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Biological properties and polyphenols content of Algerian *Cistus salviifolius* L. aerial parts

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ABSTRACT: This study evaluated the *in vitro* antioxidant properties, antibacterial and antifungal activities and *in vivo* anti-inflammatory properties, and identifying the phenolic compounds in *Cistus salviifolius*. The methanolic leaf extract showed the highest antioxidant activity with 6.1±1.60 µg/ml IC_{50} value using DPPH• and 55.5±0.20 µg/ml using Reducing Power Activity. The study revealed that the butanolic leaf extract and the aqueous leaf infusion exhibited the strongest growth-inhibiting effect against all Gram positive and Gram negative strains tested, respectively, whereas the methanolic leaf extract showed the strongest antifungal activity against the yeast tested. The MIC value for the butanolic leaf extract was 4 mg/ml against *Staphylococcus aureus, Bacillus subtilis* and *Escherichia coli*. The pharmacotoxicological tests proved the safety of the aqueous leaf infusion, which exhibit a moderate anti-inflammatory effect, with a significant inhibition of the oedema development equal to 44.7% compared to 59.3% for the reference product diclofenac sodium. Methanolic extracts of the leaf and flower buds showed varied contents of polyphenols, flavonoids, and hydrolysable tannins; which were 228.4±11.4 mg GAE/g, 34.2±0.6 mg QE/g, and 36.9±2.6 mg TAE/g of the dry weight for leaves; and 241.1±5.4 mg GAE/g, 47.6±4.5 mg QE/g, and 22.0±1.3 mg TAE/g of the dry weight for flower buds, respectively. Analysis of the ethereal and butanolic leaf extracts using Reversed Phase

High Performance Liquid Chromatographic Method coupled with a Photodiode-Array Detector identified thirteen phenolic compounds, including ascorbic acid, vanillic acid, gallic acid, quercetin, and orientin.

Keywords: Antioxidant activity; *Cistus salviifolius*; Phytochemistry; Total phenol content; Biological activity.

1. INTRODUCTION

Cistaceae, family of *Cistus* and *Helianthemum*, is an intermediate family of shrubs and sub-shrubs, more rarely herbaceous, and xerophytes that thrive in the temperate and warm regions of the Northern hemisphere; it is generally heliophilic [1, 2], and is composed of about nine genera, with no less than 170 species [3]. *Cistus* is a genus of flowering plants belonging to the Cistaceae family, and comprise of about 20 species [4]. *Cistus* is native to the Mediterranean region [5], and found across forests, scrub, dry hillsides, and rocky siliceous dry land; it is quite common in the Hill and coast. In summer, *Cistus*, which is partly quite dry, constitutes hazard as some species contain resinous substances that are highly flammable. This contributes to the rapid spread of forest fires [6]. *Cistus* often germinate first after forest fires, playing an important role in secondary successional dynamics, allowing other plants to germinate after fires [7]. *Cistus salviifolius*, known as Sage-leaved Rock-rose, blooms in sheltered places on the coast and is easily recognised by its beautiful white flowers with an orange heart, with smooth petals that are widely spread among the embossed leaves [6]. *C. salviifolius* is a xerophilous species, a thermophilous shrub that prefers sunny places with calcareous or nutrient-poor soils. The plant is cultivated as an ornamental plant, and is an important feed source for cattle. It is visited by bees, especially for pollination. The evolutionary adaptation of *Cistus salviifolius* to extreme environmental conditions mainly depends on the efficiency of its secondary metabolite, including polyphenols. In fact, it has often been reported that polyphenols effectively protect plants from environmental stresses of abiotic and biotic origin. It should be noted that polyphenols could also reduce nitrogen mobility in soils and thus play a major role in the adaptation of this shrub to slow growth on infertile soils [8-10]. The phenolic compounds in *C. salviifolius* differ from other *Cistus* species [5]. The polyphenol composition of the leaves makes this shrub a potential source of secondary metabolite that can be exploited for human health. It may also explain its distribution in the infertile soils of the Mediterranean region [8, 11].

In the genus *Cistus*, the occurrence of polyphenols, including flavonoids, is mainly based on the trichome secretion of the leaves. A study on the epicuticular resins from leaves of 16 species and three subspecies of the genus *Cistus* showed the presence of about 51 different flavonoids (including flavanones, flavones, flavonols) and two coumarin derivatives [12]. The flavonoid aglycones identified in the *Cistus* oozing liquid were also found on the soil. This supports *Cistus* allelopathy reported in dome studies [13]. *C.s salviifolius* reduces the mobility of metals by absorbing through the root system, and allowing their storage without exceeding phytotoxic concentrations [14].

Cistus salviifolius has been used as a traditional remedy, mostly due to its medicinal properties for the treatment of rheumatism, inflammatory diseases, and intestinal pain [15]. It has also been used as an analgesic and an expectorant in bronchitis, as well as an antimicrobial and antidiarrheal [16]. The importance of *Cistus* species was among the main motivations for this study.

This study aimed to provide chemical and biological knowledge of *Cistus salviifolius*, which is commonly found in Algeria, to support its uses as a natural resource for various purposes, including its use in food, pharmaceutical, and medical industries. Also, this study analyses their leaf extracts in detail, as most of the active substances are found in it.

2. MATERIALS AND METHODS

2.1. Chemicals and solvents

All chemicals used were analytical grade reagents, purchased from either Prolabo, Sigma-Aldrich or Fluka. Methanol and acetonitrile used for HPLC analysis were labelled as HPLC grade.

2.2. Plant material

The aerial part of *Cistus salviifolius* (see Figure 1), identified by researchers from the national institute of forest research of Algiers (INRF), was harvested in June 2019, in Bainem's forest (province of Algiers). This site located in Bouzareah's massif and occupying an area of about 500 hectares, is a real breath of fresh air for the capital.

