Original Research Article

High-performance liquid chromatography analysis and antimicrobial activities of Libyan *Cistus salviifolius* extract

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: analytical study
Duration: six months.
Methodology: Antimicrobial properties was performed 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.
Expected 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 that the methanol extracts had a significant antimicrobial potential particularly against *Staphylococcus aureus* and *Escherichia coli,* furthermore the extract was effective against *Aspergillus fumigatus*.
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 60cm with ovate-elliptic leaves and white flowers, the flowering

Comment [KC1]: salviifolius

Comment [KC2]: you have to summarize the plan of your study.

Comment [KC3]: you must give details of the place of study, the period and the university at which the studies were carried out.

Comment [KC4]: you must give details of the place of study, the period and the university at which the studies were carried out.

Comment [KC5]: you must give details of the place of study, the period and the university at which the studies were carried out

Comment [KC6]:

Comment [KC7]: you can set the result directly

Comment [KC8]: As you have worked on the antimicrobial activity, you should put the results of the minimum inhibitory concentrations.

Comment [KC9]: classify the references by number order.

Comment [KC10]: 60 cm

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 [16, 17]. Thus, antimicrobial agents of plant origin have become a promising alternative [14, 15]. 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 salvifolius* 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 salvifolius* L.

2. MATERIALS AND METHODS

Comment [KC11]: Material

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.

Comment [KC12]: put the plant's herbarium number.

2.2 Extraction of the plant materials

About 50gm 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.

Comment [KC13]: 50 g

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.* (2000)~~ [31]. Briefly, 5 g~~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.

Comment [KC14]: (31)

Comment [KC15]: put a space on the units.

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 37~~C°~~°C for one day in case of bacteria and 25~~C°~~°C 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.

2.4.1Determination 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

Comment [KC16]: Indicate the origin of the microorganisms.

Twelve organisms were grown to be employed in the antimicrobial assays; 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. salvifolius* L. using HPLC/UV according to the International Organization for Standardization by applying the method of Mattila *et al.* ~~(2000~~) [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 Fig. (1 and 2).

Table 1: Assessment and Identification of the major flavonoid constituents in the aerial parts of *Cistus salvifolius* 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 salvifolius* 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

