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Original Article

Composition and Antibacterial Activity of Hydro-Alcohol and Aqueous Extracts Obtained from the *Lamiaceae* Family

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ABSTRACT

Context: Plant from the *Lamiaceae* family are considered as dietary spices and medicinal herbs traditionally used in medicine for the treatment of several pathologies. **Objective:** Evaluation of the *in vitro* antibacterial activity of ethanol and aqueous extracts of nine Moroccan plants from the *Lamiaceae* family against six bacterial strains regularly implicated in toxi-infection. **Method:** The antibacterial activities of hot (HAE), cold (CAE) aqueous extracts and ethanolic extracts (EE) were evaluated using agar-well diffusion method, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and IC_{50} against six food-borne bacteria (*Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella enterica*). Total phenolic and flavonoid contents were assessed as well. **Results:** In the present study, the ethanol extracts were rich in polyphenols, with middling values of flavonoids, and relatively poor in condensed tannins. The presence of saponins, essential oils, irroides, alkaloids, anthocyanins, and aldehydes was recorded. The effect of the extracts was directly bactericidal for *B. cereus* and *L. monocytogenes*; for the other strains, the MBC value was twice higher than that of MIC. However, the ethanol extract (EE) of *O. majorana* and *T. satureioides* showed the highest antibacterial activity. With the principal component analysis, it was demonstrated that the nine *Lamiaceae* family plants possess a powerful antibacterial effect, correlated with their phenolic content. **Statistical analysis :** Analysis of variance was performed by uni-varied ANOVA in the software SPSS 22 Fr. **Conclusion :** The active compounds were thermostable and soluble in ethanol and water. The antimicrobial activities of the plants extracts investigated may contribute to understand their involvement in traditional medicine against many microbial infections.

Key words: Antibacterial Activity, *Lamiaceae* Family, Phenolic Compounds, Flavonoic Content, Hydro-Alcohol Extract, Aqueous Extract.
Key message : Results of the present study support the medicinal usage of the *Lamiaceae* family plants, that can be subjected to identification and isolation of the therapeutic antimicrobials that can be used as sources for new drugs.

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INTRODUCTION

Several aromatic and medicinal plants have been known to synthesize active secondary metabolites, that serve in plant defense mechanisms against micro-organisms and contribute to quality and nutritional value and in providing health-beneficial effects,[1] and have been known to bear antioxidant.[2] This has formed the basis for their applications in some pharmaceuticals, alternative medicines and natural therapies.[3,4] Plants from *Lamiaceae* family are well-known medicinal herbs that are widely used in pharmaceutical products and traditional medicine for the treatment of several pathologies.[5,6,7,8] However, their protective power against some strains of bacteria has not been deeply described. For further identification of substances with antimicrobial properties and streamlining their use, we evaluated in the present study the antibacterial activity of nine Moroccan plants from the *Lamiaceae* family against various bacterial species, with special attention to common germs food toxic-infections.

MATERIALS AND METHODS

Chemicals

All reagents (PCA, MH, Agarose, Resazurin, Ethanol, Folin–Ciocalteau reagent, Folin–Denis reagentsodium carbonate, Gallic acid, potassium acetate, Aluminum trichloride, Quercetin), unless otherwise stated, were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Plant collection and extract preparation

Plants were collected in March, May and Jun 2015 from different region of Morocco (Table 1). The selected parts were dried at 40°C for 15 h. All samples were then ground into a fine powder

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that was passed through an 80-mesh sieve. Aqueous extracts were obtained by extraction of samples (30 g) with distilled water (300 ml), for 60 min at 80°C (HAE) or 24 h min at 25°C (CAE). Hydro-alcohol extracts were obtained by extraction of samples (20 g) with 200 ml of ethanol solution (70%) for 24 h. The extractions were performed three times. After evaporation, the extracts obtained were autoclaved at 121°C for 15 min and stored at 4°C away from light until use. The extracts yield was determined by the following formula:[9]

$$R = (P_x / P_y) * 100$$

R: Extract yield (%), P_x: Extract weight (g), P_y: Plant weight (g).

Qualitative analysis of phytochemicals

Different groups of secondary metabolites such as aldehydes, terpenoids, polyphenols including flavonoids and tannins, alkaloids, saponins and quinone substances were investigated as used by Parekh J. *et al.,* (2007).[10]

Quantification of polyphenols and flavonoids

Determination of Total Phenolic Contents (TPC)

The TPC was determined by Folin-Ciocalteu method.[11] Briefly, 100 µl of extracts (1 mg/ml) were added to 500 µl of 1:10 Folin–Ciocalteau reagent. After 4 min, 400 µl of sodium carbonate 7.5% (m/v: 75 mg/ml) were added. The absorbance at 765 nm was measured after 30 min of incubation by spectrophotometer type VARIAN Cary 50 UV/Vis. The standard range was prepared from a solution of Gallic acid (GA) (5 mg/ml) with concentrations ranging from 0 to 150 µg/ml. Results were reported in Gallic Acids Equivalents (GAE) per g of sample.

Determination of Total Flavonoid Contents (TFC)

The TFC was determined by aluminum trichloride colorimetric method (AlCl3),[12] with modifications. Briefly, 250 µl of extracts (2 mg/ml) were added to 1.4 ml of deionized water, 50 µl of potassium acetate (1 M), 50 µl of aluminum trichloride 10% (m/v), and 750 µl of absolute ethanol. Absorbance at 415 nm was measured (VARIAN Cary 50 UV-Vis) after 30 min of incubation. The standard range was prepared from a solution of Quercetin (10 mg/ml of ethanol 80%), with concentrations ranging f rom 0 to 150 µg/ml. Results were reported in Quercetin Equivalents (QE) per g of sample.

