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Comparative Study of Antibacterial Activity of *Cistus ladanifer* L. Leaves Extracted by Ultrasound-Assisted Extraction and Maceration

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Abstract: Addressing infectious diseases poses a significant healthcare challenge due to the growing resistance of microorganisms to antibiotics. Consequently, extensive research efforts have been dedicated to identifying alternative molecules to antibiotics that can combat these infectious diseases. Notably, a wealth of bioactive compounds has been discovered in medicinal and aromatic plants, and their potential utility in the development of treatments for infectious diseases has been well-established. In this study, we employed two extraction methods, namely maceration and ultrasound-assisted extraction (UAE), and four different solvents with varying polarities to isolate bioactive compounds from *Cistus ladanifer* L. Subsequently, quantification of polyphenols was determined in all the extracted samples. Following this, we conducted both qualitative and quantitative assessments of the antibacterial activity of these extracts. Moreover, we delved into the mechanism of action of the most potent extract using scanning electron microscopy. The results revealed that methanol was the best solvent for extracting bioactive molecules and that UAE had a high extraction efficiency since it gave a higher yield of total polyphenols than the one acquired by maceration. Regarding the results of the disk diffusion assay, the extracts that were prepared by UAE were more effective against our bacterial strains than those extracted by maceration. Electron micrographs of damaged cells revealed that the methanolic extract obtained by UAE

affected the integrity of the cell membrane. These outcomes have underscored the presence of active compounds within *Cistus ladanifer* L. that could serve as potential alternatives to antibiotics against some of the most pathogenic bacterial species.

Keywords: *Cistus ladanifer*; antimicrobial activities; ultrasound-assisted extraction; minimal inhibitory concentration; scanning electron microscopy

1. Introduction

The prevalence of bacterial pathogens has contributed to a myriad of conditions, spanning gastrointestinal issues, respiratory disorders [1], and skin and soft tissue infections [2, 3], prompting a significant transformation in the approach to infectious diseases with the discovery of antimicrobial molecules. However, the indiscriminate handling of these molecules, marked by heightened usage, improper administration, and the resulting side effects, has significantly fueled the rise in bacterial resistance [4-7]. This creates a serious dilemma, rendering once-effective antimicrobial agents ineffective [8] and leading to the resurgence of infectious diseases once considered under control [1]. In the pursuit of effective alternatives, researchers are actively exploring a diverse range of sources, such as plants, animals [9], and microorganisms [10-16], to uncover bioactive molecules. This exploration is particularly focused on studying molecules of plant origin because, throughout history, humans have harnessed the phytotherapeutic properties of plants to address various health conditions [17-19]. Notably, these plants play a crucial role as therapeutic components in traditional medicine [20], finding frequent application in cases of gastrointestinal [21] and respiratory disorders as well as in addressing skin infections.

Molecules originating from plants have been the subject of research in many scientific works which were concerned with their extraction, identification, quantification, and evaluation of their in-question biological activities [22-24]. Several recent studies have demonstrated the significant potential effects of plant extracts [25] antibacterial [26-30], antifungal, insecticidal, cytotoxic [31] and antioxidant [32] either in the industrial [33] or the medical field [18, 20]. Similarly, due to their pharmacological properties that promote the cessation or regression of the cancer process, polyphenols are seen as elements of cancer prevention [34-37].

Taza's region, which is located in the northeast of Morocco, is made up of up to 42.5% (468,000 ha) of forests [38], an aspect that renders it a region of high biological diversity within our country [39]. Its flora is frequently used in traditional medicine by the local population [40].

In this context, the present work serves to highlight the antimicrobial activities of *Cistus ladanifer* L., a species of the Cistaceae family of the local flora of Taza. *Cistus ladanifer* L. is also known as "Touzal" or "Argale" in the north of Morocco and other regions [41], respectively, produces labdane, a resin that serves as a fragrance to make leathery notes and amber, and also as a natural fixative [42, 43]. Though it is an indigenous aromatic plant of

the region, *Cistus ladanifer* L. is widespread in the Mediterranean region [44]. The native communities in the north of Morocco use it as a treatment for diarrhoea and diabetes, and also as an antiacid and a spasmolytic through the decoction of its aerial segment [45, 46]. Many studies in the literature demonstrated that extracts of *Cistus ladanifer* L. have many biological activities: antifungal activity of the phenolic extract against several *Candida* species [47], the aqueous and organic extracts was an antibacterial activity against Gram-positive bacteria [48, 49], antioxidant activity [18, 49], cytotoxic activity against many types of human cancer cells [31, 49], allelopathic effect [50, 51], antihypertensive effect of the aqueous extract [52] and hypoglycemic activity [53].