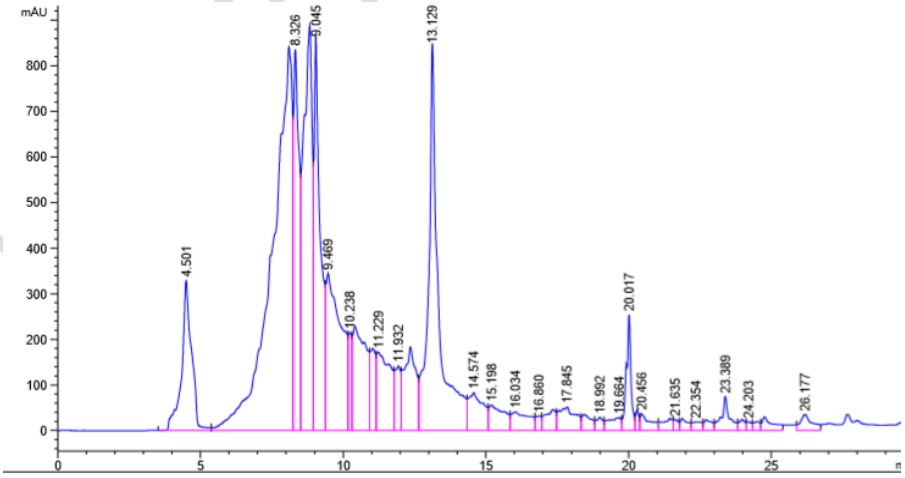

Fig1: HPLC chromatogram of 1) flavonoids in *Cistus salviifolius* L aerial parts

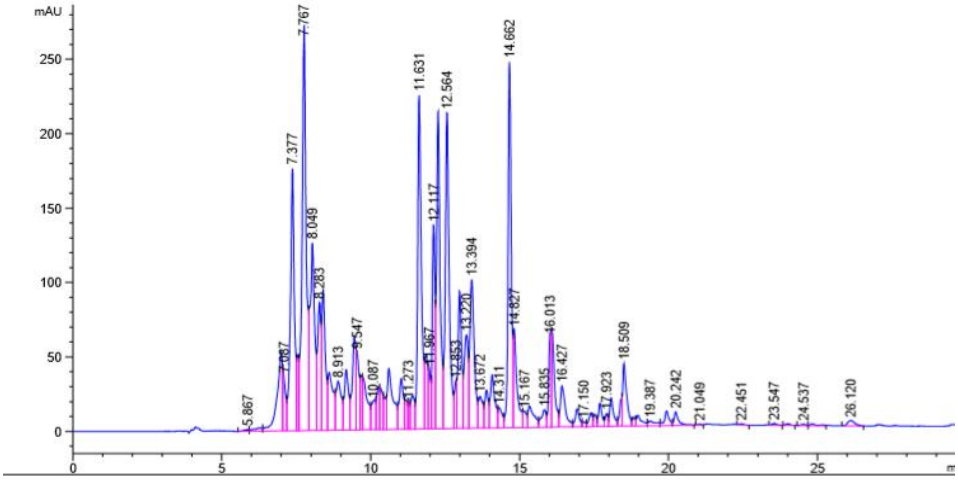

Fig2: HPLC chromatogram of Phenolics in *Cistus salviifolius* L aerial parts

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. salvifolius* 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 *C. salvifolius* 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* (~~2016~~) [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. salvifolius* [43].

3.2 Antimicrobial activity

Table 3: The antimicrobial activity of methanolic extract of the aerial part of *Cistus salvifolius* 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)

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, 45]. Bouamama *et al.* (~~2006~~) [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* (~~2005~~) [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. salvifolius* 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.* [46].

4. CONCLUSIONS

C. salvifolius 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.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. ~~The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.~~

Comment [KC17]: No need to be so explicit.

NOTE:

Comment [KC18]: you can delete this paragraph.

~~The study highlights the efficacy of "herbal medicine which is an ancient tradition, used in some parts of India. This ancient concept should be carefully evaluated in the light of modern medical science and can be utilized partially if found suitable.~~

Acknowledgment: …….

5. RFEREENCES

1. Burt, S. Essential oils: their antibacterial properties and potential applications in foods—a review. *International journal of food microbiology*, (2004). *94*(3), 223-253.

2. Sahraoui, R., Djellali, S., & Chaker, A. N. Morphological, anatomical, secondary metabolites investigation and physicochemical analysis of Cistus creticus. *Pharmacognosy Communications*, (2013). *3*(4), 58.

3. Kar, A. Pharmaocgnosy and Pharmacobiotechnology (Revised-Expanded Second Edition). *New Age International LimtedPublishres New Delhi*, (2007). 332-600.

4. Falchi, A., Paolini, J., Desjobert, J. M., Melis, A., Costa, J., & Varesi, L. Phylogeography of Cistus creticus L. on Corsica and Sardinia inferred by the TRNL-F and RPL32-TRNL sequences of cpDNA. *Molecular Phylogenetics and Evolution*, (2009). *52*(2), 538-543.

5. Madesis, P., Konstantinidou, E., Tsaftaris, A., & Nianiou-Obeidat, I. Micropropagation and shoot regeneration of Cistus creticus ssp. creticus. *Journal of Applied Pharmaceutical Science*, (2011). *1*(8), 54.

6. Guzmán, B., & Vargas, P. Historical biogeography and character evolution of Cistaceae (Malvales) based on analysis of plastid rbcL and trnL-trnF sequences. *Organisms Diversity & Evolution*, (2009). *9*(2), 83-99.

7. Post, G. E. Flora of Syria, Palestine and Sinai from the Taurus to Ras Muhammad, and from the Mediterranean Sea to the Syrian desert, (1896). 114-115.

8. Mouterde, P. Nouvelle flore du Liban et de la Syrie/2 Atlas. *Nouvelle flore du Liban et de la Syrie*., (1970). 449- 450.

9. Nicoletti, M., Toniolo, C., Venditti, A., Bruno, M., & Ben Jemia, M. Antioxidant activity and chemical composition of three Tunisian Cistus: Cistus monspeliensis Cistus villosus and Cistus libanotis. *Natural product research*, (2015). *29*(3), 223-230.

10. Al-Khalil, S. A survey of plants used in Jordanian traditional medicine. *International Journal of Pharmacognosy*, (1995). *33*(4), 317-323.

11. Yeşilada, E., Gürbüz, İ., & Shibata, H. Screening of Turkish anti-ulcerogenic folk remedies for anti-Helicobacter pylori activity. *Journal of Ethnopharmacology*, (1999). *66*(3), 289-293.

12. Haouat, A. C., Sqalli, H., Farah, A., Haggoud, A., & Iraqui, M. Activité antimycobactérienne des extraits de deux espècesmarocaines du genre Cistus. *Phytothérapie*, (2013). *11*(6), 365-372.

