plant Materials

About 50 gm of the powder was extracted with methanol 70% using soxhlet apparatus until complete exhaustion. The obtained extract was concentrated by removing the solvent under vacuum by a rotary evaporator. The residues left were weighed and kept in desiccators.

2.3 HPLC for Phenolics and Flavonoids

Phenolic and flavonoid compounds were identified using HPLC/UV technique according to the method of Mattila *et al*. [31]. Briefly, 5 gm of the dried herb was mixed with 62.5% aqueous methanol (40 ml) and centrifuged at 1000 rpm for 10 min; the formed supernatant was filtered through a 0.2 µm Millipore membrane filter. The filtrate was made up to 100 ml with methanol then 1 to 3 ml was collected in a vial for injection into a high-performance liquid chromatography system (Hewlett Packeard 1050) using a lichrosorb RP 18 column (4.0mm i.d.×250mm; particle size 5µm) (Merck, Dramastdt). Gradient separation was conducted using a mobile phase (acetonitrile and methanol 1:2) at a flow rate of one milliliter per minute. Standard flavonoids and phenolics were dissolved in the solvent system to be injected into the HPLC. Each component is determined by matching its retention time with the available authentic sample that is similarly analyzed.

2.4 The study of Antimicrobial Activity

The antimicrobial properties were evaluated using Kirby-Bauer disk diffusion sensitivity test protocol with modifications [32]. Some paper disks containing the methanolic extract of the plants (50 µl) were prepared and fixed on the surface of agar plates inoculated with the test bacteria or fungi. The same volume of DMSO was used as negative control while standard disks of ampicillin, gentamycin, and amphotericin β (antifungal agent) were used as the positive control. The plates were left inverted in the incubator at 37Cº for one day in case of bacteria and 25 Cº for two days in the case of fungi. After the process of incubation, the plates were observed to determine the zones of inhibition. Diameters less than 5 mm were recoded as no inhibition. The experiment was done in triplicate.

2.4.1 Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of an antimicrobial agent is defined as the lowest concentration capable of inhibiting the growth of microorganisms. The MIC test is a significant diagnostic tool for confirming microorganism resistance to an antimicrobial compound.

The broth micro-dilution method was used to determine the MIC. Each extract was serially diluted and mixed with broth media in a 96-well micro liter plate to obtain a final concentration range of 0.003 to 4%v/v. Following that, the plates were inoculated with a standardized suspension comprising 5 × 105 bacterial/fungal count per well. After the incubation period, the viability was assessed by measuring optical density at =600nm with a colorimeter [33].

2.4.2 Microorganisms used

A series of bacterial and fungal strains (Available in stock cultures at the Micro Analytical Center, Faculty of Science, Benghazi University) was used for susceptibility testing comprising Gram-positive bacteria; *Staphylococcus epidermidis* (RCMB010024), *Staphylococcus aureus* (RCMB010027), *Streptococcus pyogenes* (RCBM010015), and *Bacillus subtilis* (RCBM010067). Gram-negative bacteria; *Pseudomonas aeruginosa* (RCMB 010043), *Proteus vulgaris* (RCMB 010085), *Escherichia coli* (RCMB010056), and *Salmonella Typhimurium* (RCMB010315). Fungi; *Aspergillus niger* (RCMB02542), *Aspergillus funigatus* (RCMB02564), *Candida tropicalis* (RCMB 05084) and *Candida albicans* (RCMB05035).

2.5 Chemicals

All solvents and chemicals used were analytical grade and obtained from Sigma Aldrich (St. Louis, MO, USA).

3. RESULTS AND DISCUSSION

3.1 HPLC for Phenolics and Flavonoids

The present study was performed to assess the content of phenolic acids and flavonoids in the methanolic extract of the aerial parts of *C. salviifolius* L. using HPLC/UV according to the International Organization for Standardization by applying the method of Mattila *et al.* [34]. Currently, the HPLC technique is recognized as the most suitable method that facilitates the quantitative estimation of flavonoids, both the

retention times and UV spectra were used to identify the compounds. The majority of flavonoids were identified at 330 nm, while phenolic acids were detected at 280 nm.

The concentrations of the identified flavonoids and phenolics are shown in Tables (1 and 2). The chromatogram is illustrated in Figs. 1 and 2.