Characteristics of the site are summarized in Figure 2. The freshly harvested plant material was dried in the open air at an ambient temperature of 20-25 °C for not less than 3 weeks.

Figure 1. Aerial part (leaves and flowers) of *Cistus salviifolius.*

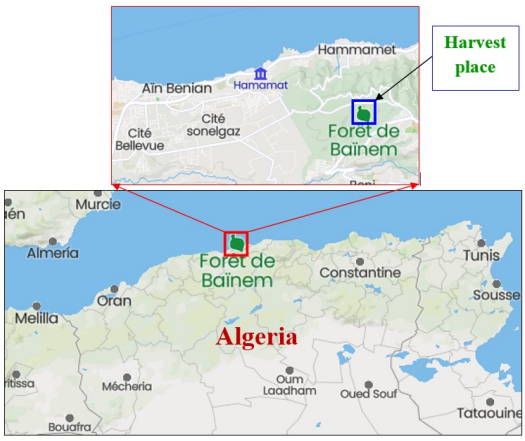

Figure 2. The plant of *Cistus salviifolius* harvesting region.

2.3. Animal material

Male Swiss albino mice for both acute toxicity and anti-inflammatory studies, weighing (20-25g), were obtained from the Research and Development Centre (RDC SAIDAL) Algiers (Algeria). Mice were maintained on a 12h light/dark cycle with a temperature of 22°C±3°C and about 50% relative humidity. Water and food (pellets from the National Office of Animal Nutrition) were provided ad libitum for the whole period of the experiment [17].

2.4. Microbial strains

Five ATCC Strains were tested, including *Staphylococcus aureus* (6538) and *Bacillus subtilis* (6633) (as Gram$^+$ bacteria), *Pseudomonas aeruginosa* (9027) and *Escherichia coli* (8739) (as Gram$^-$ bacteria), and *Candida albicans* (10231) (as yeast). The microbial strains and the culture media (Mueller-Hinton Agar for bacterial strains, Mueller-Hinton Agar supplemented with 2% glucose and 500 mg/l methylene blue for fungal strains) were obtained from the Research and Development Centre (RDC SAIDAL), Algiers (Algeria). The culture media were kept at 37°C for 48 hours before use.

2.5. Foreign matter determination

About 100 g of the plant material were spread out in a thin layer, and then examined by eye to look for foreign matter. The foreign matter was separated and weighed to calculate the percentage.

2.6. Moisture content evaluation

This test was done to know the moisture content of the plant, to help control the moisture content of the dry plant, in order to avoid any harmful effects due to excess humidity, which leads to microbial fermentation of the foliage and the development of moulds or yeasts. Since *Cistus salviifolius* contains more than 1% essential oil [18], the moisture content evaluation was carried out with toluene distillation method using Dean Stark apparatus, with some modifications. The dried plant material (20 g) was finely ground and placed in the flask. About 70 ml of toluene were added, and heating turned on. The heating was switched off when all the water was distilled. As soon as the water and toluene had completely separated, we measured the volume of water.

2.7. Analysis of ash

The total ash value is useful in determining the quality and purity of a drug as it provides information on the inorganic material; ash is also important in human nutrition [19]. Approximately 1 g of the finely ground plant material was placed in a platinum crucible and ignited in a muffle furnace at 600°C for 4 hours. The residue was then weighed.

2.8. Plant extracts

2.8.1. Aqueous Infusions

Exactly 20 g of crushed plant materials, such as leaves, flower buds, and bark, were left to infuse separately in 100 ml of boiling water for 30 minutes. The infusions were then filtered to be used for phytochemical tests.

2.8.2. Methanolic extracts

Exactly 20 g of the crushed plant materials (leaves and flower buds) were subjected to continuous hot extraction separately in a Soxhlet extractor, using 250 ml of methanol as solvent [20, 21]. Filtrates were then concentrated by distilling off the solvent under vacuum at 40°C temperature using rotary evaporator. The dry extracts were dispersed in methanol and stored in a refrigerator until subsequent use.

2.8.3. Ethereal leaf extract

Exactly 4 g of the crushed plant materials (leaves and flower), treated with 320 ml 2N HCl ere heated in 40°C water bath for 40 minutes. The filtrate was extracted by (3×50 ml) diethyl ether to obtain phenolic

acids and flavonoid aglycones extract [20, 22]. Combined dry extracts were dispersed in methanol and stored in a refrigerator until subsequent use.

2.8.4. Butanolic leaf extract

Exactly 4 g of the crushed leaves were macerated with 400 ml 70° alcohol solution for 48 hours. The filtrate, concentrated under vacuum at 40°C using rotary evaporator, was taken up by 100 ml boiling water, then extracted by 100 ml n-butanol to obtain flavonoid glycosides extract [23]. We used a rotary evaporator at 40°C temperature to obtain dry extract, which was dispersed in methanol and stored in a refrigerator until subsequent use.

2.9. Preliminary phytochemical screening

The qualitative identification tests were performed using the finely ground dried aerial parts of the plant materials separately, such as leaves, flower buds, and bark. They were subjected to aqueous infusions to identify the chemical constituents of the different parts of the plant [24, 25].

2.9.1. Test for flavonoids

The aqueous infusion was treated with magnesium foil and concentrated HCl. An orange red colour indicated the presence of flavonoids [17, 26].

2.9.2. Test for tannins

The aqueous infusion was treated with a few drops of 5% $FeCl_3$ solution. A bluish-black colour indicated the presence of tannins [24].

2.9.3. Test for condensed tannins (catechol-type tannins)

The aqueous infusion was treated with 7 ml of Stiasny reagent (formol/concentrated HCl, 2V/1V), and then heated to boiling in a water bath for 15 min. Precipitate formation indicated the presence of condensed tannins [24].

2.9.4. Test for hydrolysable tannins (gallic tannins)

The aqueous infusion saturated with sodium acetate was treated with a few drops of 5% $FeCl_3$ solution. A blue-black colour indicated the presence of gallic tannins [24].

