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Bioactive Properties of Natural Compounds Extracted from Leaves of *Cistus ladanifer*

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ABSTRACT

The main problem faced by the food industry is the deterioration of food by microorganisms and oxidation, and growing interest is widely given to identify new products with preservative characteristics with limited impact on human health. In this field, we have investigated the potential benefits of *C.ladanifer*, collected from the North of Morocco, by assessing the antibacterial, antifungal and antioxidant activities of extracts obtained from dry plant leaves. All extracts were screened against three Gram-negative and Gram-positive bacteria using the disc diffusion method and the microdilution assay. The extracts were notably active against *S. aureus* (11 to 12 mm and MIC =8.75 to 17.5 mg/ml). Antibiofilm assay revealed that all tested samples are capable of inhibiting at least 50% of the staphylococcal biofilm matrix, while the real time bacterial growth measurements proved that dichloromethane extracts can exert a bacteriostatic effect on the strain. The antifungal assay revealed the sensitivity of *B.cinerea* and *C.albicans* towards low doses of dichloromethane extracts (100% inhibition at 1.25 mg/ml for *B.cinerea* and MIC of 17.5 mg/ml for the yeast strain). On the other hand, a close relationship was obtained between the antioxidant capacity of the tested extracts and their phenolic content. The aqueous extract exhibited a strong antioxidant activity and contains a high amount of polyphenols. In conclusion, the present study revealed that the organic extracts of *C. ladanifer* have an antimicrobial activity and the aqueous extract showed an antioxidant power making this plant a strong candidate to be incorporated in new systems to prevent food contamination and extend its shelf life.

Key words: *Antibacterial effect, Antioxidant activity, Antifungal activity, Plant extracts, Cistus ladanifer, Morocco*

Introduction

For decades, the centralisation and globalisation of foods increase the likelihood of foodborne diseases and food security and safety have been considered as the main challenge to ensure food availability without risk for human health (King *et al.*, 2017; Sanders, 1999). In this field, contamination of food by microorganisms has been at the forefront of public health headlines. Food spoilage by microorganisms manifests itself in many forms such as the presence of slimy textures, change of flavour, off putting odour or acidity…. (Bautista, 2014). The occurrence of these characteristics renders food unsuitable for human consumption and therefore reduces effectively its shelf life that will affect considerably the economy of the country. Food deterioration is a result of chemical reactions between the spoilage microorganisms and food substrates (Gram *et al.*, 2002).

To prevent the proliferation of microorganisms in foods and during food processing, many kinds of additives are widely used and have an important role in preventing damage or spoilage of the foodstuff and are essential along the food chain (Surekha and Reddy, 2014). Recently, their use in food processing has been largely discussed raising a major controversy around their application. Indeed, these additives, mostly synthetics, have been the subject of numerous studies highlighting their mutagenicity, toxicity and their role in the induction of malformations on both human and animal models (Carocho *et al.,* 2014; Ciardi *et al.,* 2012; Tsay *et al.,* 2007; Yýlmaz *et al.,* 2009).

In the food industry, food oxidation is a major concern with a great impact on food quality. Food oxidation affects mainly lipids, carbohydrates, proteins and nucleic acids, leading also to food deterioration (Lopez *et al.,* 2008). To avoid food oxidation, synthetic antioxidants, including butyl-hydroxy-anisole (BHA) and butyl-hydroxy-toluene (BHT), have been widely used. Though, their use remains under strict regulations and has been discouraged since their effects after long-time consumption remains unclear (Freitas and Fatibello-Filho, 2010; Imaida *et al.,* 1983).

During the last decades, a growing interest is given to the use of green technologies aiming to limit side effects of chemicals used as additives and/or antioxidants and to enhance human wellbeing. In this field, clean labeling and establishing new substitutes most preferably deriving from natural sources have been reported as the main options for food preservation (Gedikoðlu *et al.,* 2019).

Scientific evidence has shown that extracts from aromatic plants hold a large number of antibacterial and antioxidant agents, and are considered easily degradable with several extracts enjoying the GRAS status (Generally Recognized as Safe)(Berahou *et al.,* 2007; Cowan, 1999; Duffy and Power, 2001; Mostafa *et al.,* 2018; Negi, 2012). Accordingly, numerous studies have reported the antimicrobial (Aleksic Sabo and Knezevic, 2019; Gupta *et al.,* 2016; Kim *et al.,* 2011; Tekwu *et al.,* 2012) and antioxidant (Lizcano *et al.,* 2010; Roby *et al.,* 2013; Saeed *et al.,* 2012; Skotti *et al.,* 2014) effects of many aromatic plants and highlighted the potential use of these plants in food preservation.

The *Cistaceae* family comprises of about 180 species distributed among eight genera (Guzmán and Vargas, 2009). *Cistus ladanifer* L. (*C. ladanifer*) of the *Cistus* genera is a large shrub typically found along the Mediterranean rim. It is largely abundant in the mountainous Rif region in Morocco, as well as in Beni-Znassen and the central region. Dry leaves of *C. ladanifer* are used in Moroccan traditional medicine as antidiarrheic, antiacid and antispasmodic (Zidane *et al.,* 2013). In addition, its application has also been reported for the treatment of various skin diseases, and as anti-inflammatory agents (Viuda-Martos *et al.,* 2011), therefore attesting to the important position the plant holds in Moroccan traditional practices. Thus, the present study was planned to evaluate the antimicrobial and antioxidant properties of *C. ladanifer* for its potential use in food preservation.

Materials and Methods

Chemicals and reagents used in this study

The solvents used for plant extractions, including Hexane 97%, dichloromethane 99.8% and methanol 99%, have all been provided by Sigma-Aldrich (Saint-Quentin Fallavier, France). Glycerol, DPPH, Resazurin sodium salt, Folin-Ciocalteu reagent, Gallic acid and Butylated Hydroxytoluene (BHT) were also purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). DMSO, sodium carbonate and Tween 20 have been acquired from Merck KgaA (Darmstadt, Germany). Microbial studies have been carried out using Mueller Hinton Broth (MHB), Brain Heart Infusion Broth (BHI broth), Potato Dextrose Agar (PDA) and bacteriological agar type A which were purchased from Biokar Diagnostics (Allonne, France).

Plant material and extraction

C. ladanifer was harvested from the northern region of Morocco (Beni Mansour) in February 2019. The leaves were dried in the darkness with active ventilation at 45 °C. To obtain organic extracts, dried leaves were milled and 25g of fine powder was placed in a soxhlet apparatus with 250 ml of n-hexane, as a first solvent, and the extraction took place at 50 °C until the plant material is fully exhausted by the solvent. Extraction was then continued at 100 °C with the dichloromethane, methanol and lastly distilled water. Obtained solutions were then filtered with a Whatman No.3 filter paper before undergoing in vacuum evaporation using a rotary evaporator at 50 °C. The residual solvent was additionally

dried in test tubes at 50 °C. The dry weight of each extract was measured to determine its yield and then stored in the dark at 4 °C until further use.