Determination of Total Tannins Contents (TTC)

The TTC was determined by Folin-Denis method,[13] with modifications. Briefly, 100 µl of extracts (1 mg/ml) were added to 500 µl of 1:10 Folin–Denis reagent. After 5 min, 400 µl of Na_2CO_3 7.5% (m/v) were added. After 30 min of incubation, absorbance at 760 nm was measured (VARIAN Cary 50 UV-Vis). The standard range was prepared from a solution of Tannic acid (TA) (10 mg/ml) with concentrations ranging from 0 to 150 µg/ml. Results were reported in Tannic Acids Equivalents (TAE) per g of sample.

Evaluation of antibacterial activity

Antibacterial activity was evaluated at Laboratory of Microbiology of hygiene and food safety department of the Institute Pasteur Tangier – Morocco.

Microbial strains and growth conditions

Six different reference strains and food-borne isolates were used for assessing the plant antimicrobial properties; including Gram-positive and Gram-negative bacteria (Table 2). Fresh cultures were prepared by transferring a loop of cells from the agar slant to a test tube containing 5 ml of brain heart infusion (BHI) (BioRad) and then incubated overnight at 37°C.

Disk diffusion assay

Disc-diffusion assay was used to determine growth inhibition caused by plant extracts.[14] For each strain, inoculums (10^6–10^8 CFU per milliliter), was spread on Mueller–Hinton Agar (MHA) (Bio Rad). Enumeration of bacteria was performed by measuring turbidity at 550 nm (VARIAN Cary 50 UV-Vis). Sterile Whitman's filter discs (N°40; Ø =6 mm), impregnated with 10 µl of different extracts dilutions from the initial concentration of 50 mg/ml, were deposited on the surface of each petri dish. In parallel, an empty disc and an antibiotic disc were used as a negative and positive control respectively. The petri dishes were kept at 4°C for 15 to 20 min to allow the diffusion of the extract, then incubated at 37°C for 18 to 24h, under normal atmosphere, after which, inhibition zones around each disc (> 6 mm) were measured (disc diameter included). Each test was performed in triplicate.

Determination of the Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of ethanol extracts was determined by the method of Mann and Markham, (1998),[15] using resazurin as viability indicator. Different dilutions of the extracts (50; 25; 12.5; 6.25; 3.12; 1.56; 0.8; 0.4; 0.2 and 0.1 mg/ml) were prepared from a stock solution (100 mg/ml). To each well containing 50 µl of the mixture, was added 50 µl of the bacterial suspension (10^6 to 10^8 CFU/ml) prepared in Mueller-Hinton Broth medium (MHB). Plate was then incubated at 37°C for 18 to 20h. After the first incubation step, 5 µl of resazurin (1 mg/ml) was added to each well. Reading results was carried out after further

Table 1: Plants description

Botanical name	Common name	Genre	Origin	Part used	Harvest period	Wild / Cultivated plant
Calamintha officinalis	Manta	*Calamintha*	Marrakech		May	Wild
Lavandula dentata	Khzama Beldia	*Lavandula*	Marrakech		March	Wild
Mentha pulegium	Flio	*Mentha*	Marrakech		March	Wild
Mentha routundifolia	Marceta	*Mentha*	Marrakech		March	Wild
Origanum compactum. Benth	Zaâtar	*Origanum*	Marrakech	Leaves	March	Wild
Origanum majorana	Merdeddouch	*Origanum*	Tangier		March	Cultivated
Rosmarinus officinalis. L	Azir	*Rosmarinus*	Marrakech		Jun	Wild
Salvia officinalis. L	Salmia	*Salvia*	Marrakech		March	Cultivated
Thymus satureioides	Zâitra	*Thymus*	Marrakech		March	Wild

Table 2: Used bacteria

Gram	Strains
Positive	*Staphylococcus aureus 25923*
	Listeria monocytogenes 4032
	Bacillus cereus ATCC 14579
Negative	*Escherichia coli ATCC 25929*
	Pseudomonas aeruginosa 195
	Salmonella enterica

incubation for 2 h at 37°C. The MIC corresponds to the lowest extract concentration, which does not produce change of resazurin staining. Then, the optical density at 550 nm was measured (Epoch BioTek UV-Vis) for IC_{50} determination. The following formula was used to calculate the survival germs percentage:[15]

$$S = [(df - di) / (Df - Di)] \times 100$$

S: survival percentage of germs, *di*: densimat value of experimental tube before incubation, *Di* : densimat value control tube before incubation, *Df* : densimat value after incubation control tube, *df* : densimat value of experimental tube after incubation.

Determination of the Minimal Bactericidal Concentration (MBC)

Plate counting agar (PCA) (BioRad) was seeded with 10 µl of samples from plate wells where there was no resazurin color change. Dishes were then incubated for 18 to 20 hats 37°C. The MBC corresponds to the lowest extract concentration that gives no growth. Moreover, the ratio MBC/CMI of each sample was calculated to assess the antibacterial power.