Maceration, hydrodistillation, and Soxhlet extraction are some of the methods that are used to extract phenolic compounds from plants. Nevertheless, because they need a lot of time, a high temperature and solvents' usage [54], these techniques can cause the degradation of phenolic molecules, leading to their utilization inefficient. On the other side, new techniques have been developed for extracting polyphenols, such as microwave, enzyme, and ultrasound-assisted extraction (UAE), which have demonstrated, compared to the firsts mentioned techniques, no degrading effect, higher efficiency, and simplicity in their usages for they necessitate less processing time, lower temperatures, lower energy input, and lower organic solvent consumption [55, 56].

The aims of this study were to investigate the influence of UAE and maceration extractions on bioactive compounds and, subsequently, to evaluate the antimicrobial activities of the different organic and aqueous extracts of *Cistus ladanifer* L. species from the region of Taza. For this purpose, the disk diffusion method and TTC assay were used to investigate the antimicrobial activity against five selected pathogenic bacterial strains. Moreover, scanning electron microscopy was employed to further understand the mechanism of action of the extracts, which have shown a bactericidal effect on the bacterial strains used.

2. Materials and Methods

2.1. Plant material

During the flowering phase of April 2021, *Cistus ladanifer's* aerial segments were gathered from Taza, a city in Northern Morocco (004° 52.607′ N, 004°01.190′ W and 34°09.825′ N, 004°09.850′ W). Their identification was realized by Pr. Khabbach Abdelmajid, a botanist in the LRNE (Laboratory of Natural Resources and Environment) in Polydisciplinary Faculty of Taza, Sidi Mohamed Ben Abdellah University. Then, at room temperature, plants were dried in the shade to constant weight and grounded in an electric mill to get a powder that could pass a 0.5 mm sifter.

2.2. Preparation of plant extracts

Plant powder (20 g/100 mL) of the aerial part was extracted by several solvents of increasing polarity, starting from n-Hexane (100%), then ethyl acetate (100%), methanol (100%), and up to water. Two techniques were used for the extraction: maceration with

stirring from time to time for 72 hours at room temperature and sonication using an Ultrasonication Assisted Extraction bath for 60 minutes at 25 °C. The crude extract was filtered using Whatman No. 1 paper, and then the obtained filtrate was concentrated with a rotary vacuum evaporator to get the organic extract and eliminate the solvent. The aqueous extract was lyophilized and left in the dark at +4°C until tested.

2.3.Total polyphenols estimation

The determination of polyphenols was carried out with the Folin-Ciocalteu reagent according to the method of Tan et al. [57] with few modifications. Briefly, 500 µl of each extract dissolved in methanol was added to 2.5 ml of Folin-Ciocalteu reagent (diluted ten times in methanol). Afterwards, the whole mixture was incubated in the water bath at 45 ◦C for 30 min after adding 4 mL of sodium carbonate (Na_2CO_3) (7.5%). The absorbance was measured at 765 nm by a UV-Visible spectrophotometer (Specuvisi UV/VIS Spectrophotometer, No RE1701008). Tests were performed in triplicate. The total polyphenol contents of each sample were calculated using a gallic acid calibration range from the regression equation ($Y = 0.0049X + 0.0085$; $R^2 = 0.995$). Results were expressed as mg gallic acid equivalent to per g of extract (mg GAE/g E) [58].

2.4. Antimicrobial activity

2.4.1 Bacterial Strains, Origin, and Growth Conditions

Both Gram-positive (*Staphylococcus aureus* CECT 976, *Listeria innocua* CECT 4030, *Bacillus subtilis* DSM 6633) and Gram-negative (*Escherichia coli* K12, *Proteus mirabilis,* and *Pseudomonas aeruginosa* CECT 118,) foodborne pathogenic bacteria were selected to test the antimicrobial activity of the extracts. They were provided by the Laboratory of Biology and Health (Faculty of Sciences, Tetouan).

According to the protocol of Benali et al. [59], bacterial culture was conducted: strains were cultivated for 24h at 37°C in Mueller-Hinton agar (MHA). The inoculum test concentration for our strains was fixed at 10^6 CFU/mL.