2.9.5. Test for anthocyanins

The aqueous infusion was treated with concentrated HCl. A pinkish-red coloration that changes to purplish-blue with the addition of ammonia indicated the presence of anthocyanins [23].

2.9.6. Test for saponins (foam test)

Exactly 10 ml of the aqueous infusion was shaken for a few minutes. Froth formation that persists for 60-120 seconds indicated the presence of saponins [19].

2.9.7. Test for alkaloids

Samples (5 g each) of the crushed plant material were macerated in a 50 ml mixture (ether/chloroform), (3/1; v/v) for 24 hours. The filtrate was acidified with 2N HCl and treated with Dragendroff's reagent (solution of potassium bismuth iodide). The formation of red precipitate indicated the presence of alkaloids [19, 26].

2.9.8. Test for quinones

Samples (2 g each) of the crushed plant materials were acidified with 30 ml 2N sulfuric acid and heated under a reflux condenser for 1 hour. The filtrate was treated with 20 ml chloroform and then evaporated to dryness, and 5 ml of ammonia added thereafter. A red coloration indicated the presence of quinones [25].

2.9.9. Test for coumarins

Exactly 2 g of the crushed plant materials were treated with 20 ml alcohol and heated under a reflux condenser for 15 minutes. A few drops of 2% alcoholic $FeCl_3$ solution were added to the filtrate. Deep green colour, which turned yellow on addition of concentrated HNO_3, indicated presence of coumarins [19].

2.10. Quantitative determination of phytochemicals

2.10.1. Quantitative estimation of total phenolic compounds

This test was performed using the Folin-Ciocalteu reagent. Polyphenols present in the plant extract reduce this reagent in blue tungsten oxide and molybdenum, which have an absorption maximum at 760 nm, and whose intensity is proportional to the amount of polyphenols present in the plant extract. Aliquots of each methanolic leaf and flower buds' extracts (300 µl) were well mixed with 1500 µl water-diluted Folin-Ciolcateu reagent (1/10) and incubated for 5 minutes before adding 1200 µl of 75 g/l Na_2CO_3. After agitation and incubation for 90 min at room temperature, absorbance was measured at 760 nm against blank sample, using a PerkinElmer LAMBDA 25 ultraviolet spectrophotometer [28]. Gallic acid was used to estimate the calibration curve. Samples were analyzed in triplicate. Total polyphenol content was expressed as milligrams of Gallic Acid Equivalent per Gram of dry weight (mg GAE/g of dry weight).

2.10.2. Quantitative estimation of total flavonoid compounds

The test for total flavonoid was done using aluminum chloride colorimetric method, based on the formation of a stable complex between aluminum chloride and oxygen atoms present on the carbons 4 and 5 of the flavonoids [29]. Aliquots of each methanolic leaf and flower buds extracts (1000 µl) were thoroughly mixed with an equal volume of 20 g/l $AlCl_3$ methanolic solution. After 15 minutes incubation at room temperature, absorbance was measured at 430 nm against blank sample [30]. Quercetin was used to estimate the calibration curve. Samples were analyzed in triplicate. The total flavonoid content was expressed as milligrams of Quercetin Equivalent per Gram of dry weight (mg QE/g of dry weight).

2.10.3. Quantitative estimation of total hydrolysable tannins compounds

The test for total hydrolysable tannins was done using potassium iodate colorimetric method. Hydrolysable tannins react with KIO_3 to develop a stable red coloration, with maximum absorbance between 500 nm and 550 nm [31]. Aliquots of each methanolic leaf and flower buds' extracts (1 ml each) were thoroughly mixed with 5ml of 25 g/l KIO_3 aqueous solution. The time required for a stable red color to appear differs from one plant material to another; it took 2 hours incubation at room temperature for color stabilization; absorbance was then measured at 550 nm against the blank sample [32]. Tannic acid was used to estimate the calibration curve. Samples were analyzed in triplicate. The total hydrolysable tannin was expressed as milligrams of Tannic Acid Equivalent per gram of dry weight (mg TAE/g of dry weight).

2.11. Determination of antioxidant activity

2.11.1. DPPH Scavenging Activity

The antioxidant activity was determined using DPPH• Radical Scavenging Ability. The stable, purple free radical DPPH• is reduced by antioxidant molecules to pale yellow hydrazine. The antioxidant capacity was then evaluated by following the absorbance decrease at 517 nm until constant value is obtained [33]. Samples (100 µl each) of the extracts, including methanolic, ethereal, and butanolic leaf and flower extracts, at different concentrations were added to 2 ml of freshly prepared 40 mg/l methanolic DPPH• solution. After 30 minutes incubation at room temperature, absorbance was measured at 517 nm against blank sample [34]. Ascorbic acid, BHT and BHA were used as positive control. Samples were analyzed in triplicate. Radical Scavenging Capacity (RSC) was calculated as percent inhibition (PI) using the following equation (1):

$$PI\ (\%) = [(A_{blank} - A_{sample}) / A_{blank}] \times 100 \quad (1)$$

Where A_{blank} is the absorbance value of the control reaction without extract and A_{sample} is the absorbance value of the sample.

The calibration curves, obtained by plotting the PI (%) versus concentration of the various extracts and that of ascorbic acid, allowed us to assess the half maximal inhibitory concentration (IC_{50}), the concentration required to scavenge 50% of the DPPH free radicals.