Statistical Analysis

All *in vitro* experiments were conducted in triplicate and results were expressed as mean ± SD. Analysis of variance was performed by univaried ANOVA for determination of phenolic, flavonoid and tannin contents. Statistical analysis of the antibacterial activity was performed by analysis of variance with two factors in the software SPSS 22 Fr. IC_{50} value were determined by regression analysis. The values $p \leq 0.05$ were considered significant.

RESULTS AND DISCUSSION

Detection of chemical groups

The chemical groups screening showed that the distribution of secondary metabolites differs between species. From a chemical point of view, we noted the presence of essential oils, saponins, iridoïds, alkaloids, anthocyanins, and aldehydes (Table 3).

In general, the low alkaloids content that are known for their toxicity especially in purified state, may justify the low toxicity of these plants. Alkaloids play an important role in biological structures; they are also powerful anti-cholinergic,[16] and well known for their high antibacterial power.[17] Pharmacological activities have been reported about saponins such as antibiotic, antifungal, antiviral, hepatoprotective anti-inflammatory and anti-ulcer.[18,19] While for essential oils, their presence is equated with a bacteriostatic effect, though some of their chemical constituents seem to have bactericidal properties.[20-26] In general, the harvest area and other parameters as the pH and its richness in organic matter, influence greatly the production of alkaloids and other chemical compounds in the plant.[21]

Total Phenolic, Flavonoid and Tannin Contents

These assays were performed to characterize the ethanol extracts (EE) from our samples. The choice of these substances was due to the fact that polyphenols, such as tannins and flavonoids as epigallocatechin, catechin, myricetin, quercetin[21] and luteolin[22] are important antibacterial substances.

Ethanol extracts of the *Lamiaceae* family plants were rich in polyphenols, with average values of flavonoids, and relatively poor in condensed tannins (Table 4). These results support partly those of the literature. However, there was a difference in levels reported by some authors that were very low compared to our results.

This difference was probably due to the difference of the standard used, and the fact that many compounds can react with the Folin-Ciocalteu reagent.[27] The phenolic content of a plant depends also on many intrinsic and extrinsic factors.[28] In general, flavonoids are plant nutrients that when consumed in the form of fruits and vegetables are non-toxic as well as potentially beneficial to the human body. Tannins are basically used

Table 3: Qualitative detection of chemical groups

Plants	Alkaloids	Leucoantho-cyanins	Irridoids	Saponins	Anthraquinone	Anthocyanins	Deoxyoses	Aldehydes	Essential oils
C. officinalis	+	-	+	++	-	-	-	+	++
L. dentata	+	-	+	+	-	-	-	+	-
M. pulegium	+	-	+	+	-	-	-	+	++
M. rotundifolia	++	+	+	+	-	+	-	+	-
O. compactum.	+	-	+	-	-	+	-	-	++
O. majorana	+	-	+	++	-	+	-	+	++
R. officinalis. L	+	-	+	++	-	+	+	++	++
S. officinalis. L	+	-	+	++	-	+	+	++	++
T. satureioides	+	-	+	++	-	-	-	++	++
Total (N)	9	1	9	8	0	5	2	8	7
Total (%)	100	11.1	100	88.9	0	55.5	22.2	88.9	77.8

(-): Negative test; (+): Positive test

Table 4: Polyphenols and flavonoids content of ethanol extracts and their correspondence in literature

Plants	Polyphenols (mg AG/g Ms)	Flavonoids (mg Qu/g Ms)	Total tannins (mg AT/g Ms)	Correspondence in literature		
				Polyphenols (mg AG/g Ms)	Flavonoids (mg Qu/g Ms)	Reference
C. officinalis	176.68±0.45	83.11±0.74	1.80±0.0011	-	-	-
L. dentata	145.61±0.85	74.65±0.95	19.41±0.004	-	-	-
M. pulegium	160.58±0.45	80±0.65	42.43±0.49	206.58± 4.54	77.12± 2.93	[23]
M. rotundifolia	162.06±0.35	68.77±0.85	26.18±0.072	4.6 ± 0.1	3.3 ± 0.1	[24]
O. compactum.	178.12±0.55	89.14±0.98	45.93±0.27	259.12±1.74	109.56±2.68	
O. majorana	170.14±0.24	70.21±0.64	50.22±0.14	4.65±1	-	
R. officinalis. L	148.87±0.45	92.78±0.78	29.78±0.19	190	-	[25]
S. officinalis. L	162.23±0.36	98.66±0.65	30.02±0.025	5.8±1	-	
T. satureioides	167.70±0.19	82±0.74	37.71±0.18	475.00 ± 8.30 456.73±6.94	182.79 ± 3.23 172.79±2.12	[26]

for the treatment of inflammation, leucorrhoea, gonorrhea, burn, piles, diarrhea and antidote in the treatment of alkaloidal poisoning.[29] Despite the observed difference between these plants, the *Lamiaceae* family remains one of the richest families on polyphenols and flavonoids.[30]

Antibacterial activity

Measure of the ethanol extracts pH showed values between 5.47 and 6.67 (n = 3). The normal pH of MH medium according to the manufacturer is 6.6 ± 0.2. These values were close statistically (p> 0.05). Therefore, addition of ethanol extracts does not alter significantly the pH of the MHB medium. For evaluation of antimicrobial potential of these extracts, we preferred to use multiple targets that have, each of them, a particular cell structures and metabolism. Results of antibacterial power against these strains helped to plot the sensitivity curves and determine the different antimicrobial parameters ($IC_{50,}$ MIC and MBC) (Table 5). Results obtained by disk diffusion assay were expressed as mean ± SD. Antibacterial activity was estimated according to the inhibition zone diameter (D) generated by the extract.[31]

By disk diffusion assay, it appears that inhibition diameters varied depending on the ethanol extract used and the bacterial strain tested. Thus, an extract was considered active if it produces an inhibition diameter superior or equal to 15 mm.[31] In another study, an inhibition zone superior or equal to 10 mm sufficient to determine the effectiveness of a sample.[32] Thus, only *O. majorana* and *R. officinalis* followed by *S. officinalis* and *T. satureioides* were active.