2.4.2 Antimicrobial Activity

The disk diffusion method was used to evaluate the antibacterial activity as described by Benali et al. [59] and Kelen and Tepe [60]with minor modifications. Briefly, Whatman paper discs (6 mm diameter) were placed in previously inoculated agar plates, and then 20 µL (50 mg/mL) of each extract was put in the discs. Moreover, Gentamicin (15 µg) was used as a positive control and for the negative control, the 10% DMSO was used since it was employed in the solubilization of the extracts. The plates were afterwards incubated for 24 hours at 37 °C, to allow the diffusion of our extracts. The plates were kept for 2 hours at room temperature. The diameter of the zone of inhibition that surrounded the disc reflected the capacity of the extract to inhibit the growth of the tested bacteria. Tests were performed in triplicate.

2.4.3. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The active extracts were tested using the microdilution technique to determine the Minimum inhibitory concentrations (MICs) according to the protocol of Gulluce et al. [61] with minor adjustments. Briefly, 100 µL of Mueller–Hinton broth (MHB) was distributed in all wells of the sterile 96-well microplate except for the first well of every line. In this last, the stock solution, which was previously prepared by dissolving the extract with 10% DMSO in MHB, was deposited with a final concentration of 50 mg/mL. Then, an intake of 100 µL of the solution was transferred in each forward well by taking it from its previous in a gradual scale of concentration from the first well (50 mg/mL) to the ninth (0.019 mg/mL). Subsequently, 10 µL of the bacterial suspension (10^6 CFU/mL) was added after the removal of 10 µL from the medium of each well. The tenth well, containing neither the extract solution nor the microorganisms, was considered a sterile medium. The eleventh well served as a positive control to the microbial growth, while the twelfth one, containing 10% DMSO/MHB plus the bacteria to be tested, acted as a negative control to ensure that DMSO had no effect against our bacteria.

After incubation at 37 ºC and for 24 hours, the plates were re-incubated for 2 to 4 hours at 37°C after the addition of 20 uL (5 mg/mL) of an indicator of bacterial growth, Triphényl tétrazolium Chloride (TTC), to each well. TTC has the particularity of remaining colourless where there is no growth of bacteria. The lowest concentration of the extract, which inhibited the visible growth of microorganisms corresponded to the MIC.

To determine the minimum bactericidal concentration (MBC), 20 µL of broth from the uncoloured well (corresponding to the well with no visible growth) was inoculated in MHA, and the dishes were incubated for 24h at 37°C. The smallest concentration of extract that was in the well from which the broth yielded less than 3 colonies when inoculated in MHA corresponded to the MBC. Tests were performed in triplicate.

2.4.4. Scanning Electron Microscopy (SEM)

The morphology of the cells treated with the extract which had a bactericidal effect, was visualized by scanning electron microscopy (SEM) to understand its mechanism of action as reported in the literature [62-64]. Only bacterial strains that were sensitive to the extracts were subject to this test. For this, after adjustment to McFarland 1 standard, the cells from the overnight cultures were treated with the extract, which was the most efficient at the corresponding MIC values that were determined previously. Subsequently, the cells were centrifuged at 7000 rpm for 15 min at 4° C after the incubation time. A sterile solution of potassium nitrate was used to wash the bacterial pellet twice; this was resuspended afterwards in the same solution. On a microscope slide, 20 µL of each suspension was spread and left to dry in the air. For the negative control, cultures of our bacteria without treatment with the extracts were employed. Finally, a microscopic examination with the SEM (ISM-IT500HR) was carried out after the samples were deposited on the conductive and adhesive stainless-steel slides and after they were covered with a layer of gold under vacuum [62-64].

2.5. Statistical analysis

The results of the tests were explored by means of statistical analysis after they were repeated three times. XLSTAT Version 2016.02.28451 was used to determine the mean and the standard error.

3. Results

3.1. Total polyphenols estimation

The test of Fisher's Least Significant Difference (LSD) presents a significant difference in polyphenols' concentration depending on the solvents used and the extraction techniques used. Ethyl acetate extracts, however, were an exception for not displaying any significant difference in the resulting concentration of polyphenols whether extracted by UAE method or by maceration. Moreover, these results proved that the sonication technique allowed a more efficient extraction of polyphenols compared to maceration, since the methanolic, aqueous and hexanic extracts obtained by the former method contained a high level of total polyphenols (145 ± 4.55 mg EAG /g E, 124 ± 0.62 mg EAG / g E, and 21 ± 1.78 mg EAG / g E), compared to the extracts obtained by the later technique (131 ± 5.50 mg EAG / g E, 116 ± 1.67 mg EAG/g E, 15 ± 1.78 mg EAG/g E) (Figure 1).