Antibacterial activity

Test microorganisms and growth conditions

In this study, antibacterial activity of *C. ladanifer* was performed on 3 gram positive strains: *Enterococcus faecalis* (ATCC19433), *Listeria monocytogenes* (ATCC19144) and *Staphylococcus aureus* (B1), and 3 gram negative strains: *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC14028) and *Escherichia coli* (ATCC25922). All strains were sustained in BHI broth with 25% glycerol and stored at -20°C. Before each testing, all cultures were cultivated in BHI broth for 24 hours and adjusted to a concentration of approximately 10^8 UFC/ml using McFarland 0.5 standard.

***In vitro* screening of plant extracts on pathogenic strains using the well diffusion method**

Aliquots of 100 µl of each bacterial inoculum were introduced and spread uniformly over Mueller Hinton agar. Each plant extract was prepared in three concentrations: 30 mg/ml, 60 mg/ml and 100 mg/ml. For testing, 50 µl of each concentration is incorporated into wells of 5mm in diameter and the inoculated plates were first incubated at +4°C during 2h to ensure total diffusion in the medium, and then transferred to 37°C for an overnight incubation. For each test extract the corresponding negative control was used (10% DMSO, Tween20 + Distilled water [2:1] and sterile distilled water). The antibacterial effects were assessed by measuring the inhibition zones obtained by the active extracts (well diameter not included).

Screening for minimal inhibitory and minimal bactericidal concentrations with microdilution assay

Active extracts, displaying aninhibitory effect during the initial testing, were additionally analysed to evaluate the minimal inhibitory and bactericidal concentrations (MIC/MBC). MIC/MBC evaluations were done using the Mann and Markham method with some modifications (Mann and Markham, 1998). Tested extracts were diluted several times to obtain the extracts' concentrations ranging from 4.37 to 40 mg/ml. Then, 180 µl of each concentration was introduced in wells of a 96-well microtitration plate along with 20 µl of each bacterial suspension. After an overnight incubation at 37 °C,10 µl of resazurin sodium salt at 0.01% was added, and then the plates were left for 30 min. MIC is defined as the lowest concentration giving a bacteriostatic effect illustrated by the non-discoloration of the resazurin dye. MBC is defined as the lowest concentration giving a bactericidal effect and was assessed by sub-culturing the broth dilution of ≥ MIC tests on agar plates. For each test, DMSO and Tween 20 were used as negative controls.

Real-time antibacterial kinetics

The antibacterial kinetics of *C. ladanifer* hexanic, dichloromethane and methanolic extracts have been determined using a bioreactor to determine real time bacterial growth in the presence of super-inhibitory concentrations of microbiologically active extracts (2MIC). Extracts were re-suspended in their respective solvents and then added to 10 ml of MH broth containing the pathogen of interest ($1x10^6$ CFU/ml). The bioreactor in allows measurements of bacterial growth to be taken at real-time by means of OD_{850} taken at 15 min intervals. The bioreactor is programmed at 2000 rpm and a reverse tube spin of 1s at 37 °C. The plotting of $\log_{10}$(OD) obtained over 24h allows the determination of kinetic parameters using the following equations:

Bacterial growth rate (µexpo): $\mathbf{Y_f = A_i e^{k\Delta t}}$ **(with k= µexpo)**

Generation time (G): $\mathbf{G = \ln 2 / \mu expo}$

µexpo: Growth rate in the presence of plant extract obtained from the exponential growth phase (h^{-1}).

G: Generation time or the average time interval allowing the doubling of the microbial population (h).

Anti-cell adhesion assay

Anti-cell adhesion assay was performed on *S. aureus* and *C. albicans* using the modified protocol of Bazargani and Rohloff (2016). Overall, 100 µl of the extract prepared in Mueller-Hinton broth at concentrations corresponding to 2MIC; MIC; ½ MIC were added to an equal volume of *S. aureus* ($1x10^6$ CFU/ml) and *C. albicans* ($1x10^9$ CFU/ml) in a 96-flat bottom titration plates. After incubation at 37°C during 24h for *S. aureus* and 48h for *C. albicans*, the supernatants were discarded and the wells were washed 3 times and dried at 60 °C for 1h. Then, 200 µl of 0.4% crystal violet was added to each well and left for staining during for 15 min at room temperature.

Wells were then washed twice and 150 µl of ethanol was added to elute the dye from the formed biofilms. Finally, 100 µl of the obtained solution was transferred to a new plate and served to measure the optical density at 590 nm using a microplate reader (SPECTROstarNano, BMGlabtech). Cell suspensions with extract-free medium and sterile MH broth served as control and blank respectively. The biofilm inhibition rate (IR) was evaluated as fellow:

$$IR(\%) = \frac{OD\ (Negative\ control) - OD\ (Test\ sample)}{OD\ (Negative\ control)} \times 100$$

Antifungal activity of plant extracts

The antifungal effect was performed on 3 fungal strains: *Candida albicans*, *Botrytis cinerea* and *Fusarium oxysporum,* using the macro-dilution assay (Onaran and Saglam, 2016). For *C. albicans*, the antifungal activity has been explored using the well diffusion method as described previously. For *B. cinerea* and *F. oxysporum*, the percentage of the fungal growth inhibition has been determined according to the protocol reported by Onaran and Saglam. Tested extracts, prepared at concentration ranging from 0.02 mg/ml to 10 mg/ml, were added to a 2 ml sterile PDA, kept at a temperature of 50 °C to avoid agar solidification, and then plotted onto 7 mm Petri-dishes. After the solidification, 6 mm fungal discs were added to the center of the plates. The inoculated plates were incubated at 25°C for 7 days, the fungal growth was identified by measuring the average radial growth of the mycelia on a daily basis and the percentage of the mycelial growth inhibition (I%) was calculated using the following formula:

$$I(\%) = \frac{Dc - Dt}{Dc} \times 100$$

Where: $\mathbf{D_c}$ corresponds to the growth diameter of the negative control (PDA medium supplemented with DMSO alone or Tween 20+distilled water). $\mathbf{D_T}$ corresponds to the growth diameter of the test samples. Tests have been performed in duplicate and the results are expressed as means ± SD.