13. Shariff, Z. U. *Modern herbal therapy for common ailments*. Spectrum Books, (2001). 9-84.

14. Cohen, M. L. Epidemiology of drug resistance: implications for a post—antimicrobial era. *Science*, (1992). *257*(5073), 1050-1055.

15. Czechowska, K., McKeithen-Mead, S., Al Moussawi, K., & Kazmierczak, B. I. Cheating by type 3 secretion system-negative Pseudomonas aeruginosa during pulmonary infection. *Proceedings of the National Academy of Sciences*, (2014). *111*(21), 7801-7806.

Comment [KC19]: Classify the references by alphabetic order. I cant see Mattila et al 2000. Put the doi of this all reference article

16. Li Bassi, G., Rigol, M., Marti, J. D., Saucedo, L., Ranzani, O. T., Roca, I., ... & Torres, A. A novel porcine model of ventilator-associated pneumonia caused by oropharyngeal challenge with Pseudomonas aeruginosa. *Anesthesiology*, (2014). *120*(5), 1205-1215.

17. Ali, M. M. M., Ahmed, S. F., Klena, J. D., Mohamed, Z. K., Moussa, T. A., & Ghenghesh, K. S. Enteroaggregative Escherichia coli in diarrheic children in Egypt: molecular characterization and antimicrobial susceptibility. *The Journal of Infection in Developing Countries*, (2014). *8*(05), 589-596.

18. Smith, S., Wang, J., Fanning, S., & McMahon, B. J. Antimicrobial resistant bacteria in wild mammals and birds: a coincidence or cause for concern. *Irish Veterinary Journal*, (2014). *67*(1), 1-3.

19. Dib, M. A., Paolini, J., Bendahou, M., Varesi, L., Allali, H., Desjobert, J. M., ... & Costa, J. Chemical composition of fatty acid and unsaponifiable fractions of leaves, stems and roots of Arbutus unedo and in vitro antimicrobial activity of unsaponifiable extracts. *Natural product communications*, (2010). *5*(7), 1934578X1000500721.

20. Ferreira, S., Santos, J., Duarte, A., Duarte, A. P., Queiroz, J. A., & Domingues, F. C. Screening of antimicrobial activity of Cistus ladanifer and Arbutus unedo extracts. *Natural product research*, (2012). *26*(16), 1558-1560.

21. Bouamama, H., Noel, T., Villard, J., Benharref, A., & Jana, M. Antimicrobial activities of the leaf extracts of two Moroccan Cistus L. species. *Journal of ethnopharmacology*, (2006). *104*(1-2), 104-107.

22. Barrajón-Catalán, E., Fernández-Arroyo, S., Saura, D., Guillén, E., Fernández-Gutiérrez, A., Segura-Carretero, A., & Micol, V. Cistaceae aqueous extracts containing ellagitannins show antioxidant and antimicrobial capacity, and cytotoxic activity against human cancer cells. *Food and Chemical Toxicology*, (2010). *48*(8-9), 2273-2282.

23. Tomás-Menor, L.; Morales-Soto, A.; Barrajón-Catalán, E.; RoldánSegura, C.; Segura-Carretero, A.; Micol, V. Correlation Between the Antibacterial Activity and the Composition of Extracts Derived from Various Spanish Cistus Species. Food Chem. Toxicol. 2013, 55, 313–322. DOI: 10.1016/j.fct.2013. 01.006.

24. Guinoiseau, E., Luciani, A., de Rocca Serra, D., Quilichini, Y., Berti, L., & Lorenzi, V. Primary mode of action of Cistus ladaniferus L. essential oil active fractions on Staphylococcus aureus strain. *Advances in Microbiology*, (2015). *5*(13), 881-890.

25. Chinou, I., Demetzos, C., Harvala, C., Roussakis, C., & Verbist, J. F. Cytotoxic and antibacterial labdane-type diterpenes from the aerial parts of Cistus incanus subsp. creticus. *Planta medica*, (1994). *60*(01), 34-36.

26. Kalpoutzakis, E., Aligiannis, N., Mitaku, S., Chinou, I., Charvala, C., & Skaltsounis, A. L. New hemisynthetic manoyl oxide derivatives with antimicrobial activity. *Chemical and pharmaceutical bulletin*, (2001). *49*(7), 814-817.

27. Demetzos, C., Angelopoulou, D., & Perdetzoglou, D. A comparative study of the essential oils of Cistus salviifolius in several populations of Crete (Greece). *Biochemical Systematics and Ecology*, (2002). *30*(7), 651-665.

28. Güvenç, A., Yıldız, S., Özkan, A. M., Erdurak, C. S., Coşkun, M., Yılmaz, G., ... & Okada, Y. Antimicrobiological studies on turkish Cistus. species. *Pharmaceutical biology*, (2005). *43*(2), 178-183.