2.11.2. Reducing Power Ability Scavenging Activity

Put 1 ml of each extract of different concentrations (10-500 µg/ml) were mixed with 2.5 ml of a phosphate buffer solution (0.2 M, pH 6.6) and 2.5 ml of a solution of 1% of potassium ferricyanide $K_3Fe(CN)_6$. The whole is incubated at 50°C for 20 min, then cooled to room temperature. 2.5 ml of 10% trichloroacetic acid was added to stop the reaction, and then the tubes are centrifuged at 3000 rpm for 10 min. To a 2.5 ml aliquot of supernatant was added 2.5 ml of distilled water and 0.5 ml of an aqueous solution of 0.1% iron chloride ($FeCl_3$). Absorbances are read against a blank at 700 nm using a UV-Visible spectrophotometer. The positive control is represented by standard antioxidant solutions; (ascorbic acid BHT and BHA), at the same concentrations and under the same operating conditions as the samples. The increase in antioxidant activity corresponds to a high $Fe3^+$ reduction capacity. This results in an increase in absorbance at 700 nm [35]

2.12. High-performance liquid chromatography (HPLC) analysis of polyphenols

The identification was carried out by HPLC system. This comprises WATERS e2695 pump equipped with an auto-sampler, in-line degasser, column oven, LC 2998 diode array detector, and connected to EMPOWER 3 software for data acquisition. Separation was carried out using a 5 µm Hypersil GOLD column (250×4.6 mm; Thermo Scientific) [36, 37], kept at 25°C [38]. Two linear gradients were used; the first one to identify both phenolic acids and flavonoid aglycones (flavones and flavonols), and the second for identification of flavonoid glycosides (flavone and flavonol glycosides, and flavanone) [22]. The eluents used as mobile phase were 2% acetic acid AcOH solvent (A), methanol MeOH solvent (B), and acetonitrile ACN/water H_2O/acetic acid AcOH:78/20/2 solvent (C). The linear gradient regarding phenolic acids and flavonoid aglycones, using solvent (A) and (B), was as follows: 0-30 min, 5-70% B; 30-40 min, 70% B; 40-44 min, 70-75% B; 44-50 min, 5% B. For the identification of flavonoid glycosides, linear gradient using solvent (A) and (C) was as follows: 0-20 min, 12-17% C; 20-30 min, 17-22% C; 30-45 min, 22-37% C; 45-60 min, 37-40% C; 60-65 min, 40-70% C; 65-68 min 70%C, 68-73min 70-72% C; 73-83 min 12% C. For the re-

equilibration of the linear gradients, the initial conditions were recovered in 5 min before ending the run [39]. The mobile phase was filtered through HA filters (0.45 µm). A flow rate was set at 0.8 ml/min [38]. Analytes were monitored by DAD from 200 to 400 nm [36], and detection was performed at 260 nm, 365 nm, and 380 nm, the wavelengths used for the identification of phenolic acids, flavonoid aglycones, and flavonoid glycosides respectively [37, 38]. Prior to analysis, ethereal and butanolic leaf extracts, along with the standard solutions were filtered through a 0.45 µm PTFE membrane filter. The sample injection volume was 10 µl. The dentification was carried out by comparing retention times and photodiode array detection (DAD) spectra of the eluted compounds with those obtained with standards [40].

2.13. Evaluation of antimicrobial activity

2.13.1. Agar discs diffusion method

Antimicrobial activities of aqueous leaf infusion as well as methanolic, butanolic, and ethereal leaf extracts were carried out using agar discs diffusion method, which requires measuring the diameter of inhibition zone around the disc [41, 42]. Saline suspensions of isolated colonies selected from 24 hours agar plate for bacteria and 48 hours agar plate for yeast were made, and then adjusted to standardize the inoculum density in order to get an absorbance of 0.08 to 0.13 at 625 nm, which corresponds to 0.5 McFarland turbidity. Final inoculum size obtained was (1 to 2)$\times 10^8$ CFU/mL for bacteria and (1 to 5)$\times$ 10^6 CFU/mL for yeasts. Suspensions were used within 10 minutes [43]. About 9 mm diameter sterile filters papers discs containing 10 µl of each extract, were placed on the agar surface previously inoculated with the standardized inoculum of the target microorganism. The Petri dishes were then incubated during 18 hours at 37°C for bacteria and 24 hours at 37°C for yeasts [41]. Inhibition zones were than measured.

2.13.2. Agar dilution method

MIC determination of the butanolic leaf extract was carried out by incorporating variable concentrations of the antimicrobial agent to agar medium, followed by inoculation of the target microbial inoculum on the agar plate surface [41]. To perform tests, a series of agar medium were prepared to whom various extract's concentrations ranging from 0,06 mg/ml to 4 mg/ml were added. They were then inoculated with 2 µl of the target microorganism adjusted to 10^4 CFU/ml within 15 minutes of preparation. After incubation for 18 hours at 37°C, MIC, the lowest concentration of antimicrobial agent that completely inhibits growth was determined.

2.14. Acute oral toxicity test

Acute oral toxicity occurs if adverse effects are observed after a single or multiple oral dose of the substance given over a 24-hours period. It was carried out using OECD-423 guidelines [44]. Three groups of five mice each were prepared. They fasted 16 hours prior to dosing with free access to water and then weighed. Each group 1, 2 and 3 received orally a single dose of 275 mg/kg; 1562 mg/kg and 2555 mg/kg respectively of the aqueous leaf infusion using a stainless steel cannula, then food was withheld for an additional 1 to 2 hours. Mice were observed daily for 14 days for behavioural changes, toxic reactions, and mortality.