Table 1. Assessment and Identification of the major flavonoid constituents in the aerial parts of *Cistus salviifolius* L. using HPLC

No	Flavonoid	Flavonoid Conc. mg/100 g extract	R.T
1	Narengin	74.50	4.501
2	Rutin	199.12	8.1
3	Hesperidin	176.80	8.326
4	Quercetrin	152.45	9.045
5	unkown	3.63	9.467
6	Quercetin	17.26	10.238
7	Kaempferol	2.6	11.229
8	Hesperitin	3.2	11.932
9	catetchin	200.4	13.129
10	7-OH flavone	21.2	20.017

ppm= part per million

Table 2. Assessment and Identification of the major phenolic constituents in the aerial parts of *Cistus salviifolius* L. using HPLC

No	Phenolics	Phenolic Conc. mg/100 g extract	R.T
1	Cinnamic acid	84.11	7.377
2	Gallic acid	180.03	7.767
3	4-aminobenzoic acid	14.73	8.049
4	Protocatechuic acid	8.09	8.283
5	Catechol	10.11	8.654
6	Epicatecheine	23.35	9.547
7	p-hydoxy benzoic acid	19.82	10.087
8	Caffeic acid	11.25	10.784
9	Vanillic acid	5.53	11.273
10	Alph-Coumaric	95.9	11.631
11	Chlorogenic acid	91.39	12.324
12	P-Coumaric	99.20	12.564
13	Ferulic acid	149.59	14.662
14	Ellagic acid	7.36	14.827
15	E-vanillic acid	3.31	15.167
16	Benzoic acid	5.57	15.835
17	3,4,5-trimethoxy cinnamic	2.98	16.427
18	Salicylic acid	3.55	17.923
19	Ellagic acid	7.36	18.509

ppm= part per million

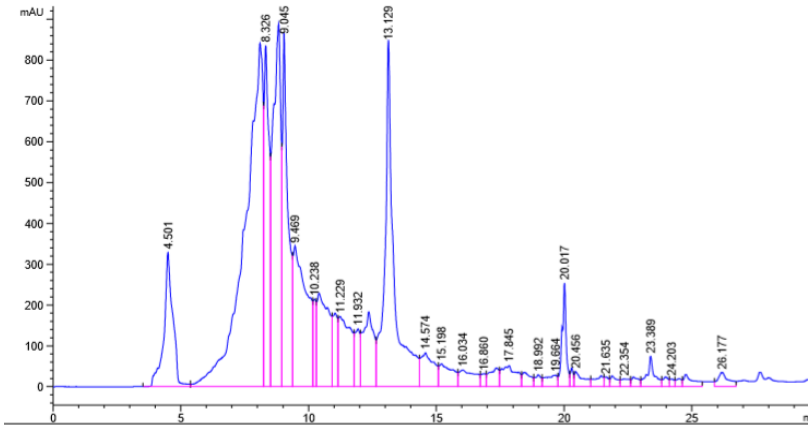

Fig. 1. HPLC chromatogram of 1) flavonoids in *Cistus salviifolius* L aerial parts

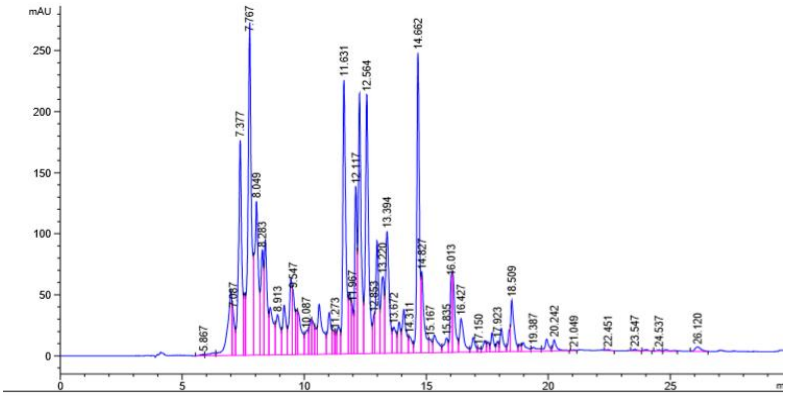

Fig. 2. HPLC chromatogram of Phenolics in *Cistus salviifolius* L aerial parts

Table 3. The antimicrobial activity of methanolic extract of the aerial part of *Cistus salviifolius* L

Tested microorganisms	Effect of *Cistus salvifolius*	MIC of extract	Effect of standard	MIC of standard (µg/ml)
Fungi			Amphotericin B	
Aspergillus fumigatus (RCMB02564)	22.2±1.2	0.98	23.7±0.63	0.49
Aspergillus niger (RCMB02542)	20.6±0.63	1.95	21.9±0.58	0.98
Candida albicans (RCMB05035)	18.3±1.2	7.81	26.4±0.72	0.49
Candida tropicalis (RCMB05084)	NA	NA	25.4±1.5	0.49
Gram Positive Bacteria			Ampicillin	
Staphylococcus aureus (RCMB010027)	16.3±1.5	15.63	28.9±1.2	0.24
Staphylococcus epidermidis (RCMB010024)	20.3±2.1	3.9	25.4±0.63	0.49
Streptococcus pyogenes (RCBM010015)	NA	NA	26.4±0.34	0.49
Bacillus subtilis (RCBM010067)	23.3±0.63	0.98	32.4±1.2	0.24
Gram Negative Bacteria			Gentamycin	
Proteus vulgaris (RCMB 010085)	NA	NA	23.4±0.58	0.49
Pseudomonas aeruginosa (RCMB 010043)	19.3±1.2	3.9	17.3±0.63	15.63
Salmonella Typhimurium (RCMB010315)	NA	NA	24.8±0.63	0.49
Escherichia coli (RCMB010056)	26.3±0.58	0.49	25.3±0.18	0.49