Antioxidant activity

The antioxidant potential of *C. ladanifer* extracts was evaluated using the DPPH free radical assay following the protocol reported by Mensor *et al.*(2001). Overall, 1 ml of DPPH solution in methanol (0.3mM) was added to 2.5 ml of a dilution series of test solutions ranging from 2 mg/ml to 0.0005mg/ml (diluted in methanol). The absorbance of each test concentration is measured at 518nm after 30min of incubation in the dark. In this study, BHT was used as a positive control. For each test, blanks consisting of 1 ml of methanol and 2.5ml of each dilution concentration were included and are used for the evaluation of the antioxidant activity (AA%) according to the following formula:

$$AA(\%) = \frac{(A)\ control - (A)\ sample}{(A)\ control} \times 100$$

Total polyphenol content

The phenolic content of the four extracts was determined with the Folin-Ciocalteu method using the protocol reported by the International Organization for Standardization (ISO) (ISO 14502-1: 2005., 2005) (Anesini *et al.,* 2008). The Folin-Ciocalteu's reagent was prepared at a ratio of 1:10; 5 ml of this solution was added to tubes containing 1 ml of each diluted test sample. The solution was then left for 8 min before being supplemented with 4 ml of sodium carbonate 7.5% (w/v). The final mixture was incubated at room temperature for 1h. The absorbance was measured at 765 nm with distilled water as a blank. The results were obtained through the standard curve of gallic acid (10 to 50 µg/ml) and are expressed as a mg of gallic acid equivalent per 1g of dry material (mg GAE /g material).

Statistical analysis

Tests were conducted in triplicates and results were presented as means ± Standard Deviation (SD).The means were analysed using a one-way ANOVA analysis with student's t-test along with Fisher's Least Significant Difference (LSD) test on Statigraphic software (Fisher, 1935).

Results

Extract yield

Extraction yields of *C. ladanifer* were expressed in percentage of dry extracts, obtained from each extraction, and are presented in Table 1. Results indicate that polar solvents produced higher amounts of extracts than non-polar solvents. The highest yield was obtained with the aqueous extracts (21.24%) followed by methanol (15.84%). The lowest yield was obtained with the hexane extraction (6.56%).

Table 1. Extraction yield of *C. ladanifer* organic extracts

Plant extracts	Yield (%)
Hexanic	6,56
Dichloromethane	12,82
Methanolic	15,84
Aqueous	21,24

Qualitative and quantitative evaluation of the antibacterial activity

The antibacterial activity of *C. ladanifer* extracts against the 6 selected bacterial species representatives of gram-negative and gram-positive strains was qualitatively and quantitatively measured using the well diffusion and microdilution assays. The results obtained are illustrated in Table 2 (inhibition zone) and Table 3 (MIC/MBC values). The results obtained revealed that most tested strains are resistant to the actions of the tested extracts apart from *S.aureus B1* which showed sensitivity to hexanic (11.67±0.57 mm) dichloromethane (10.6±1.15 mm) and methanolic extracts (13±0 mm). Results of the quantitative testing showed low MIC with hexanic and methanolic extracts (8.75 mg/ml). Of particular interest, the dicholoromethane exhibited both bacteriostatic and bactericidal effects at 17.5 mg/ml.

Antimicrobial kinetics

To further highlight the efficiency of *C. ladanifer* extracts on tested strains, we have proceeded to evaluate the effect of extracts on microbial growth parameters. Table 4 details the results obtained which indicate that the dichloromethane extract exerts a bacteriostatic effect on both sensitive strains (*S. aureus* and *C. albicans*) and the methanolic extract is bacteriostatic only on *C. albicans*. The hexane extract action mainly affects the growth kinetics by deceleration of the microbial growth speed (μ) and consequently delaying the generation time. Indeed, in presence of hexanic extract, *S. aureus* grew with a generation time of 2.51±0.17h and maximal growth speed of 0.277±0.02h^{-1}. However, the methanolic ex-

Table 2. Antibacterial activity of *C. ladanifer* extract against bacterial strains tested based on the well Diffusion Method (mm)

	Gram negative bacteria									Gram positive bacteria								
	EC[1]			ST[2]			PA[3]			EF[4]			SA[5]			LM[6]		
Concentration (mg/ml)	30	60	100	30	60	100	30	60	100	30	60	100	30	60	100	30	60	100
Hexanic	0	0	0	0	0	0	0	0	0	0	0	0	0	0	11.67±0.57	0	0	0
Dichloromethane	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10.6±1.15	0	0	0
Methanolic	0	0	0	0	0	0	0	0	0	0	0	0	0	0	13±0	0	0	0
Aqueous	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[1] *E. coli* ATCC25922; [2] *S. typhimurium* ATCC14028; [3] *P.aeruginosa* ATCC 27853; [4] *E.faecalis* ATCC 19433; [5] *S. aureus* B1; [6] *L. monocytogenes* ATCC19144

Table 3. MIC and MBC values of *C. ladanifer* organic fractions against *S. aureus*

Plant extracts					
Hexanic		Dichloromethane		Methanolic	
MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
8.75	35	17.5	17.5	8.75	> 40

Table 4. Effect of *C. ladanifer* extracts of on microbial kinetic parameters

Plant extract/control	*S. aureus*		*C. albicans*	
	μ_{expo} h^{-1}	G(h)	μ_{expo} h^{-1}	G (h)
Control (Tween20/Distilled water)	0.74	0.94	0.6	1.15
Control (DMSO)	0.6	1.16	0.16	4.28
Hexanic	0.277±0.02	2.51±0.17	-	-
Dichloromethane	0	0	0	0
Methanol	0.047±0.005	14.85±1.7	0	0

tract displayed a stronger action by reducing bacterial reproduction at a rate of 0.047±0.005h^{-1} and extending the doubling time to 14.85±1.7h.

Anti-cell adhesion assay

In this study, inhibition of biofilm formation was estimated by the analysis of initial cell attachment. All tested extracts exerted an effect on tested biofilms with a significant dose-effect, with inhibition rates exceeding 50% at high concentrations (Table 5). The hexanic extracts showed the most significant damage on *S. aureus* biofilms at its highest concentration of 17.5 mg/ml (83.57±0.33%) followed by dichloromethane extracts with 67.61±3.36% inhibition at 35 mg/ml whilst the methanolic extract revealed the lowest effect at 17.5mg/ml (51.9±2.02%).

For *C. albicans*, biofilm attachment has been reduced by both methanolic and dichloromethane extracts with inhibition rates exceeding 70% at 17.5mg/ml and 35 mg/ml respectively. Methanolic extract is more likely to be more active against *C. albicans* biofilm formation, inducing a biofilm inhibition at a rate of 74.26% at 17.5 mg/ml whereas a 71.02% inhibition rate was obtained with the dichloromethane at 35 mg/ml.