In liquid medium, the action of the extracts tested was directly bactericidal for certain bacteria strains (MBC = MIC and MBC/MIC = 1). For others, the MBC value was twice that MIC (Table 5). For both Gram-positive and gram-negative bacteria, the microplate dilution method was more reliable than solid medium.

The comparison of numerical values of our results with other publications was often qualitative, because authors express their results with different units, in µl/ml.[33] in % (v/v) [34] or in mg/ml,[35] which makes quantitative comparison of these results difficult. Qualitatively, our results were correlated with those of the literature. In general, the EEs of all plants were found to be active against all strains tested. The lower antibacterial parameters values were obtained with *L. monocytogenes*; therefore, it was the most sensitive strain. Whereas, *S. aureus 25923* and *P. aeruginosa* 195 were the most resistant strains with high MICs and IC_{50} values, which was consistent with the study of Sqalli *et al.,* 2007[36] on six *Lamiaceae* family plants. Intermediate values were recorded for *E. coli* and *B. cereus*.

We also noted that the activity report MBC/MIC for *P. aeruginosa 195* was still > 2, except for *O. majorana* and *T. satureioides*. Therefore, these extracts were the only ones to have a bactericidal effect in liquid medium against all tested strains and especially against *P. aeruginosa*.

By tukey test, we noticed that there was a significant difference between the IC_{50} of the nine plants (table 6). The plants groups with the same letters did not differ significantly. In addition, by this test, it seems that the specie with the best antibacterial power was *O. majorana*, while the lower effect was attributed to *L. dentata*. For a threshold α = 5%, Fisher's table provides high critical values. This means that the significant difference between IC_{50} mean depended on the plant species used.

The difference between the effects of these extracts, was probably due to differences in the chemical composition, the nature and composition of the microorganisms tested membrane [32] and the influence of the medium. In addition, the results were affected by the method used to evaluate the antibacterial activity. Indeed, Fazeli *et al.* (2007)[37] founded that the diffusion method from wells on agar was more suitable for studying the activity of aqueous and organic extracts than the disc agar diffusion method.

To study the response of the bacterial strains to other types of extracts, especially aqueous one, a comparative study was conducted on the basis of IC_{50}; plants that showed the highest antibacterial potency were selected for this purpose.

Results indicated that EE of *O. majorana* was the most active on the six strains followed by CAE, while HAE showed the higher IC_{50} (Table 7 and figure 1). This was in agreement with results of O. Ertürk (2006)[38] which showed that the ethanol extract of cumin was active against *E. coli ATCC* and *P. aeruginosa 195*, compared to the hydro-methanol crude extract. However, a different result was obtained for *T. satureioides*.

These results indicate that the antibacterial activity depends on the extraction method, the solvent used for extraction, but also on the phytochemical composition of each extract.[37-39] Thermo ability of active compounds can also explain the result of *O. majorana*. In fact, during high-temperature treatments, the phenolic structure of polyphenols can be degraded. In addition, thermal processes may lead to the formation of o-quinone and o-semiquinones, very reactive molecules that can react with nucleophilic groups of proteins and / or polysaccharides.[40] A recent study shows good stability of polyphenols and flavonoids in the plant matrix during high-temperature treatments commonly applied in food. The degradation kinetics observed were slow in comparison with the duration of the extraction.[41]