29. Ustün, O., Ozçelik, B., Akyön, Y., Abbasoglu, U., & Yesilada, E. Flavonoids with anti-Helicobacter pylori activity from Cistus laurifolius leaves. *Journal of Ethnopharmacology*, (2006). *108*(3), 457-461.

30. Abouzeed, Y. M., Elfahem, A., Zgheel, F., & Ahmed, M. O. Antibacterial in-vitro activities of selected medicinal plants against methicillin resistant Staphylococcus aureus from Libyan environment. *J Environ Anal Toxicol*, (2013). *3*(6), 1-10.

31. M Ezzat, S., S El-Hawary, S., El Shabrawy, A. E. R., & AA El-Shibani, F. Evaluation of the phenolic and flavonoid contents, antimicrobial and cytotoxic activities of some plants growing in Al Jabal Al-Akhdar in Libya, *International Journal of Pharmacognosy and Phytochemical Research*, (2016). 8(7); 1083-1087.

32. Hudzicki, J. Kirby-Bauer disk diffusion susceptibility test protocol, (2009)..

33. Wikler, M., A, Performance standards for antimicrobial susceptibility testing: Sixteenth informational supplement, Clinical and Laboratory Standards Institute. (2006).

34. Mattila, P., Astola, J., & Kumpulainen, J. Determination of flavonoids in plant material by HPLC with diode-array and electro-array detections. *Journal of Agricultural and Food Chemistry*, (2000). *48*(12), 5834-5841.

35. Dixon, R. A., & Paiva, N. L. Stress-induced phenylpropanoid metabolism. *The plant cell*, (1995). *7*(7), 1085.

36. Aerts, R. The advantages of being evergreen. *Trends in ecology & evolution*, (1995). *10*(10), 402-407.

37. Bassman, J. H. Ecosystem Consequences of Enhanced Solar Ultraviolet Radiation: Secondary Plant Metabolites as Mediators of Multiple Trophic Interactions in Terrestrial Plant Communities¶. *Photochemistry and photobiology*, (2004). *79*(5), 382-398.

38. Vogt, T., Proksch, P., & Gülz, P. G. Epicuticular flavonoid aglycones in the genus Cistus, Cistaceae. *Journal of plant physiology*, (1987). *131*(1-2), 25-36.

39. Chaves, N., Ríos, J. J., Gutierrez, C., Escudero, J. C., & Olías, J. M., Analysis of secreted flavonoids of Cistus ladanifer L. by high-performance liquid chromatography–particle beam mass spectrometry. *Journal of Chromatography A*, (1998). *799*(1-2), 111-115.

40. Chaves, N., Sosa, T., & Escudero, J. C., Plant growth inhibiting flavonoids in exudate of Cistus ladanifer and in associated soils. *Journal of Chemical Ecology*, (2001). *27*(3), 623-631.

41. Kada, S., Bouriche, H., Senator, A., & Gul, F., Phytochemical screening, antioxidant and antimicrobial activities of Algerian Cistus salvifolius extracts. *Advances in Environmental Biology*, (2016). *10*(1), 23-33.

42. Gürbüz, P., Koşar, M., Güvenalp, Z., Kuruüzüm Uz, A., & Demirezer, L. Ö., Simultaneous determination of selected flavonoids from different Cistus species by HPLC-PDA. *Marmara Pharmaceutical Journal*, (2018). 22(3): 405-410

43. Qa'dan, F., Nahrstedt, A., & Schmidt, M., Isolation of two new bioactive proanthocyanidins from Cistus salvifolius herb extract. *Die Pharmazie-An International Journal of Pharmaceutical Sciences*, (2011). *66*(6), 454-457.

44. Mahboubi, M., & Haghi, G., Antimicrobial activity and chemical composition of Mentha pulegium L. essential oil. *Journal of ethnopharmacology*, (2008). *119*(2), 325-327.

45. Boukhebti, H., Chaker, A. N., Belhadj, H., Sahli, F., Ramdhani, M., Laouer, H., & Harzallah, D., Chemical composition and antibacterial activity of Mentha pulegium L. and Mentha spicata L. essential oils. *Der Pharmacia Lettre*, (2011). *3*(4), 267-275.

46. Mahmoudi, H., Aouadhi, C., Kaddour, R., Gruber, M., Zargouni, H., Zaouali, W & Hosni, K., Comparison of antioxidant and antimicrobial activities of two cultivated Cistus species from Tunisia. *Bioscience Journal*, (2016). *32*(1).