Antifungal activity

The antifungal activity of the organic extracts is reported in Figure 1. Overall, the dichloromethane extract of *C. ladanifer* was the most active inducing sig-

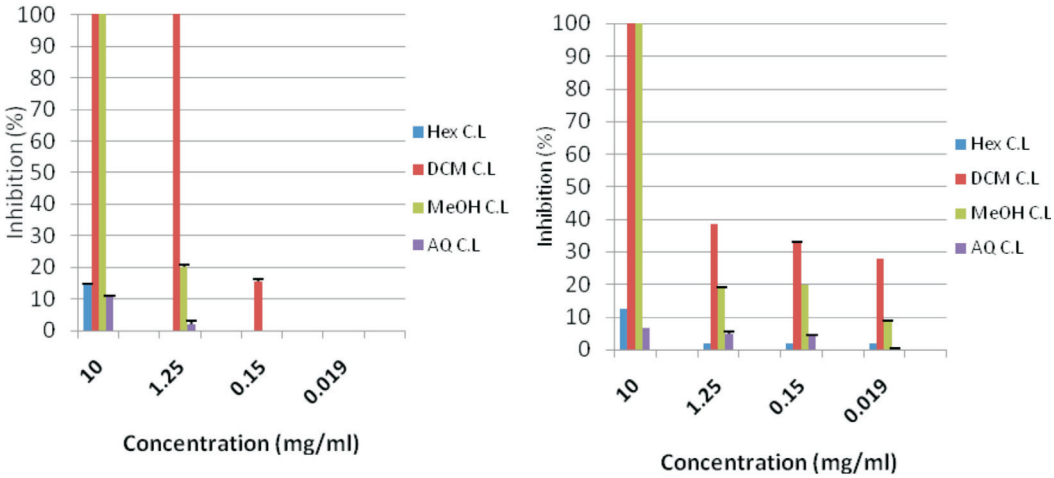

Fig. 1. Percentage of fungal inhibition of *C. ladanifer* extracts against the selected fungal strains: *B. cinerea*(A) and *F. oxysoporum*(B)

Table 5. Effect of hexanic, dichloromethane and methanolic extracts on biofilm formation

Extract	Strain	Concentration (mg/ml)	Anti-biofilm effect (%)
Hexanic	*S.aureus*	17.5	83.57±0.33
		8.75	78.1±0
		4.38	63.3±0
Dichloromethane	*S.aureus*	35	67.61±3.36
		17.5	55.47±4.37
		8.75	14.76±4.04
	C.albicans	35	71.02±0.9
		17.5	70.53±0
		8.75	61.78±4.8
Methanol	*S. aureus*	17.5	51.9±2.02
		8.75	22.61±3.7
		4.38	26.9±7
	C. albicans	17.5	74.26±0.27
		8.75	72.2±2.9
		4.38	45.1±0.8

nificant growth inhibition of both tested fungi. The dichloromethane exhibited an 18% mycelia inhibition of *B.cinerea* at 0.15 mg/ml and total inhibition at 1.25 mg/ml, whereas on *F. oxysoporum* a dose-response inhibition was obtained with a total inhibition at 10 mg/ml. The methanolic extract was also active and showed total inhibition at 10 mg/ml on both fungal strains. Hexanic and aqueous extracts showed a reduced activity on fungal mycelia growth with an inhibition not exceeding 15% at higher doses.

For all tested concentrations, mycelia inhibition has been recorded for both strains since the 2nd day after incubation and the fungal response to *C. ladanifer* extracts was dose dependant. At the concentration of 10 mg/ml both dichloromethane and methanolic extracts showed a fungistatic effect against the two fungal strains as it is illustrated in Figures 2 and 3.

Dichloromethane and methanolic extracts were also the active extracts against *C.albicans* with inhibition zones of 13.3 mm and 10.6 mm, respectively (Table 6). The methanolic extract was more fungicide and fungistatic with a MIC and MBC of 8.75 mg/ml. The MIC and MBC obtained with dichloromethane was significantly higher (17.5 mg/ml).

Total phenol content and antioxidant activity

Table 7 presents the results of the antioxidant activity of the *C. ladanifer* extracts molecule. Overall, a close dose-dependent relationship was obtained with all extracts. At a high dose (2mg/ml), the 4 extracts exhibited an interesting antioxidant activity equivalent to that obtained with BHT, used as reference molecule. At 1 mg/ml, aqueous extract was the most active followed by the dichloromethane, with antioxidant activities of 86.34±0.03 and 69.92±0.009 respectively. Of particular interest, at lower doses, the methanolic extract exhibited a significant high antioxidant activity, greater than that obtained with the BHT.