Table 5: The antimicrobial activity parameters

Plants	Bacterial strain	Ø of zone inhibition (mm) for 10 µl extract (50 mg/ml)	Qualitative decision	MIC (mg/ml)	MBC (mg/ml)	Report CMB/MIC	Decision
C. officinalis	*E. coli*	6	No inhibitory	25	100	4	Bacteriostatic
	S. aureus	6	No inhibitory	12.5	12.5	1	Bactericidal
	L. monocytogenes	6	No inhibitory	25	25	1	Bactericidal
	P. aeruginosa	6	No inhibitory	50	>100	>2	Bacteriostatic
	B. cereus	8±0.13*	No inhibitory	6.25	6.25	1	Bactericidal
	S. enterica	6	No inhibitory	50	>100	>2	Bacteriostatic
L. dentata	*E. coli*	6	No inhibitory	12.5	25	2	Bactericidal
	S. aureus	6	No inhibitory	12.5	50	4	Bacteriostatic
	L. monocytogenes	6	No inhibitory	12.5	50	4	Bacteriostatic
	P. aeruginosa	6	No inhibitory	25	100	4	Bacteriostatic
	B. cereus	6	No inhibitory	25	25	1	Bactericidal
	S. enterica	6	No inhibitory	50	>100	>2	Bacteriostatic
M. pulegium	*E. coli*	9.3±0.21*	No inhibitory	25	50	2	Bactericidal
	S. aureus	6	No inhibitory	25	25	1	Bactericidal
	L. monocytogenes	6	No inhibitory	6.25	6.25	1	Bactericidal
	P. aeruginosa	6	No inhibitory	100	>100	>1	Bacteriostatic
	B. cereus	9±0.22*	No inhibitory	3.12	6.25	2	Bactericidal
	S. enterica	6	No inhibitory	25	25	1	Bactericidal
O. compactum	*E. coli*	13±0.17*	Slight inhibitory	25	100	4	Bacteriostatic
	S. aureus	6	No inhibitory	12.5	25	2	Bactericidal
	L. monocytogenes	6	No inhibitory	3.12	6.25	2	Bactericidal
	P. aeruginosa	6	No inhibitory	25	>100	>4	Bacteriostatic
	B. cereus	6	No inhibitory	6.25	12.5	2	Bactericidal
	S. enterica	6	No inhibitory	12.5	50	4	Bacteriostatic
M. rotundifolia	*E. coli*	6	No inhibitory	25	100	4	Bacteriostatic
	S. aureus	6	No inhibitory	25	25	1	Bactericidal
	L. monocytogenes	6	No inhibitory	25	50	2	Bactericidal
	P. aeruginosa	8±0.11*	No inhibitory	25	>100	>4	Bacteriostatic
	B. cereus	7±0.5*	No inhibitory	25	25	1	Bactericidal
	S. enterica	6	No inhibitory	25	25	1	Bactericidal
S. officinalis	*E. coli*	6	No inhibitory	50	100	2	Bactericidal
	S. aureus	16.3±0.057**	High inhibitory	1.56	3.12	2	Bactericidal
	L. monocytogenes	17±0.28*	High inhibitory	3.12	6.25	2	Bactericidal
	P. aeruginosa	6	No inhibitory	25	>100	>4	Bacteriostatic
	B. cereus	13±0.11*	Slight inhibitory	1.56	3.12	2	Bactericidal
	S. enterica	11±0.51	Slight inhibitory	25	50	2	Bactericidal
T. satureioides	*E. coli*	6	No inhibitory	25	50	2	Bactericidal
	S. aureus	6	No inhibitory	6.25	12.5	2	Bactericidal
	L. monocytogenes	8.1±0.31*	No inhibitory	6.25	12.5	2	Bactericidal
	P. aeruginosa	10±0.043*	No inhibitory	25	50	2	Bactericidal
	B. cereus	13.2±0.23*	Slight inhibitory	<0.5	1	2	Bactericidal
	S. enterica	6	No inhibitory	12.5	25	2	Bactericidal

Continued...

Table 5: Cont'd

Plants	Bacterial strain	Ø of zone inhibition (mm) for 10 µl extract (50 mg/ml)	Qualitative decision	MIC (mg/ml)	MBC (mg/ml)	Report CMB/MIC	Decision
O. majorana	*E. coli*	17±0.1**	Moderate inhibitory	25	50	2	Bactericidal
	S. aureus	15.3±0.1*	Slight inhibitory	1.56	3.12	2	Bactericidal
	L. monocytogenes	22.3±0.09**	High inhibitory	3.12	3.12	1	Bactericidal
	P. aeruginosa	15.2±0.065**	Slight inhibitory	25	50	2	Bactericidal
	B. cereus	13±0.15*	Slight inhibitory	3.12	3.12	1	Bactericidal
	S. enterica	12±0.22*	Slight inhibitory	12.5	25	2	Bactericidal
R. officinalis	*E. coli*	10.6±0.11*	Slight inhibitory	50	100	2	Bactericidal
	S. aureus	8.3±0.057*	No inhibitory	12.5	50	4	Bacteriostatic
	L. monocytogenes	10.2±0.06**	No inhibitory	6.25	12.5	2	Bactericidal
	P. aeruginosa	8±0.19*	No inhibitory	50	>100	>2	Bacteriostatic
	B. cereus	12.2±0.09**	Slight inhibitory	25	50	2	Bactericidal
	S. enterica	10.2±0.09**	Slight inhibitory	25	50	2	Bactericidal

Table 6: Comparative study of antibacterial effect of nine species EE from the *Lamiaceae* family.

	IC_{50} mean ± Er.Std (mg/ml)					
	P. aeruginosa	E. coli	S. aureus	S. enterica	B. cereus	L. monocytogenes
C. officinalis	12,313±0,20 (b)	20,896±0,28 (e)	31,421±0,28 (c)	20,928±0,38 (d)	7,405±0,14 (c)	8,666±0,11 (c)
L. dentata	86,933±0,22 (g)	61,453±0,23 (f)	41,482±0,23 (e)	53,932±0,48 (e)	10,441±0,17 (d)	21,222±0,24 (g)
M. pulegium	12,405±0,15 (b)	11,411±0,28 (c)	54,222±0,24 (f)	16,17±0,21 (c)	17,37±0,25 (g)	13,296±0,22 (e)
M. rotundifolia	20,33±0,66 (d)	12,358±0,36 (c)	86,48±0,14 (g)	21,306±0,30 (d)	36,443±0,25 (h)	17,531±0,23 (f)
O. compactum	81,556±0,26 (f)	18,046±0,23 (d)	95,466±0,15 (h)	22,142±0,44 (d)	13,202±0,31 (e)	10,697±0,24 (d)
O. majorana	6,546±0,24 (a)	1,892±0,27 (a)	13,506±0,44 (a)	10,484±0,30 (a)	1,3487±0,08 (a)	1,327±0,13 (a)
R. officinalis	22,652±0,24 (e)	18,3±0,95 (d)	31,769±0,26 (c)	17,375±0,28 (c)	6,683±0,32 (c)	3,445±0,20 (b)
S. officinalis	17,373±0,15 (c)	8,395±0,24 (b)	34,421±0,15 (d)	14,469±0,20 (b)	14,265±0,17 (f)	8,13±0,25 (c)
T. satureioides	16,519±0,21 (c)	11,752±0,24 (c)	28,752±0,26 (b)	9,305±0,18 (a)	2,467±0,10 (b)	8,698±0,26 (c)
TOTAL	52,947±22,3	18,278±1,72	46,391±2,75	20,679±1,33	12,180±1,05	10,334±0,63
FISHER	20562,56 (p<0,000) **	1749,44 (p<0,000) **	11190,43 (p<0,000) **	1631,67 (p<0,000) **	2298,84 (p<0,000) **	823,09 (p<0,000) **