MIC= Minimum inhibitory concentration (µg/ml)

Various natural compounds of different molecular families derived from plants may provide a wide range of medicinal properties. Ethno-botanical evidence revealed that the herb designated in this work is utilized in several traditional treatments [10-12]. Few scientific investigations have provided results that support the medicinal value of *C. salviifolius* L. The evolutionary adaption of Cistus species to harsh habitats has relied heavily on effective secondary metabolites. Polyphenols, in particular, have been shown to efficiently protect plants from both abiotic and biotic environmental stress. [35-37].

The process of identification and quantification of polyphenols, predominantly flavonoids, in the genus Cistus have mostly targeted the exudates or the substances secreted from the outer compact covering of leaf trichomes [38-40]. As phenolic compounds (involving several flavonoids) comprise the phenolic hydroxyl groups, their extraction in a polar solvent such as methanol is reasonable. Ten types of flavonoids were identified and quantified in the obtained extract. Rutin and catechin were the major known flavonoids with concentrations of 199 and 200 mg/100g respectively. Quercetin and hesperidin were also found in considerable amounts.

The findings confirmed the presence of nineteen phenolic ingredients; the gallic and ferulic acids were the most abundant phenolic compounds in the methanolic extract of the aerial parts of Algerian *C. salviifolius* L with a concentration of 180 and 149mg/100g D.W respectively. While the least abundant phenolic was salicylic acid. All these results are supported by the study carried by Kada, *et al* [41]. According to the published paper, a mono-coumaroyl kaempferol glucoside, was found to be the most abundant flavonoid in *C. salviifolius* [42]. In another study, epigallocatechin derivatives were isolated from the air-dried herb of *C. salviifolius* [43].

3.2 Antimicrobial Activity

Because of undesirable adverse effects and the emergence antibiotic resistant pathogens, much attention has been recently directed to natural extracts and bioactive phytochemicals isolated from herbs species utilized in herbal medicine.

The results revealed that the methanolic extract of the aerial part of the studied plant has significant activity against gram-positive bacterial strains particularly *Staphylococcus epidermidis* and *Bacillus subtilis* while showed no effect against *Streptococcus pyogenes* and displayed a considerable action against Gram-negative bacteria; the strongest antibacterial effect were observed on *Escherichia coli.* On other hand, it did not exhibit any antimicrobial properties against *Proteus vulgaris* and *Salmonella Typhimurium.* Concerning antifungal activity, the extract was effective against *Aspergillus fumigatus*, *Aspergillus niger* and *Candida albicans* while show no effect against *Candida tropicalis*.

Previous studies also indicated that gram-positive bacteria are more sensitive to herbal extracts than gram-negative bacteria [44]. Bouamama *et al*. [21] reported that organic and aqueous extracts of *C. villosus* and *C. monspeliensis* differed clearly in their antimicrobial activities since *C. villosus* extracts exerted stronger activity than C. *monspeliensis* when tested on *Candida glabrata* (MIC 0.2 mg/ml) and *Staphylococcus aureus* (MIC 0.8 mg/ml). Güvenç *et al* [28], demonstrated that the water, methanol, chloroform, ethyl acetate, and butanol extracts of five Cistus species; *C. laurifolius*. L., C. *creticus*. L., *C. monspeliensis*. L. and *C. salviifolius. L.* have revealed at least some activity against *B. cereus* and *B. subtilis*. In another study, the lyophilized extracts of *C. salviifolius* L. exhibited the highest activity against *S. aureus* while butanol extracts of *C. creticus* leaves and fruits showed good inhibitory effect against *S. subtilis, B. subtilis, S. faecalis., B. cereus.,* and *E. coli*., whereas all extracts were not effective against *C. albicans* and *P. aeruginosa.* [45].

4. CONCLUSIONS

C. salviifolius obtained from Libya is highly rich in phenolic and flavonoid compounds, which are recognized by their antimicrobial activity. This research supports the idea that Cistus species can be a significant source of natural constituents that can be utilized in the pharmaceutical drug industry to manufacture antimicrobial products.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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