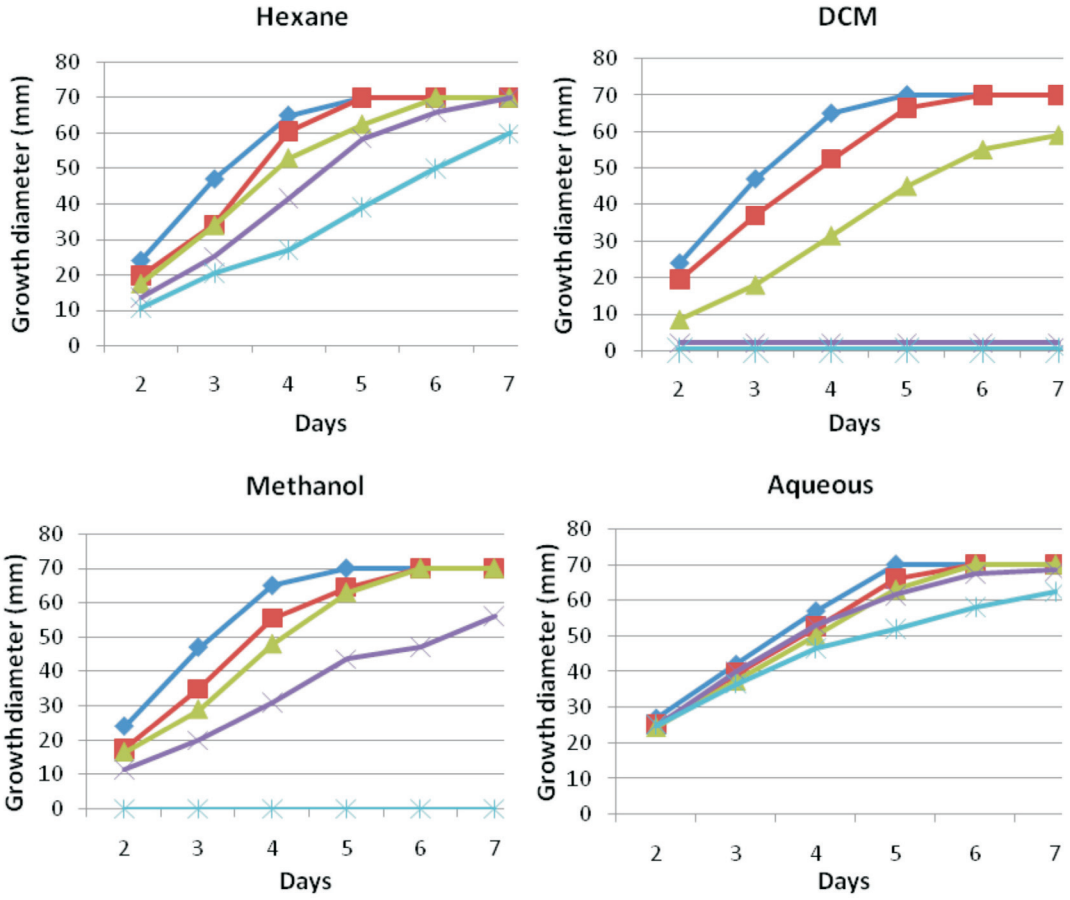

Fig. 2. Mycelial growth kinetics of *B. cinerea* in presence of *C. ladanifer* extracts

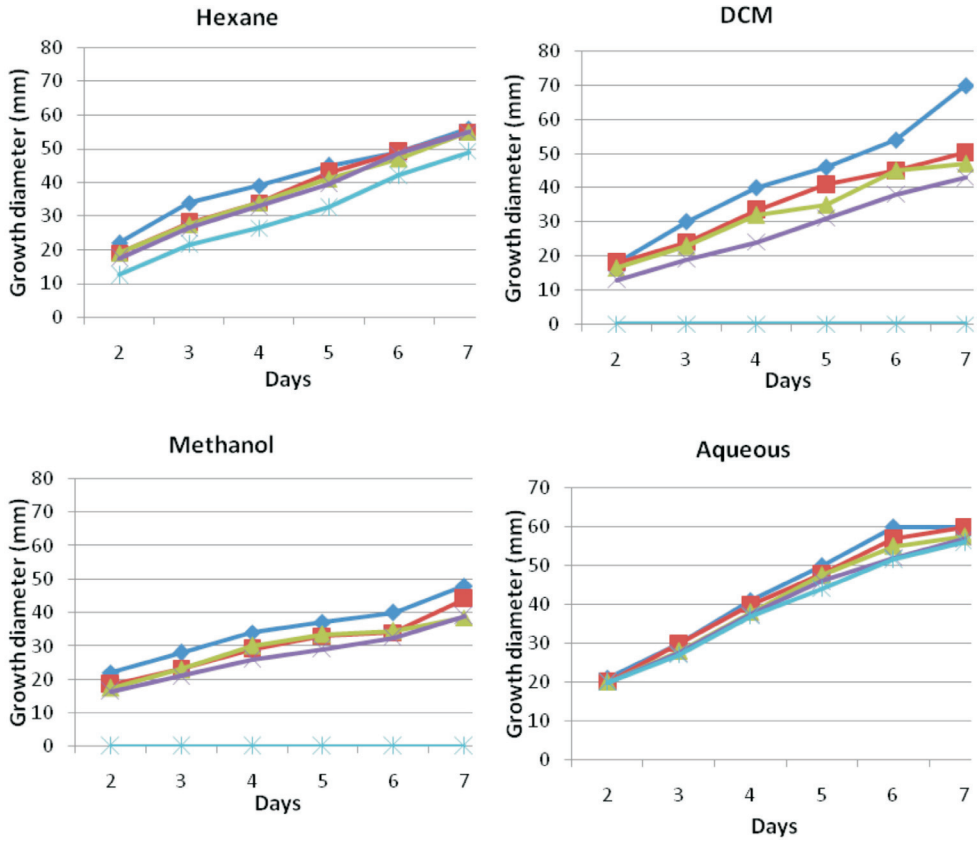

Fig. 3. Mycelial growth kinetics of *F. oxysporum* in presence of *C.ladanifer* extracts

Polyphenolic analysis showed that the aqueous extract displayed the highest levels of phenol compounds (60.67±0.056 GAE). Dichloromethane and methanolic extracts presented moderate levels of polyphenolic contents: 16.53±0.015 GAE and 9.8±0.012 GAE, respectively (Figure 4). Comparison between total phenol content and the antioxidant activity showed a close relationship between the

Table 6. Antifungal effect of *C. ladanifer* extracts against C. albicans strain

Plant extracts	Concentration (mg/ml)			MIC	MBC
	30	60	100		
Hexanic	0	0	0	-	-
Dichloromethane	0	13.3±2.8	-	17.5	17.5
Methanolic	0	10.6±0.57	-	8.75	8.75
Aqueous	0	0	0	-	-

Table 7. Antioxidant potential and efficiency (IC_{50}) of C.ladanifer extracts in comparison with BHT

Concentration (mg/ml)	2mg/ml	1mg/ml	0.0312mg/ml	0.00048mg/ml	IC_{50} (mg/ml)
Hexanic	95.87±0.01^{a}	36.52±0.27^{b}	10.8±0.03^{b}	10.15±0.02^{b}	1.04
Dichloromethane	93.02±0.005^{a}	69.92±0.009^{b}	15.88±0.01^{c}	6.9±0.02^{d}	0.84
Methanolic	93.69±0.001^{a}	42.79±0.064^{b}	39.64±0.01^{b}	17.12±0.04^{b}	0.81
Aqueous	95.15±0.02^{a}	86.34±0.03^{b}	26.81±0^{c}	16,51±0.01^{d}	0.6
BHT	93.81± 0.002^{a}	92.86±0.003^{a}	27.48±0.01^{b}	10.61±0.1^{c}	0.6

A,b,c,d:Means presented in the same row with different letters denote a statistically significant difference according to Fisher's Least Significant Difference test ($p < 0.05$).

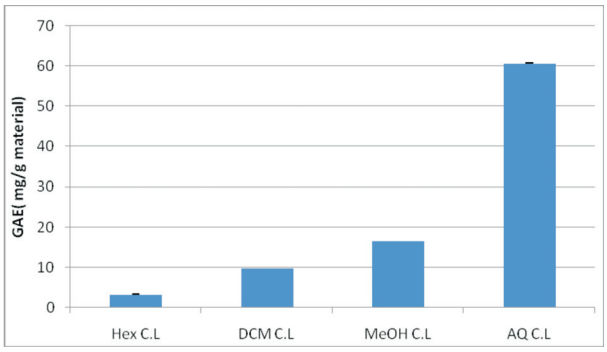

Fig. 4. Total polyphenol content of *C. ladanifer* extracts in GAE (mg/g material)

phenolic status of the extract and free radical scavenging ability.