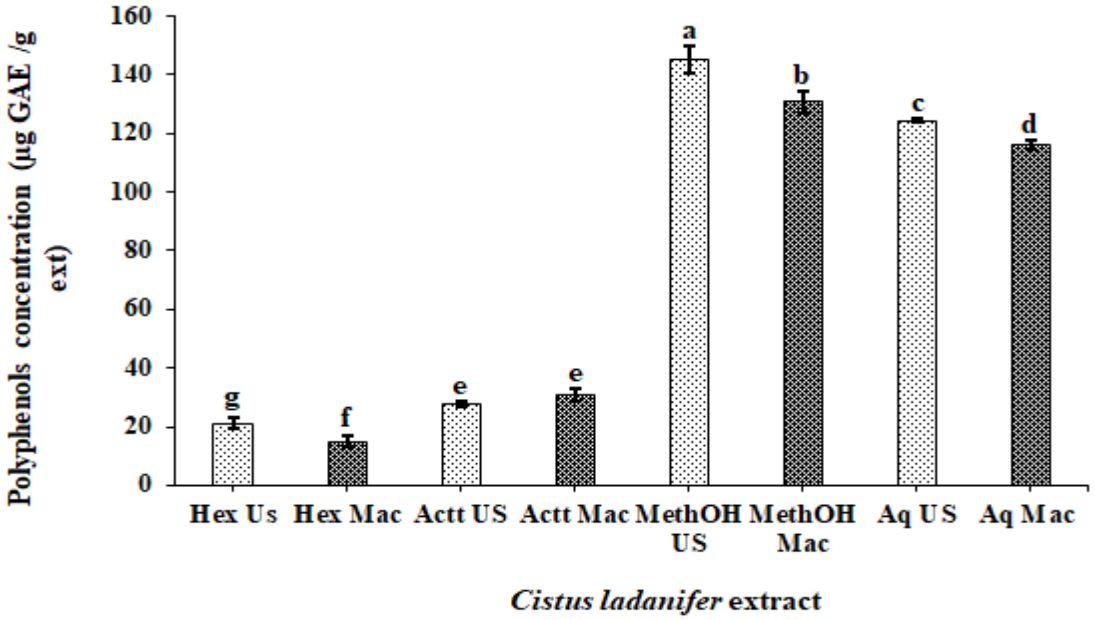

Figure 1. Total polyphenol contents of different extracts of *Cistus ladanifer* L. Results are presented as mean (n = 3) ± SD. Means that are significantly different (at 5%, LSD-Fisher) are represented by different letters. **Hex US:** hexanic extract obtained by Ultrasound-assisted extraction, **Hex Mac:** hexanic extract obtained by maceration, **Actt US:** ethyl acetate extract obtained by Ultrasound-assisted extraction, **Actt Mac:** ethyl acetate extract obtained by maceration, **MetOH US:** methanolic extract obtained by Ultrasound-assisted extraction, **MetOH Mac:** methanolic extract obtained by Maceration, **Aq US:** aqueous extract obtained by Ultrasound-assisted extraction, **Aq Mac:** aqueous extract obtained by maceration.

3.2. Antimicrobial activity

The antimicrobial activity of *Cistus ladanifer* L. extracts was qualitatively and quantitatively tested against foodborne pathogenic bacteria using disk diffusion and the TTC assays.

3.2.1. Disk diffusion method

The qualitative results of the antimicrobial activity of the extracts are presented in Table 1.

Table 1. Results of the disk diffusion test of the extracts from *Cistus ladanifer* L. against foodborne pathogens bacteria.

Extracts	**Inhibition zone diameter (mm)**				
	Bacillus subtilis	***Proteus mirabilis***	***Escherichia coli***	***Staphylococcus aureus***	***Pseudomonas aeruginosa***
Hex US	-	12 ± 0.6	11.0 ± 0.0	-	-
Hex Mac	-	-	-	-	-
Actt US	-	12 ± 1.0	14.3 ± 0.6	-	-
Actt Mac	10.7 ± 0.6	13.7 ± 1.3	-	-	-
MetOH US	14.3 ± 1.2	17 ± 0.0	12.0 ± 0.0	-	-
MetOH Mac	13.0 ± 1.0	15.0 ± 1.0	-	-	-
Aq US	-	-	-	-	-
Aq Mac	-	-	-	-	-
Gentamicin	29 ± 0.0	26 ± 0.0	27 ± 0.0	27 ± 0.0	28 ± 0.0

Hex US: hexanic extract obtained by Ultrasound-assisted extraction, **Hex Mac:** hexanic extract obtained by maceration, **Actt US:** ethyl acetate extract obtained by Ultrasound-assisted extraction, **Actt Mac:** ethyl acetate extract obtained by maceration, **MetOH US:** methanolic extract obtained by Ultrasound-assisted extraction, **MetOH Mac:** methanolic extract obtained by Maceration, **Aq US:** aqueous extract obtained by Ultrasound-assisted extraction, **Aq Mac:** aqueous extract obtained by maceration. (-): inhibition zone less than 6 mm.