Groups with the same letters do not differ significantly by tukey test. Er.Std: standard error; **: Very highly significant difference.

Based on IC_{50} of *T. satureioides* extract, the effectiveness ratios IC50-EE/ IC50-HAE for the six strains were between 1 and 4, while IC50-CAE/ IC 50_{HAE} varied between 2 and 8; this means that in the case of *T. satureioides*, HAE was active respectively until 4 to 8 times more than EE and CAE. For *O. majorana* extract, IC50-HAE/ IC50-EE varied between 1 and 135, and IC50-CAE/ IC50-EE were between 2 and 224. Thus, the EE had activity respectively 135 and 224 times more than the HAE and CAE. Results of Mohsen and Ammar (2009)[42] showed that ethanol was the best solvent for phenolic compounds extraction, followed by methanol and finally water. This could explain the difference mentioned below. The active compounds were soluble in ethanol but also in water and their activity does not disappear after treatment of the extract 15 min at 100°C.

The different values obtained allowed to plot the sensitivity curves of each strain for the nine-ethanol extracts (Figure 2). In general, this sensitivity was reflected by a decrease of the overall appearance of all the sensitivity curves. Therefore, there was a significant progressive decrease in the number of germs colonies. Curves were canceled between 20 and 40 mg/ml for *E. coli 25922* and *S. enterica*, between 20 and 50 mg/ml for *S. aureus 25923* and *P. aeruginosa 195*, and between 10 and 30 mg/ml for *L. monocytogenes 4032* and *B. cereus 14579*. The negative correlation between the extract concentration and survival of the six bacterial strains indicated that these ethanol extracts were active in different degrees with a dose-response relationship (Figure 2).

According to these curves and figure 3, it seems that the most resistant strain was *S. aureus* and *P. aeruginosa*, followed by *E. coli* 195 and *S. enterica*. Whereas, *L. monocytogenes* and *B. cereus* showed a remarkable sensitivity towards the majority of extracts.

The high standard error recorded in the case of *P. aeruginosa* indicated that, from a plant to another, there was a significant difference in the survival of this strain (Figure 3). Which means that this biological activity

Table 7: Comparison of the antimicrobial activity of ethanol and aqueous extracts of O. majorana and T. satureioides

Plant	Bacterial strain	IC_{50} (mg/ml)			$IC50_{EE}/IC50_{HAE}$	$IC50_{CAE}/IC50_{HAE}$	$IC50_{CAE}/IC50_{EE}$	$IC50_{HAE}/IC50_{EE}$
		EE	HAE	CAE				
T. satureioides	*E. coli*	11.88±0.7	11.82±0.41	78.11±1.60	1.08	6.6	-	-
	S. aureus	28.66±0.75	6.69±0.55	11.26±0.46	4.28	1.68	-	-
	L. monocytogenes	8.27±0.024	3.88±0.15	24.62±0.40	2.13	6.34	-	-
	P. aeruginosa	16.37±0.85	4.26±0.37	33.63±0.86	3.84	7.89	-	-
	B. cereus	2.35±0.12	3.8±0.41	4.93±0.072	0.61	1.29	-	-
	S. enterica	9.1±0.86	11.76±0.79	7.64±0.69	0.77	0.64		
O. majorana	*E. coli*	1.13±0.07	253.14±3.11	152.48±2.27	-	-	224.01	134.93
	S. aureus	13.96±0.35	13.99±0.97	3.68±0.20	-	-	1.56	0.41
	L. monocytogenes	1.18±0.21	26.57±0.57	21.54±0.42	-	-	22.51	18.25
	P. aeruginosa	6.13±0.96	36.33±0.52	22.5±0.64	-	-	5.92	3.67
	B. cereus	1.13±0.12	30.7±0.25	20.34±1	-	-	27.16	18
	S. enterica	10.55±0.79	11.94±0.6	16.60±0.55	-	-	1.57	1.13

EE: Ethanol Extract; HAE: Aqueous extract at 80°C; CAE: Aqueous extract at 25°C.