Discussion

In Morocco, medicinal and aromatic plants are widely used for their potential as functional food, as vegetable oil and as for primary care. Moreover, growing interest is given to identify plants with potential added values that could be used for human wellbeing and to identify plants as sources of pharmaceutical active substances(Aghraz *et al.,* 2018; Bouyahya *et al.,* 2020). Thus, this study interferes with the efforts made across the country for the promotion of medicinal and aromatic plants and participates in the socio-economic development of the country.

In this perspective, we have evaluated the antibacterial activity of *C. ladanifer*. Results have clearly shown that aqueous extract didn't exhibit any effect on selected bacteria but hexanic, dichloromethane and methanolic extracts have an interesting effect on *S. aureus*, a gram positive bacterium. The resistance of selected Gram-negative bacteria could mainly be due to their lipopolysaccharide cell wall limiting the diffusion of the hydrophobic compounds (Erdogrul, 2002; Ouattara *et al.,* 1997). In a previous study, Benayad *et al.* reported that hexanic, dichloromethane and methanol extracts of *C. ladanifer*, collected from the Middle-Atlas region of Morocco, inhibited *S. aureus* growth. However, they have reported that the methanolic extract has an inhibitory effect also on *E. coli* and *P. aeruginosa* (Benayad *et al.,* 2013). This difference could be explained by the tested concentrations of the extract and also the chemical composition that was reported to be different according to the climate and soil composition (Messaoudi *et al.,* 2019). Additionally, the variable sensitivity profiles displayed by bacterial strains towards chemical substances pertain to different resistance levels between the strains (Erdogrul, 2002).

Of particular interest, dichloromethane extract exerted a bacteriostatic effect on *S. aureus* strains and explains the traditional medical application of *C. ladanifer* to treat various infections caused by this microbe.

Given the interest in biofilms and their role in contaminations associated with the food industry (Galié *et al.,* 2018), a great interest is currently given to identify natural compounds with anti-biofilm potentialin the perspective to set up alternative options for food preservation that will limit the risk of food deterioration on human health. In this context we have evaluated the ability of *C.ladanifer* extracts to reduce biofilm formation. For instance, the hexanic extract exhibited the highest effect on *S. aureus* biofilm at 17.5 mg/ml and the activity decreased in low concentrations. Interestingly, *C. ladanifer* extracts are able to interfere with the molecular process of biofilm initiation even at low doses and highlight the potential use of these extracts to limit the initiation and formation of biofilm and therefore prevent spoilage of specific foods.

On the other hand, *C. ladanifer* extracts have shown also an interesting antifungal activity on *B. cinerea*, *F. oxysporum* and *C. albicans*. Dichloromethane and methanolic extracts were the most active extracts and have exhibited total growth inhibition of both selected fungi. These results are in agreement with already published data (Barros *et al.,* 2013; Greche *et al.,* 2009), confirming the antifungal capacity of *C. ladanifer.* Therefore. Dichloromethane and methanolic extracts of *C. ladanifer* could be considered as a significant source of mycotoxins and their application may offer a renewable source for potentially effective, safe and environment friendly fungicides. Their use as antifungal preservatives may be of great interest to prevent fungal induced spoilage.

Scientific evidence has shown that plants are a source of numerous antioxidant agents. In this context, we have evaluated the antioxidant power of *C. ladanifer* and results clearly showed a highly significant antioxidant activity of the aqueous extract, with an IC50 similar to that obtained with BHT used as a reference product (0.6 mg/ml). The extracts obtained from *C. ladanifer,* collected from different re-

gions, have been previously documented as strong antioxidant candidates (Amensour *et al.,* 2010; Andrade *et al.,* 2009; Barrajón-Catalán *et al.,* 2010; Zidane *et al.,* 2013).

Moreover, it's widely accepted that there's a strong association between antioxidant activity and total phenol content, and a positive correlation between phenolic content and free radical scavenging activity (DPPH) has been reported (Khorasani Esmaeili *et al.*, 2015). In this context, we have assessed the polyphenolic status of *C. ladanifer* extracts and found, as expected, that the aqueous extract is the richest source of polyphenols (TPC= 60. 67 mg GAE/g). As already reported, a close relationship was obtained between the antioxidant capacity of the tested extracts and their phenolic content, confirming once again that the antioxidant activity of plant extracts is mainly due to the presence of polyphenols.

In the present study, results have shown that dichloromethane and methanolic organic extracts have antibacterial and/or antifungal activities whereas the aqueous extract is rich in polyphenols and exhibit a strong antioxidant activity. Extraction with dichloromethane and methanol, known for their low polarity, leads to obtain organic compounds like sesquiterpene lactones, saponins, tannins, and alkaloids widely reported for their antibacterial and antifungal activities (Ahmed and Abdelgaleil, 2005; Barrero *et al.,* 2000; Sieberi *et al.,* 2020). On the other hand, the aqueous extraction leading to select products with high polarity is used selectively to obtain polyphenols widely reported as antioxidant agents (Guerrero *et al.,* 2008).

The present study is very instructive and brings a lot of information of great interest: (1) *C. ladanifer* has multiple biological activities; (2) Each extract has a range of specific activities; (3) extraction with non-polar solvents will give extracts with antimicrobial activity whereas the extraction with a polar solvent will lead to extract polyphenols with antioxidant activity. However, the main limitation of the study is the absence of the chemical composition of each extract. Indeed, chemical composition will inform on the available chemicals on each extract which would be of great interest to identify the active compounds, alone or under a synergic action, for the specific activity.

Conclusion

The present study revealed that *C. ladanifer* contains 2 panels of products: organic compounds with antibacterial and antifungal activities and polyphenols with an interesting antioxidant activity, suggesting the high opportunity of using this plant as an antimicrobial agent and/or free radical scavenge rfor control of plant diseases and food preservation.

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References

Aghraz, A., Gonçalves, S., Rodríguez-Solana, R., Dra, L.A., Di Stefano, V., Dugo, G., Cicero, N., Larhsini, M., Markouk, M. and Romano, A. 2018. Antioxidant activity and enzymes inhibitory properties of several extracts from two Moroccan *Asteraceae* species. *South African Journal of Botany.* 118: 58–64.

Ahmed, S. M. and Abdelgaleil, S. A. M. 2005. Antifungal Activity of Extracts and Sesquiterpene Lactones from *Magnolia grandiflora* L. (*Magnoliaceae*). *Int. J. Agric. Biol*. 7(4): 1–6.