2.15. Evaluation of anti-inflammatory activity

Anti-inflammatory activity of the aqueous leaf infusion was carried out in vivo, using the carrageenan-induced paw oedema method with slight modifications [17, 45]. Three groups of six mice each

were prepared. They fasted 16 hours prior to test with free access to water and then weighed. For each mice, the initial diameter (D_0) of the left rear paw was measured using a Mitutoyo digital caliper 150 mm stainless steel. First group, the negative control received orally 0.5 ml of physiological water at 9‰. Second group, the positive control received orally 0.5 ml of diclofenac sodium at a dose of 10 mg/kg body weight. The third group, the test control received orally aqueous leaf infusion at a dose of 2000 mg/kg body weight. Thirty minutes later, a volume of 0.025 ml of the 1% carrageenan suspension in physiological water at 9‰ was injected to all mice under the plantar aponeurosis of the left rear paw. The diameters of the injected paws were measured hourly (D_t) for 6 hours after carrageenan injection. The Oedema thickness percentage was calculated using the following equation (2):

$$\% \text{ Oedema thickness} = [(Dt - D0) / D0] \times 100 \quad (2)$$

The Anti-inflammatory activity: evaluated as percent inhibition of oedema (% IO), was than calculated using the following equation (3):

$$\% \text{ IO} = [(\% \text{ Oedema thickness}_{\text{negative control}} - \% \text{ Oedema thickness}_{\text{treated}}) / \% \text{ Oedema thickness}_{\text{negative control}}] \times 100 \quad (3)$$

Where % IO: oedema thickness $_{\text{negative control}}$ is the oedema thickness percentage of the negative control, and % oedema thickness $_{\text{treated}}$ is the oedema thickness percentage of either positive or test control.

3. RESULTS

3.1. Foreign matter

As plant material must be free of any visible signs of contamination, we first proceeded with the determination of foreign matter. It was carried out by visual inspection, resulting in an evaluation of no more than 2.3%. This result is in line with the report of Mignacca et al. [46]. Foreign matter is any form of external contaminant introduced to foods or food products at any point in their production/distribution.

3.2. Moisture content

We also ensured that the plant dried properly, because water can cause polyphenols degradation resulting from Oxidation, and can also interfere with the determination of the extraction yield. The moisture content was 8.9%.

3.3. Analysis of ash

Total ash determination allowed us to evaluate the amount of inorganic matter, resulting from the plant tissue (such as inorganic salts), as well as any elements which may adhere to the plant. The result obtained for total ash content was 6.5%, thus allowing us to rule on the purity of the plant. This result agrees with the result reported by Lisiecka et al. [47], which was 7.70 g/100 g for *Cistus incanus* species.

3.4. Plant extracts

Ensuring the best extraction of phenolic compounds by minimizing losses and alteration of their chemical structure is mainly based on the selection of extraction conditions. Extraction yield depend also on the solvent polarity therefore, the highest yields are generally obtained using ethanol and methanol. The Soxhlet extraction was more efficient in terms of yield, with 19.1% and 22.3% for methanolic leaf and methanolic flower buds extracts, respectively. El Euch et al. [48], published similar results, with the yields obtained being 21.80% and 30.20% for methanolic leaf and methanolic flower buds extracts, respectively [48]. The results of the extraction yields are presented in Table 1.

Table 1. Extraction yields.

Extracts	Yields
EMCL	19.1%
EMCF	22.3%
CE	1.7%
CEFB	1.2%
CB	6.6%
CBFB	4.9%

EMCL: Methanolic leaf extract, EMCF: Methanolic flower buds extract, CE: Ethereal leaf extract, CB: Butanolic leaf extract, CEFB: Ethereal flower buds extract, CB: Butanolic flower buds extract.

3.5. Preliminary phytochemical screening

The results of the qualitative phytochemical analysis of *Cistus salviifolius* showed on the one hand, the plant's richness in flavonoids and tannins, and on the other hand, alkaloids were not detected. The leaves had higher levels of condensed tannins, hydrolysable tannins, anthocyanins, and coumarins than the flower buds or bark; while the flower buds had the highest content of saponins. We also noticed a low concentration of quinones in the leaves and bark of the plant. Table 2 shows the results of the qualitative phytochemical screening of different aerial parts of *C. salviifolius*. These results are in agreement with those obtained these literatures [49-51]; relative to the richness of the plant in flavonoids, condensed tannins, and hydrolysable tannins in *C. salviiflius* leaves.

Table 2. Phytochemical screening of *Cistus salviifolius.*

Tests	Leaves	Flower buds	Stem bark
Flavonoids	+++	+++	++
Tannins	+++	+++	+++
Condensed tannins	++	+	+
Hydrolysable tannins	+++	++	++
Anthocyanins	+++	+	+
Saponins	++	+++	+
Alkaloids	-	-	-
Quinones	+	-	+
Coumarins	++	+	+

(+++): Abundant, (++): Modest presence, (+): Low presence, (-): Absence.

3.6. Quantitative determination of phytochemicals

Despite the fact that spectrophotometric methods may not allow the separation of phenolic compounds or the individual determination of their content, they provide valuable information on their quantification. Methanolic extracts of the leaf and flower buds were chosen for the quantitative determination of phytochemicals compounds, viz total polyphenols, total flavonoids, and total hydrolysable tannins as they showed the best extraction yields. The results are presented in Table 3.

First, it should be noted that the highest levels of total polyphenols and flavonoids were those of the methanolic flower buds extract, while the leaf extract was richer in hydrolysable tannins. For methanolic leaf extract, the result of the total polyphenol content was similar very to the results reported by El Euch et al. [48], but lower than the results reported by Sayah et al. [52]; and Rebaya et al. [15]; and significantly higher than that of Mahmoudi et al. [53].

Table 3. Quantitative determination of phytochemicals.

Extracts	Total polyphenols (GAE)*	Total flavonoids (QE)*	Total hydrolysable tannins (TAE)*
EMCL	228.4 ± 11.4	34.2 ± 0.6	36.9 ± 2.6
EMCF	241.1 ± 5.4	47.6 ± 4.5	22.0 ± 1.3

GAE: Gallic acid equivalent, QE: Quercetin equivalent, TAE: Tannic acid equivalent. *mg/g of dry weight. EMCL: Methanolic leaf extract, EMCF: Methanolic flower buds extract. Values are expressed as means ±Standard Deviation (SD), n=3.