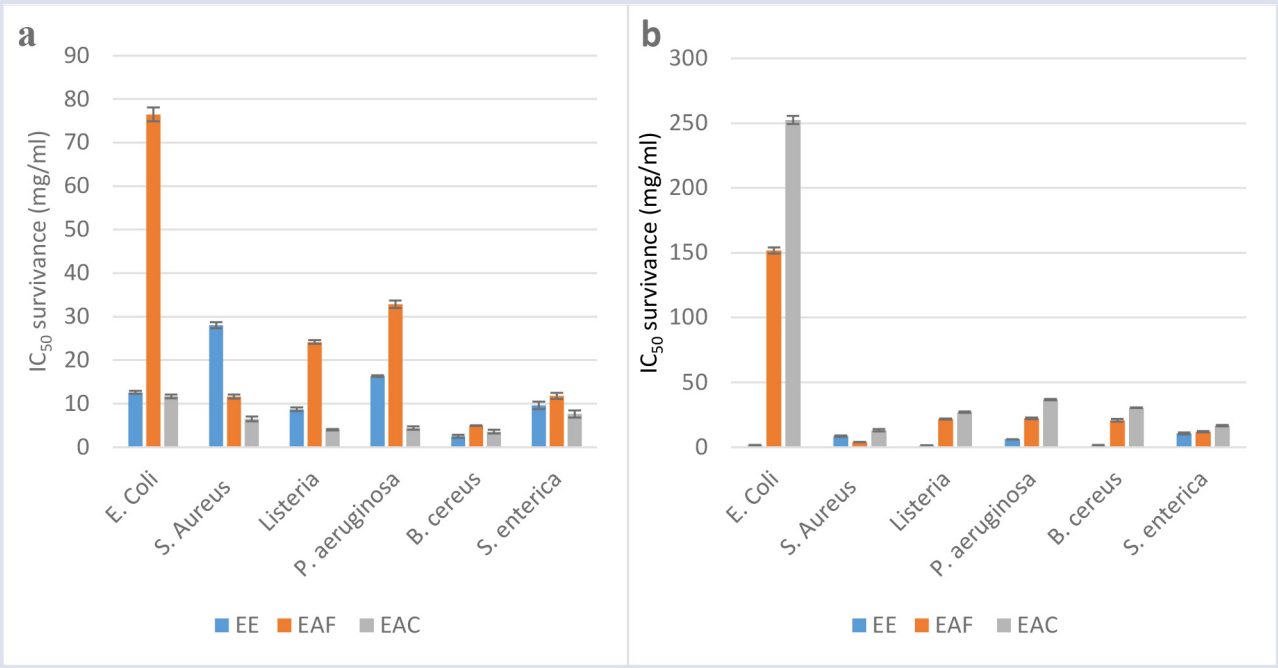

Figure 1: Comparison of the IC_{50} of ethanol and aqueous extracts of *T. satureioides* (a) and *O. majorana* (b). EE: Ethanol Extract; EAC: Aqueous extract at 80°C; EAF: Aqueous extract at 25°C.

was not due to an active principle present in all the *Lamiaceae* family plants, but rather to one or several specific compounds present in each species. While the response of the other strains was almost the same regardless of the plant used. A highly significant difference ($p < 0.000$) was found by comparing the IC_{50} of the six bacterial strains paired two by two (Table 8).

The hypersensitivity of *L. monocytogenes* and *B. cereus* can be explained by the sensibility of Gram (+) to the external environmental changes (temperature, pH, and natural extracts…), due to the absence of the outer membrane.[43] While, even if *S. aureus* is a Gram (+), it showed some resistance to the extracts tested. Some studies have not revealed selective antimicrobial activity towards the Gram (+) and Gram (-).[44] The