Aleksic Sabo, V. and Knezevic, P. 2019. Antimicrobial activity of *Eucalyptus camaldulensis Dehn*. plant extracts and essential oils: A review. *Industrial Crops and Products.* 132 : 413–429.

Amensour, M., Sendra, E., Pérez-Alvarez, J. A., Skali-Senhaji, N., Abrini, J. and Fernández-López, J. 2010. Antioxidant Activity and Chemical Content of Methanol and Ethanol Extracts from Leaves of Rockrose (*Cistus ladaniferus*). *Plant Foods for Human Nutrition.* 65(2): 170–178.

Andrade, D., Gil, C., Breitenfeld, L., Domingues, F. and Duarte, A. P. 2009. Bioactive extracts from *Cistus ladanifer* and *Arbutus unedo L. Industrial Crops and Products.* 30(1) : 165–167.

Anesini, C., Ferraro, G. E. and Filip, R. 2008. Total polyphenol content and antioxidant capacity of commercially available tea *(Camellia sinensis*) in Argentina. *Journal of Agricultural and Food Chemistry*. 56(19): 9225–9229.

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

Barrero, A. F., Oltra, J. E., Álvarez, M., Raslan, D. S., Saúde, D. A. and Akssira, M. 2000. New sources and anti-

fungal activity of sesquiterpene lactones. *Fitoterapia*. 71(1): 60–64.

Barros, L., Duenas, M., Alves, C. T., Silva, S., Henriques, M., Santos-Buelga, C. and Ferreira, I. 2013. Antifungal activity and detailed chemical characterization of *Cistus ladanifer* phenolic extracts. *Industrial Crops and Products.* 41: 41–45.

Bautista, D. A. 2014. *Spoilage problems | Problems Caused by Bacteria.* In: C.A. Batt and M.L. Tortorello. Encyclopedia of Food Microbiology (Second Edition),Academic Press.p. 465–470.

Bazargani, M. M. and Rohloff, J. 2016. Antibiofilm activity of essential oils and plant extracts against *Staphylococcus aureus* and *Escherichia coli* biofilms. *Food Control*. 61: 156–164.

Benayad, N., Mennane, Z., Charof, R., Hakiki, A. and Mosaddak, M. 2013. Antibacterial activity of essential oil and some extracts of *Cistus ladaniferus* from Oulmes in Morocco. *Journal of Materials and Environmental Science.* 4(6): 1066–1071.

Berahou, A., Auhmani, A., Fdil, N., Benharref, A., Jana, M. and Gadhi, C. A. 2007. Antibacterial activity of *Quercus ilex* bark's extracts. *J. Ethnopharmacol*. 112(3): 426–429.

Bouyahya, A., Belmehdi, O., Benjouad, A., Ameziane El Hassani, R., Amzazi, S., Dakka, N. and Bakri, Y. 2020. Pharmacological properties and mechanism insights of Moroccan anticancer medicinal plants: What are the next steps?. *Journal of Ethnopharmacology*. 147 : 112-198.

Carocho, M., Barreiro, M. F., Morales, P. and Ferreira, I. C. F. R. 2014. Adding Molecules to Food, Pros and Cons: A Review on Synthetic and Natural Food Additives. *Comprehensive Reviews in Food Science and Food Safety*. 13(4) : 377–399.

Ciardi, C., Jenny, M., Tschoner, A., Ueberall, F., Patsch, J., Pedrini, M., Ebenbichler, C. and Fuchs, D. 2012. Food additives such as sodium sulphite, sodium benzoate and curcumin inhibit leptin release in lipopolysaccharide-treated murine adipocytes *in vitro*. *The British Journal of Nutrition*. 107(6) : 826–833.

Cowan, M. M. 1999. Plant Products as Antimicrobial Agents. *Clinical Microbiology Reviews.* 12(4) : 564–582.

Duffy, C. F. and Power, R. F. 2001. Antioxidant and antimicrobial properties of some Chinese plant extracts. *International Journal of Antimicrobial Agents*. 17(6): 527–529.

Erdogrul, Ö. T. 2002. Antibacterial Activities of Some Plant Extracts Used in Folk Medicine. *Pharm Biol*. 40(4): 269–273.

Fisher, R. A. 1935. *The Design of Experiments.* Oliver & Boyd, Edinburgh.

Freitas, K. H. G. and Fatibello-Filho, O. 2010. Simultaneous determination of butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) in food samples using a carbon composite electrode modified with Cu3(PO4)2 immobilized in polyester resin. *Talanta*. 81(3) : 1102–1108.

Galié, S., García-Gutiérrez, C., Miguélez, E. M., Villar, C. J. and Lombó, F. 2018. Biofilms in the Food Industry: Health Aspects and Control Methods. *Frontiers in Microbiology.* 9.

Gedikoðlu, A., Sökmen, M. and Çivit, A. 2019. Evaluation of *Thymus vulgaris* and *Thymbra spicata* essential oils and plant extracts for chemical composition, antioxidant, and antimicrobial properties. *Food Science & Nutrition.* 7(5) : 1704–1714.

Gram, L., Ravn, L., Rasch, M., Bruhn, J. B., Christensen, A. B. and Givskov, M. 2002. Food spoilage—Interactions between food spoilage bacteria. *Int. J. Food Microbiol*. 78 (1): 79–97.

Greche, H., Mrabet, N., Zrira, S., Ismaïli-Alaoui, M., Benjilali, B. and Boukir, A. 2009. The Volatiles of the Leaf Oil of *Cistus ladanifer* L. var. *Albiflorus* and Labdanum Extracts of Moroccan Origin and their Antimicrobial Activities. *Journal of Essential Oil Research.* 21(2) : 166–173.

Guerrero, M. S., Torres, J. S. and Nuñez, M. J. 2008. Extraction of polyphenols from white distilled grape pomace: Optimization and modelling. *Bioresource Technology.* 99(5) : 1311–1318.

Gupta, D., Dubey, J. and Kumar, M. 2016. Phytochemical analysis and antimicrobial activity of some medicinal plants against selected common human pathogenic microorganisms. *Asian Pacific Journal of Tropical Disease.* 6(1): 15–20.

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

Imaida, K., Fukushima, S., Shirai, T., Ohtani, M., Nakanishi, K. and Ito, N. 1983. Promoting activities of butylated hydroxyanisole and butylated hydroxytoluene on 2-stage urinary bladder carcinogenesis and inhibition of ã-glutamyl transpeptidase-positive foci development in the liver of rats. *Carcinogenesis*. 4(7): 895–899.