Regarding total flavonoid content, it was much higher than those obtained by Mahmoudi et al. [53]; and lower than the contents obtained by El Euch et al. [48]; Sayah et al. [54]; and Rebaya et al. [15]. The contents of total polyphenols and total flavonoids of methanolic flower buds extract were less than the results reported by El Euch et al. [48] and Rebaya et al. [15]. Numerical results obtained by the different authors are shown in Table 4.

Table 4. Overview of the results obtained by the different authors.

Extracts	Harvest site	Total polyphenols*	Total flavonoids*	IC_{50} (µg/ml)		Authors
				DPPH	Reducing Power Ability	
Methanolic leaf extract	Nahli mount, Tunisia	286.99 ± 2.96[a]	65.58 ± 0.86[b]	6.48 ± 0.19	77.35 ± 0.32	[48]
	Setif region, Algeria	414.71 ± 0.01[a]	14.13 ± 0.03[b]	6.79	5.29	[15]
	Maâmoura Forest, Morocco	336.51 ± 1.22[a]	188.66 ± 2.90[c]	3.30 ± 0.25	-	[52]
Ethanolic leaf extract	Sidi Mechreg, Tunisia	536.2 ± 0.38[a]	278.4 ± 0.02[d]	3.52	-	[15]
	Ghardimaou, Tunisia	49.98 ± 3.39[a]	7.00 ± 1,80[d]	0.13 ± 0.04	-	[53]
n-butanol leaf extract	Sidi Mechreg, Tunisia	-	-	20.74	-	[15]
Methanolic flower buds extract	Nahli mount, Tunisia	305.30 ± 4.68[a]	76.21 ± 1.26[b]	5.11 ± 0.53	59.27 ± 0.13	[48]
Ethanolic flower buds extract	Sidi Mechreg, Tunisia	382 ± 0.17[a]	264 ± 0.35[d]	11.79	-	[15]
n-butanol flower buds extract	Sidi Mechreg, Tunisia	-	-	29.16	-	[15]

[a] Gallic Acid Equivalent (GAE), [b] Quercetin Equivalent (QE), [c] Rutin Equivalent (RE), [d] Catechin Equivalent (CE), *mg/g of dry weight extract.

3.7. Antioxidant Activity

Antioxidant activity was estimated by comparing half maximal inhibitory concentration (IC_{50}) of methanolic, ethereal, and butanolic leaf extracts of *Cistus salviifolius* to that of ascorbic acid. The smallest value of IC_{50} corresponds to the most important antioxidant activity. The results expressed as IC_{50} are shown in Table 5.

These results allowed us to classify, in decreasing order, the extracts according to their antioxidant power compared to positive control, ascorbic acid: EMCL > CE > CB. In fact, with the lowest IC_{50}, 7.4±1.20 µg/ml, methanolic leaf extract has an antioxidant activity which results in an excellent antiradical effect but remains less effective than that of ascorbic acid, BHA and BHT.

Similar results were also reported by El Euch et al. [48]; regarding methanolic leaf extract, with an IC_{50} corresponding to 6.48±0.19 and 6.79 µg/ml respectively, while Sayah et al. [52]; Rebaya et al. [15] and Mahmoudi et al. [53], reported a much lower IC_{50} corresponding to 3.30±0.25, 3.52 and 0.13±0.04 µg/ml respectively. For butanolic leaf extract, the result was in agreement with the one obtained by Rebaya et al.

[15] corresponding to 20.74 µg/ml. Numerical results obtained by the different authors are summarised in Table 4. The differences and similarities in these results could be due to the slight differences in methodology and/or extraction conditions.

Table 5. Antioxidant activity performed using DPPH• and Reducing Power Ability.

Extracts	DPPH•	Reducing Power Ability
	IC_{50} (µg/ml)	IC_{50} (µg/ml)
EMCL	7.4±1.2	61.3±0.3
EMCF	6.1±1.6	55.5±0.2
CE	12.5±2.4	68.0±0.1
CB	20.5±3.0	81.9±0.3
CEFB	61.7±4.3	104.2±0.4
CBFB	12.6±0.3	70.5±0.2
Positive control - ascorbic acid	3.7±1.9	33.2±0.1
BHT	0.035±0.001	15.6±0.1
BHA	0.14±0.001	19.2±0.5

EMCL: Methanolic Leaf Extract, EMCF: Methanolic flower buds extract, CE: Ethereal Leaf Extract, CB; Butanolic Leaf Extract, CEFB: Ethereal flower buds extract, CB: Butanolic flower buds extract. Values are expressed as means ± (SD), n=3.

3.8. High-performance liquid chromatography analysis of polyphenols

HPLC with UV-Vis detection is a valuable technology used in the separation of phenolic compounds over the last four decades [37], and remains a widely used technique at present [40]. Analysis of ethereal and butanolic leaf extracts by means of Reversed Phase High-Performance Liquid Chromatography (RP-HPLC) revealed the richness of the extracts in flavonoids. Gradient elution is the method used mostly in highlighting the chromatographic profiles complexity of phenolic compounds in plants [37].

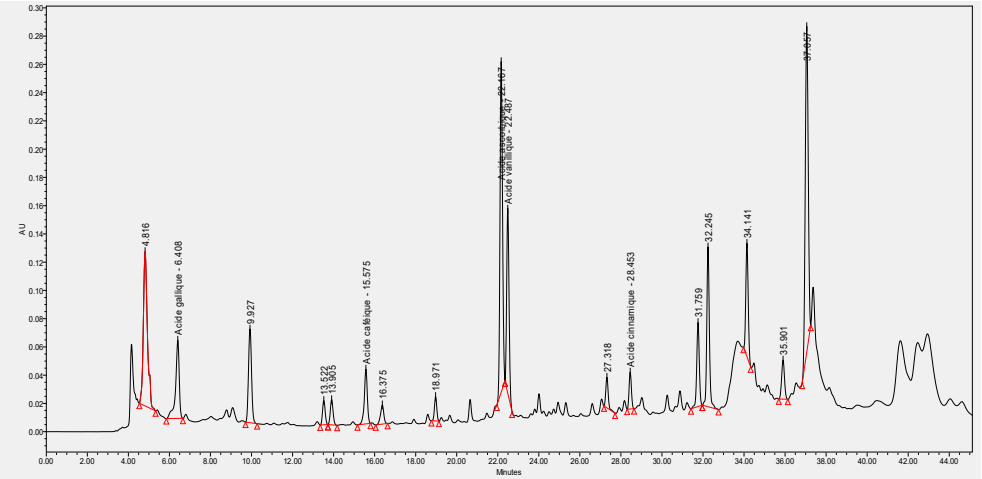

Figure 3. HPLC chromatogram of the ethereal leaf extract detected at λ=260 nm.