As shown in Table 1, the organic extracts from *Cistus ladanifer* L. had an antibacterial effect against three foodborne pathogenic bacteria: *Proteus mirabilis, Bacillus subtilis* and *Escherichia coli*. *Proteus mirabilis* was the most sensitive bacteria to the extracts of both methods of extraction: The inhibition zones varied between 12 mm for the hexanic and ethyl acetate extract and 17 mm for the methanolic extracts, all acquired by sonication; while the diameters belonging to the inhibition zones of the extracts obtained by maceration were in the range of 13.7 mm for the ethyl acetate extracts and 15 mm for the methanolic extracts; The hexanic extracts obtained by maceration as well as the aqueous extracts, however, did not have an effect against *Proteus mirabilis* whatever the technique of extraction that has been used. Unexpectedly, the effect of ethyl acetate extract acquired by maceration was higher than the one that resulted from the UAE technique. Regarding *Bacillus subtilis*, only the methanolic and ethyl acetate extracts of maceration had an effect against this strain, with the

highest inhibition zone corresponding to the methanolic extract prepared by UAE. On the other side, the extracts of maceration did not show any effect against the strain of *Escherichia coli*, whereas the ultrasound extracts exhibited an effect against it with an inhibition zone at the order of 11 mm for the hexanic extract, followed by 12 mm for the methanolic extract, and the bigger zone corresponded to the ethyl acetate extracts with a value of 14.3 mm. No activity was observed for any of the extracts against *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

3.2.2. Determination of MIC and MBC

MIC was determined only for the strains that presented a susceptibility towards the extracts of *Cistus ladanifer* L.: *P. mirabilis*, *E. coli* and *B. subtilis.*

Table 2. MIC and MBC (mg/mL) of extracts of *Cistus ladanifer* L.

Extract	MIC (mg/mL)			MBC (mg/mL)		
	Bacillus subtilis	***Proteus mirabilis***	***Escherichia coli***	***Bacillus subtilis***	***Proteus mirabilis***	***Escherichia coli***
Hex US	-	0.39 ± 0.0	50 ± 0.0	-	>50	>50
Actt US	-	0.78 ± 0.0	25 ± 0.0	-	>50	>50
Actt Mac	6.25 ± 0.0	1.56 ± 0.0	-	>50	>50	-
Meth US	3.125 ± 0.0	0.78 ± 0.0	6.25 ± 0.0	>50	1.56 ± 0.0	50 ± 0.0
Meth Mac	3.125 ± 0.0	1.56 ± 0.0	-	50 ± 0.0	50 ± 0.0	-

Hex US: hexanic extract obtained by Ultrasound-assisted extraction, **Actt US:** ethyl acetate extract obtained by Ultrasound-assisted extraction, **Actt Mac:** ethyl acetate extract obtained by maceration, **MetOH US:** methanolic extract obtained by Ultrasound-assisted extraction, **MetOH Mac:** methanolic extract obtained by maceration.
(-): Not tested.

For *B. subtilis*, the MIC was minimal for the methanolic extract of both used techniques with a value of 3.125, followed by the ethyl acetate extracts that resulted from maceration with a concentration of 6.25 mg/mL. All the extracts had a bacteriostatic effect against this strain since the MBC/MIC ratio of all these extracts was greater than 4.

Regarding *Escherichia coli,* only the extracts prepared by sonication were effective: the MIC ranged from 6.25 mg/ml for the methanolic extract, followed by the ethyl acetate extract with a concentration of 25 mg/ml, then the higher MIC was associated with the Hexanic extract (50 mg/ml). Thus, these extracts also resulted in a bacteriostatic effect against *Escherichia coli* since their MBC/MIC ratio was greater than 4.

The most sensitive bacteria were *Proteus mirabilis.* The highest MIC was 1.56 mg/ml associated with the methanolic extract obtained by maceration, and the lowest one was 0.39 mg/mL of the hexanic extract prepared by sonication. In fact, extracts of UAE technique were the ones with the lowest MIC value compared to those of the maceration method. All extracts had a bacteriostatic effect for the corresponding MBC, which were greater than or equal to 50 mg/ml, so the ratios MBC/MIC were higher than 4. As an exception, the methanolic extract obtained by UAE had a bactericidal effect with a MIC value equal to 0.78 and an MBC equal to 1.56 (Table 2).