ISO 14502-1: 2005. 2005. Determination of substances characteristic of green and black tea–part 1: Content of total polyphenols in tea–colorimetric method using Folin-Ciocalteu reagent. *International Organization for Standardization.*

Khorasani Esmaeili, A., Mat Taha, R., Mohajer, S. and Banisalam, B. 2015. Antioxidant Activity and Total Phenolic and Flavonoid Content of Various Solvent Extracts from *In Vivo* and *In Vitro* Grown *Trifolium pratense* L. (Red Clover). *Bio Med Research International.* 1–11.

Kim, S. Y., Kang, D. H., Kim, J. K., Ha, Y. G., Hwang, J. Y., Kim, T. and Lee, S.-H. 2011. Antimicrobial Activity of Plant Extracts Against *Salmonella Typhimurium*, *Escherichia coli* O157:H7, and *Listeria monocytogenes*

on Fresh Lettuce. *Journal of Food Sci.* 76(1): 41–46.
King, T., Cole, M., Farber, J. M., Eisenbrand, G., Zabaras, D., Fox, E. M. and Hill, J. P. 2017. Food safety for food security: Relationship between global megatrends and developments in food safety. *Trends in Food Science & Technology.* 68: 160–175.
Lizcano, L. J., Bakkali, F., Begoña Ruiz-Larrea, M. and Ignacio Ruiz-Sanz, J. 2010. Antioxidant activity and polyphenol content of aqueous extracts from Colombian Amazonian plants with medicinal use. *Food Chemistry.* 119(4): 1566–1570.
Lopez, V., Akerreta, S., Casanova, E., García-Mina, J., Cavero, R. and Calvo, M. 2008. Screening of Spanish medicinal plants for antioxidant and antifungal activities. *Pharmaceutical Biology.* 46(9): 602–609.
Mann, C. M. and Markham, J. L. 1998. A new method for determining the minimum inhibitory concentration of essential oils. *Journal of Applied Microbiology.* 84(4): 538–544.
Mensor, L. L., Menezes, F. S., Leitão, G. G., Reis, A. S., dos Santos, T. C., Coube, C. S. and Leitão, S. G. 2001. Screening of Brazilian plant extracts for antioxidant activity by the use of DPPH free radical method. *Phytotherapy Research.* 15(2): 127–130.
Messaoudi, M., Laglaoui, A., Amzazi, S., Hammani, K., Benbacer, L. and El Mzibri, M. 2019. Cytotoxic, antioxidant and antimicrobial activities of *Nerium oleander* collected in Morocco. *Asian Pacific Journal of Tropical Medicine.* 12(1): 32.
Mostafa, A. A., Al-Askar, A. A., Almaary, K. S., Dawoud, T. M., Sholkamy, E. N. and Bakri, M. M. 2018. Antimicrobial activity of some plant extracts against bacterial strains causing food poisoning diseases. *Saudi Journal of Biological Sciences.* 25(2): 361–366.
Negi, P. S. 2012. Plant extracts for the control of bacterial growth: Efficacy, stability and safety issues for food application. *International Journal of Food Microbiology.* 156(1) : 7–17.
Onaran, A. and Saðlam, H. D. 2016. Antifungal activity of some plant extracts against different plant pathogenic fungi. *International Journal of Advances in Agricultural and Environmental Engineering*. 3(2) : 2349–1523.
Ouattara, B., Simard, R. E., Holley, R. A., Piette, G. J. and Bégin, A. 1997. Antibacterial activity of selected fatty acids and essential oils against six meat spoilage organisms. *International Journal of Food Microbiology.* 37(2–3): 155–162.
Roby, M. H. H., Sarhan, M. A., Selim, K. A.-H. and Khalel, K. I. 2013. Evaluation of antioxidant activity, total phenols and phenolic compounds in thyme (*Thymus vulgaris* L.), sage (*Salvia officinalis* L.), and marjoram (*Origanum majorana* L.) extracts. *Industrial Crops and Products.* 43: 827–831.
Saeed, N., Khan, M. R. and Shabbir, M. 2012. Antioxidant activity, total phenolic and total flavonoid contents of whole plant extracts *Torilis leptophylla* L. *BMC Complementary and Alternative Medicine.* 12(1): 221.
Sanders, T. A. B. 1999. Food production and food safety. *British Medical Journal.* 318(7199): 1689–1693.
Sieberi, B. M., Omwenga, G. I., Wambua, R. K., Samoei, J. C. and Ngugi, M. P. 2020. Screening of the Dichloromethane: Methanolic Extract of *Centella asiatica* for Antibacterial Activities against *Salmonella typhi, Escherichia coli, Shigella sonnei, Bacillus subtilis,* and *Staphylococcus aureus. The Scientific World Journal.* 1–8.
Skotti, E., Anastasaki, E., Kanellou, G., Polissiou, M. and Tarantilis, P. A. 2014. Total phenolic content, antioxidant activity and toxicity of aqueous extracts from selected Greek medicinal and aromatic plants. *Industrial Crops and Products.* 53 : 46–54.
Surekha, M. and Reddy, S. M. 2014. PRESERVATIVES | Classification and Properties. p. 69–75 In: C. A. Batt and M. L. Tortorello (Eds.). *Encyclopedia of Food Microbiology (Second Edition).* Academic Press.
Tekwu, E. M., Pieme, A. C. and Beng, V. P. 2012. Investigations of antimicrobial activity of some Cameroonian medicinal plant extracts against bacteria and yeast with gastrointestinal relevance. *Journal of Ethnopharmacology.* 142(1) : 265–273.
Tsay, H. J., Wang, Y. H., Chen, W. L., Huang, M. Y. and Chen, Y. H. 2007. Treatment with sodium benzoate leads to malformation of zebrafish larvae. *Neurotoxicology and Teratology.* 29(5) : 562–569.
Viuda-Martos, M., Sendra, E., Pérez-Alvarez, J. A., Fernández-López, J., Amensour, M. and Abrini, J. 2011. Identification of Flavonoid Content and Chemical Composition of the Essential Oils of Moroccan Herbs: Myrtle (*Myrtus communis* L.), Rockrose (*Cistus ladanifer L.*) and Montpellier cistus (*Cistus monspeliensis* L.). *Journal of Essential Oil Research*. 23(2) : 1–9.
Yýlmaz, S., Ünal, F. and Yüzbaþýoðlu, D. 2009. The *in vitro* genotoxicity of benzoic acid in human peripheral blood lymphocytes. *Cytotechnology*. 60(1–3): 55–61.
Zidane, H., Elmiz, M., Aouinti, F., Tahani, A., Wathelet, J., Sindic, M. and Elbachiri, A. 2013. Chemical composition and antioxidant activity of essential oil, various organic extracts of *Cistus ladanifer* and *Cistus libanotis* growing in Eastern Morocco. *African Journal of Biotechnology.* 12(34) : 5314–5320.