HPLC chromatograms showed thirteen phenolic compounds were present; including five phenolic acids (gallic, caffeic, ascorbic, vanillic and cinnamic acids) (see Figure 3), three flavonols (myricetin, quercetin, and kaempferol), a flavone, luteolin (see Figure 4), two flavone glycosides (orientin and luteolin-7-O-glucoside), a flavonol glycosides, quercetin-3-β-D-glucoside, and a flavanone, hesperidin (see Figure 5).

These results were in agreement with those obtained by Barrajón-Catalán et al. [55]; Gürbüz et al. [56]; regarding gallic acid, quercetin glucoside, cinnamic acid, and myricetin [55, 56].

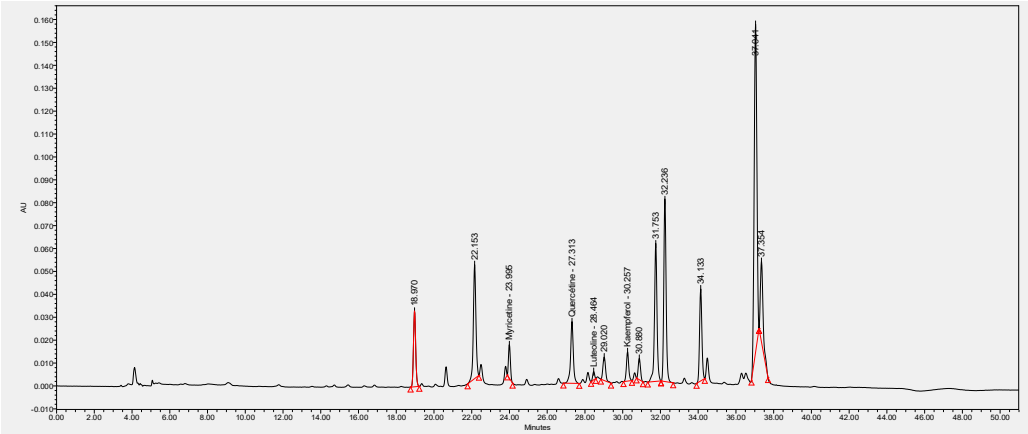

Figure 4. HPLC chromatogram of the ethereal leaf extract detected at λ=365 nm.

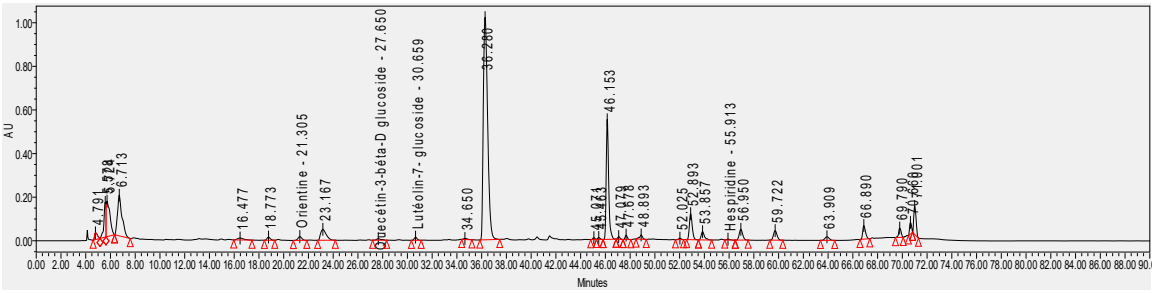

Figure 5. HPLC chromatogram of the butanolic leaf extract detected at λ=380 nm.

3.9. Antimicrobial activity

The results of the antimicrobial analysis of the aqueous leaf infusion, methanolic, butanolic and ethereal leaf extracts are reported in the Table 6.

Table 6. Results of antimicrobial activity.

Bacteria strains	Inhibition zone (mm)			
	ALI	**EMCL**	**CE**	**CB**
Staphylococcus aureus	21.4 ± 0.05	21.3 ± 0.09	18.2 ± 0.05	25.3 ± 0.09
Bacillus subtilis	9.0 ± 0.03	16.1 ± 0.05	15.1 ± 0.03	21.0 ± 0.1
Pseudomonas aeruginosa	23.1 ± 0.05	9.0 ± 0.03	12.1 ± 0.05	19.1 ± 0.09
Escherichia coli	27.1 ± 0.05	13.0 ± 0.05	14.1 ± 0.05	24.6 ± 0.2
Candida albicans	*	16.1 ± 0.03	9.0 ± 0.03	12.3 ± 0.03

ALI: Aqueous leaf infusion, *EMCL:* Methanolic leaf extract, CE: Ethereal leaf extract, CB: Butanolic leaf extract. Values are expressed as means ± (SD), n=3. *Not study.