3.2.3. Mechanism of action of Cistus ladanifer's extract against Proteus mirabilis (SEM analysis)

To better understand the mechanism of action of the methanolic extract of *Cistus ladanifer* L. obtained by UAE technique and to visualize the morphology of *Proteus mirabilis* when treated with this extract, SEM analysis was performed.

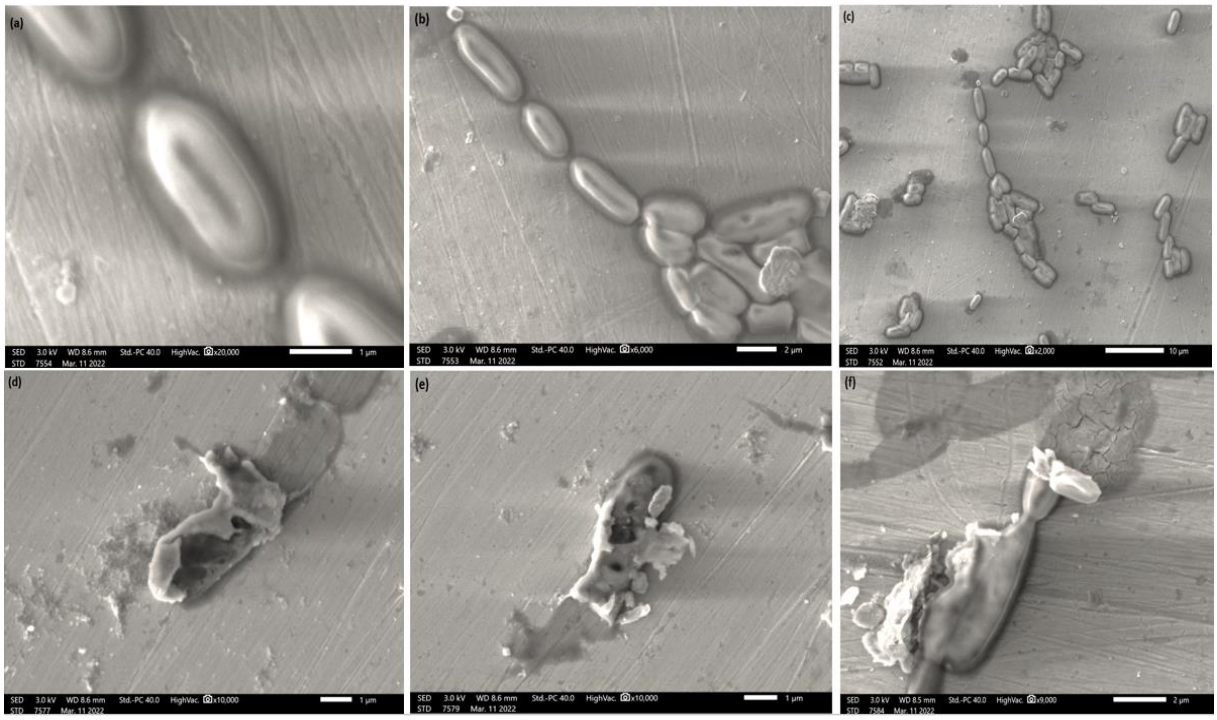

Figure 2. Scanning electron micrographs of *Proteus mirabilis* cells after treatment with methanolic extract of the leaves of Moroccan *Cistus ladanifer* L. prepared by UAE method. Images (a), (b), and (c) represent untreated cells with gradual descending magnification (× 20,000, × 6,000, and × 2,000); (d), (e) and (f) are images represent cells treated with the methanolic extract at MIC value (magnification × 10,000).

Electron micrographs of the negative control (untreated) and the treated cells with methanolic extract are shown in Figure 2. In the untreated culture (Figure. 2: a, b, and c), SEM analysis showed normal rod-shaped images of *Proteus mirabilis*. Nonetheless, and as demonstrated in the remaining figures (d, e, and f), the cells that were treated with methanolic extract at the MIC value exhibited morphological destruction. This data suggested that the extract has a disruptive effect on the cell wall of *Proteus mirabilis*.