According to the results obtained, *Staphylococcus aureus* strain for the Gram^{+} bacteria and *Escherichia coli* strain for the Gram^{-} bacteria, proved to be the most sensitive to all the extracts studied. With an inhibition zone ranging from 12 to 25 mm, Butanolic leaf extract showed an antimicrobial activity against all the microbial strains tested. These results were much higher than those reported by Güvenç et al. [16]; for

Staphylococcus aureus, *Bacillus subtilis,* and *Escherichia coli*. Also, Güvenç et al. [16], reported that the butanolic leaf extract was not effective against *Pseudomonas aeruginosa* and *Candida albicans*. Aqueous leaf infusion showed the strongest activity against all $Gram^-$ tested with an inhibition zone between 23 and 27 mm. Güvenç et al. [16], also reported that the aqueous extract was not effective against *P. aeruginosa* and *E. coli.* For $Gram^+$ bacteria, the results obtained were similar to those reported by Güvenç et al., for aqueous extract [16], The results obtained for the methanolic leaf extract were in accordance with those obtained by Mahmoudi et al. [53]; which revealed similar activity of ethanolic extract against *S. aureus, P. aeruginosa, E. coli and C. albicans*. On the other hand, Güvenç, et al. [16]; reported that methanolic extract had no activity against *S. aureus, B. subtilis, P. aeruginosa, E. coli* and *C. albicans*. For aqueous leaf infusion, methanolic and ethereal leaf extracts, we will note that no activity against *B. subtilis, P. aeruginosa* and *C. albicans* respectively, was observed. Numerical results obtained by the different authors are resumed in Table 7. Since butanolic leaf extract was the most sensitive against all the microbial strains tested, it was chosen for MIC determination. MIC value carried out by agar dilution method was equal to 4 mg/ml against *S. aureus, B. subtilis* and *E. coli* strains. This antimicrobial effect can be attributed to flavonoids [57].

Table 7. Overview of the results obtained by the different authors relating to the antimicrobial activity.

Extracts	Inhibition zone (mm)					Authors
	Staphylococcus aureus	***Bacillus subtilis***	***Pseudomonas aeruginosa***	***Escherichia coli***	***Candida albicans***	
Aqueous extract	15	10	-	-	-	[16]
Methanolic extract	9.5	-	-	-	-	[16]
Ethanolic extract	17.5 ± 0.5	*	10.0 ± 0.5	18.0 ± 0.5	17.0 ± 0.5	[53]
Butanolic extract	15	10	-	11	-	[16]

*Not study.

3.10. Acute oral toxicity

During the 14 days, after oral administration of the aqueous leaf infusion at different level doses (275 mg/kg; 1562 mg/kg and 2555 mg/kg), mice were observed daily. No visible toxicity signs or behavioural changes such as mice's body weight loss, hyperactivity, irritability, or mortality were seen; which may explain the consumption of infusions of most cistus specie's of in Turkey [16]. Therefore, the approximate median lethal oral dose (LD_{50}) of *Cistus salviifolius* aqueous leaf infusion was estimated to be higher than 2555 mg/kg.

3.11. Anti-inflammatory activity

Inflammation is characterized by classic symptoms, such as heat, redness, swelling, and pain. The measurement of oedema is therefore an excellent tool to estimate skin inflammation, induced by inflammation-inducing agents such as carrageenan. Measuring the diameter of the mice, left rear paws hourly for six hours, allowed us to estimate the percentage increase in oedema thickness as a function of time. Results are summarized in Table 8.

An increase in oedema thickness percentage of all groups were noted from the first hour of the experiment. It reaches after one hour, a maximum of 84.8% for the negative control, and 80.0% for the test control while for the positive control, the maximum is reached in the first hour with 72.3%, and therefore we should notice that diclofenac sodium administration at a dose of 10 mg/kg prevented a greater evolution of oedema. Results of percentage inhibition of oedema calculation are summarized in Table 9.

Table 8. Evolution of oedema thickness percentage over time (hours).

Time	1h	2h	3h	4h	5h	6h
Negative control	92.5±1.0	84.8±1.0	76.6±0.9	68.4±0.6	57.2±0.6	47.4±0.12
Positive control	72.3±0.7	56.4±0.2	38.1±0.7	33.3±0.4	26.5±0.9	19.3±0.8
Test control	79.2±0.9	80.0±0.6	46.6±0.2	41.2±0.3	32.9±0.6	26.2±0.3

Values are expressed as means ± (SD), n=6.

Table 9. Evolution of percent inhibition of oedema over time (hours).

Time	1h	2h	3h	4h	5h	6h
Positive control	12.3	33.5	50.3	51.3	53.7	59.3
Test control	4.0	5.6	39.2	39.8	42.5	44.7

First, diclofenac sodium at a dose of 10 mg/kg body weight, does not only act from the first hour, but also has the most important inhibition after 6 hours. On the other hand, the aqueous leaf infusion of *Cistus salviifolius* at a dose of 2000 mg/kg body weight does not begin to be active until the third hour, and even if it is slightly lower than that of diclofenac sodium, it remains of value. This anti-inflammatory activity is mainly due to the richness of the plant in phenolic compounds [57]. These results are in line with Sayah et al. [52], findings; regarding the inhibition power of *Cistus salviifolius* aqueous extract [52].

4. CONCLUSION

The results obtained in our study were very encouraging and showed that *Cistus salviifolius* is rich in phenolic compounds, with promising antioxidant, antimicrobial and anti-inflammatory properties; therefore, we recommended its use in traditional and modern medicine. The findings open up new horizons for its use as a potential source of natural antioxidant and antimicrobial agent that can be exploited for medical, food, pharmaceutical, and cosmetic applications. However, work is ongoing on other harvesting sites and in other areas, in order to assess the great potential of this plant by highlighting the relationship between phenolic compounds and biological activities. More studies are strongly recommended to establish the clinical significance of this plant in relation to the properties studied in this study

Authors' Contributions: SB, SIM and AH conceived and designed the experiment. MM, SB and AkR studied and analyzed the data. SB helped sample preparation and data collection. MM and SB wrote the manuscript. CGA, OAO and BS performed the proof reading and final editing. All authors read and approved the final manuscript.

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