4. Discussion

The UAE method yielded higher polyphenol content than the maceration method for the methanolic, aqueous and hexanic extracts. This result may be explained by the principle of extraction of UAE technique: it has been demonstrated that this technique allows a better penetration of the solvent into the cell by reducing the size of the particles and breaking the plant cell wall, which improves the mass transfer and so facilitates the extraction of bioactive molecules compared to conventional techniques [65]. Also, the temperature of the water bath increases by the sound radiation if the exposure time is high [66]; this increase in temperature

improves the extraction due to the reduction in the viscosity of the solvent and the improvement of the coefficient of diffusion and solubility [67].

In contrast, the risk of oxidation of the phenolic compounds increases if the time of extraction is long, which leads to a drop in the polyphenol content [68, 69]. Furthermore, the ultrasonic vibrations induce cavitation, which may sometimes modify the conformation and the molecular structure of bioactive molecules [70]. This may explain the small yield of polyphenols of the ethyl acetate extract prepared by the UAE technique compared to the one that resulted from maceration. Besides, the low polyphenol content in most extracts obtained by maceration can be linked to the fact that this method takes a long time to extract the bioactive molecules because the solvent takes a longer time to penetrate the surface of the sample [71].

Our results were similar to the study of Zannou et al. [72], which demonstrated that UAE presented a higher extraction efficiency for the polyphenolic compounds. It should be mentioned that in our study, the maceration extraction necessitated more time (72 h) to dissolute the phenolic compounds from *Cistus ladanifer* L. leaves in the solvents compared to UAE (60 min), which produced more interesting results. This result is similar to the one of Cassiana Frohlich et al. [73], Tambun et al. [74] and Yılmaz et al. [75], who have proven that the new extraction methods, such as UAE, significantly diminish the time of extraction compared to maceration.

Though the extraction techniques have significantly impacted the biological activities of the extracts of plants because of their influence over the phenolic content, the choice of the solvent has also influenced the resulting phenolic molecules in terms of quantity and quality. Indeed, it is well known that the enhancement of the solubility of phenolic compounds depended mostly on the pH, solvent polarity, and hydrophilicity of the solvent [72]. In our study, methanol was the best solvent that allowed the extraction of the greatest number of polyphenols. The study of Benali et al. [58] has come to the same conclusion since it has been demonstrated that the methanolic and aqueous extracts were rich in polyphenols. This may be explained by the fact that the polarity of methanol has a high affinity for molecules with similar polarity, like flavonoids. Similarly, several studies have shown that methanol has been the best choice for the efficient extraction of antimicrobial substances [76] from various natural sources, including plants, over other solvents such as hexane, water, and ethanol [77-79]. Indeed, it has been demonstrated that the type of solvent used in the extraction largely affected the success of compound isolation from plant material [80].

According to the results of the antibacterial activity of the different extracts of *Cistus ladanifer* L., most of the extracts have significant inhibitory activity against the growth of some strains of the bacteria with different degrees. Moreover, the extracts did not show any selective antimicrobial activity based on whether the strains were gram-negative or gram-positive; this may be explained by some molecules in the extracts that are effective against both strains. These results are similar to the ones obtained by Gulluce et al. [61]. Besides, all the extracts, independently of the method of extraction, had a bacteriostatic effect apart from the methanolic extract prepared by UAE technique, which displayed a bactericidal effect.

Also, the extracts obtained by UAE method were efficient against *E. coli*, while those prepared by maceration did not have any effect against this strain; this indicates that the extracts obtained by UAE technique were more potent than those obtained by maceration. These results can be explained by the differences in the chemical compositions of these extracts that change depending on the type of solvent and the extraction method used. In fact, many factors influence the isolation of bioactive molecules, notably the extraction method, temperature, time, and the solvent used [72]. This idea can explain the difference in the molecules of polyphenol among the extracts of maceration and those of UAE in our study. The active extract was the methanolic one prepared by UAE, which displayed a high polyphenol content and exhibited great antibacterial activity.

Our results agreed with the study of Benali et al. [59] concerning the effectiveness of the methanolic extract against pathogenic strains of *Proteus mirabilis* and *Bacillus subtilis*. More precisely, the methanolic extract obtained by the UAE method was the most active against both Gram-negative strains (*Proteus mirabilis*, *Escherichia coli*) and Gram-positive (*Bacillus subtilis*), with better activity on the first [81]. This may be explained by the fact that methanol can release some active ingredients such as saponins, bryophyline and phenolic compounds [82, 83], alkaloids, steroids, terpenoids, and other secondary metabolites which possess antimicrobial activity [84]. In addition to that, according to Masoko et al. [80] and Serkedjieva et al. [85], aromatic or saturated organic compounds have been the most identified antimicrobial compounds in plants, and they were mostly obtained by a first extraction with ethanol or methanol. On the other hand, our results were in contradiction with those of Barrajón-Catalán et al. [49], who proved that the aqueous extracts of *Cistus ladanifer* L. were less effective against Gram-negative than Gram-positive bacteria. This high sensitivity of Gram-positive bacteria to plant extracts has been demonstrated by other authors [86].

Besides, the other solvents were not efficient for extracting active molecules since the extracts presented moderate to low antibacterial activity (Table 2), and this was proved by the small amount of polyphenols in the extracts prepared by hexane and ethyl acetate. This can be explained by the low affinity of the solvents to the active compounds of *Cistus ladanifer* L. and by the effect of the drying process of the plant, which caused conformational changes in some molecules [78]. However, although the aqueous extracts yielded a high content of polyphenols, they did not have an antibacterial activity against the explored bacterial strains. This can be explained by the lack of antibacterial properties of the extracted molecules or because the molecules that have antibacterial properties were present but at very low concentrations to have an effect [87].

Several studies have shown that essential oils and crude extracts of medicinal plants had antimicrobial activity due to their content of molecules with antimicrobial properties [88-91]; but the mechanism of action has not been elucidated yet [59, 92, 93]. In fact, these antimicrobial compounds from plants act on the coagulation of cellular contents, active transport, proton motive force destabilization, and cytoplasmic membrane disruption [84].

The adverse effect on the morphology of *Proteus mirabilis* treated with methanolic extract obtained by UAE at the MIC is evident, as shown on the electron micrographs of the

bacteria, which have incomplete and deformed shapes. This effect may have happened because the extract denatured the cell membrane, which caused an increase in cell permeability and a disruption of the membrane integrity, which finally led to a rupture and lysis of the cells. A similar study by Lv et al. [63] has demonstrated that essential oils act mainly on the cytoplasmic membrane of the cells of microorganisms. The work of Ceylan and Fung [94] has revealed that the major active components of essential oils are terpenes, phenols, aldehydes, and ketones; these molecules are secondary metabolites synthesized by plants to defend themselves against environmental stress. According to the research of Valares Masa et al. [95], flavonoids, especially kaempferol 3,7-di-O-methyl ether, kaempferol 3-methyl ether, apigenin, apigenin 41 -methyl ether, apigenin 7-methyl ether, and diterpenes are the two families of secondary metabolites that characterize *Cistus ladanifer* L. This work has also proved that the variation in the chemical composition of extracts from individuals of the same species of *Cistus ladanifer* L. was only quantitative and that kaempferol 3,7-di-O- methyl ether and oxocativic acid were the main component in flavonoids and diterpenes, respectively [95]. Based on the same study, we can explain our results by the presence, at a sufficient concentration, of kaempferol and apigenin in our methanolic extract obtained by UAE, a molecule that is characterized by an antimicrobial activity among other biological ones [96-101]. Furthermore, based on the study of Rauha et al. [102], kaempferol had no activity against *E. coli,* and it was slightly active against *B. subtilis*. These results may also be explained by the presence of apigenin in the *Cistus ladanifer* L. extracts. It was the second molecule among the flavonoids that were extracted, and it had strong activity against Gram-negative bacteria [97, 99] with MIC values ranging from 4 mg/L and 128 mg/L against *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Enterobacter aerogenes*, *Salmonella typhi*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter cloaceae* [103]. Also, the study of Öksüz et al. [104] has revealed that the MICs of apigenin isolated from *Centaurea* species and that have been tested against *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, and *Proteus vulgaris* were ranging from 55 mg/L to 219 mg/L. Moreover, it has been demonstrated that flavonoids have many properties: the ability to bind with extracellular proteins and bacterial wall proteins [91, 92], and to inhibit enzymes, bacterial quorum sensing toxins and signal receptors [91].

5. Conclusions

To sum up, the methanolic extract of *Cistus ladanifer* L. obtained by sonication revealed a wide spectrum of antibacterial activities against many bacteria, with the highest activity against *Proteus mirabilis*, responsible for the most common infectious diseases. This study has displayed the scientific basis for some therapeutic uses of this plant in traditional medicine. This basis was revealed to be associated with the composition of the plants on bioactive molecules, which have enormous therapeutic potential, such as antimicrobial activity. In fact, this promising extract, on the one hand, provides an initial platform for further pharmacological and phytochemical studies, as well as opens the possibility of finding new clinically effective antibacterial compounds.

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