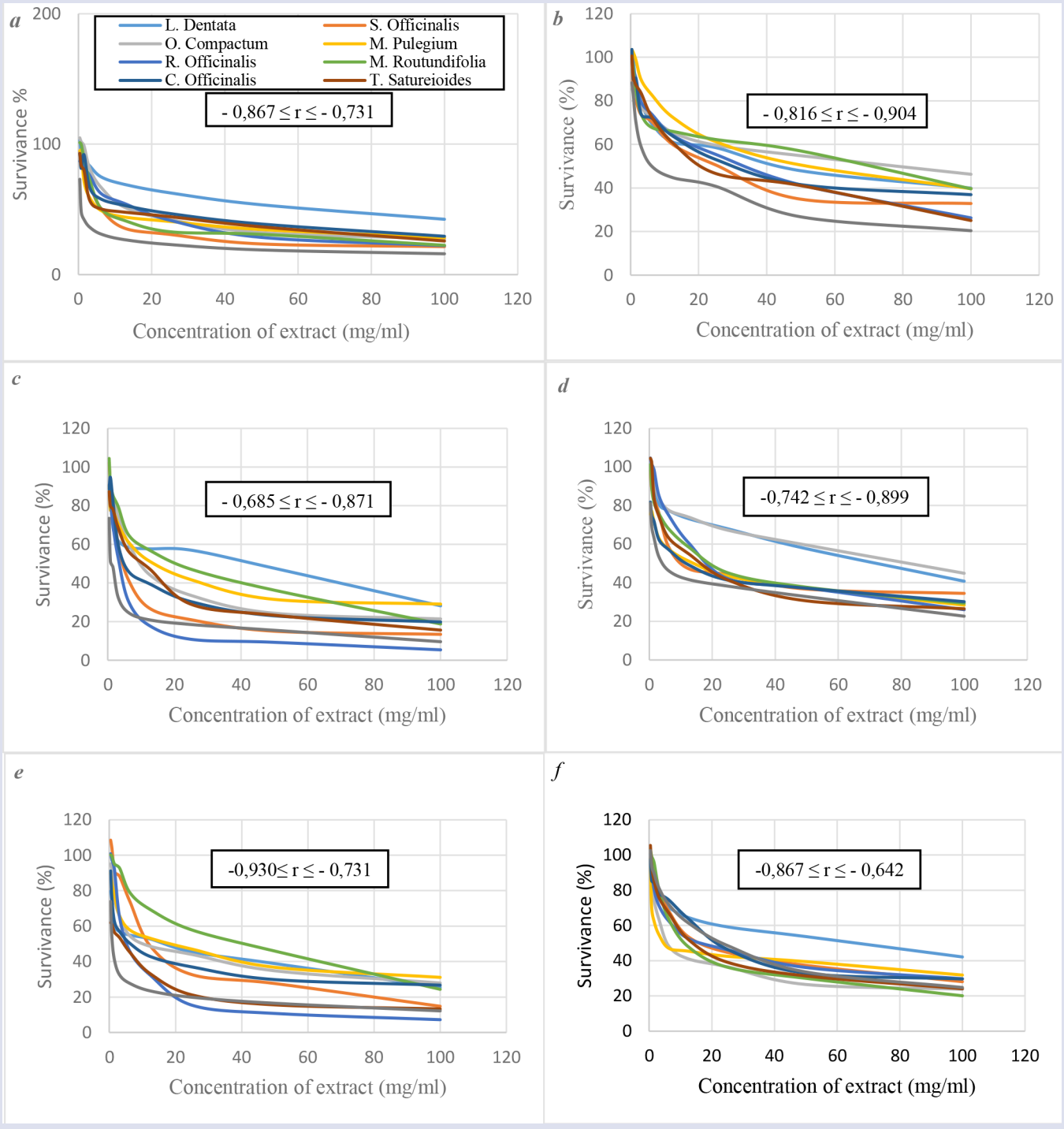

Figure 2: Effect of ethanol extracts on the *in vitro* growth of *E. coli* (a), *S. aureus* (b), *L. monocytogenes* (c), *P. aeruginosa* (d), *B. cereus* (e), *S. enterica* (f).

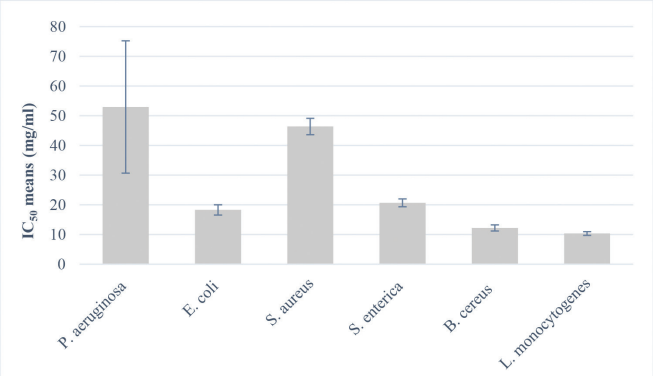

Figure 3: The IC_{50} means representation of ethanol extracts of the *Lamiaceae* family plants for the six strains studied.

Table 8: Comparison of means two by two of the ethanol extracts effect of the different bacterial strains by the student test

Paired samples		T student	Signifiance
P. aeruginosa	*E. coli*	5,88	*p<0,000* **
	S. aureus	5,25	*p<0,000* **
	S. enterica	4,55	*p<0,000* **
	B. cereus	5,79	*p<0,000* **
	L. monocytogenes	7,35	*p<0,000* **
E. coli	*S. aureus*	8,88	*p<0,000* **
	S. enterica	4,15	*p<0,000* **
	B. cereus	2,97	*p<0,004* **
	L. monocytogenes	5,74	*p<0,000* **
S. aureus	*S. enterica*	9,1	*p<0,000* **
	B. cereus	16,32	*p<0,000* **
	L. monocytogenes	14,58	*p<0,000* **
S. enterica	*B. cereus*	5,39	*p<0,000* **
	L. monocytogenes	10,74	*p<0,000* **
B. cereus	*L. monocytogenes*	2,2	*p<0,03* *

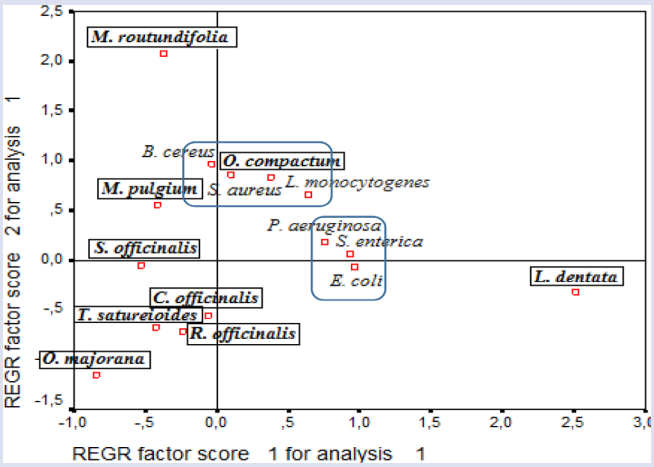

Figure 4: Projection of different variables of the Principal Component Analysis (PCA) on the factorial graph.

resistance of *S. aureus* can be attributed to the ability of the antibacterial agent to diffuse uniformly in the agar [45] while for *P. aeruginosa*, resistance can be assigned to its outer membrane composed of lipopolysaccharides, which form an impermeable barrier to hydrophobic compounds.[46]

To facilitate the analysis of various factors and detect correlation and all independence relationships between different variables, we conducted a principal component analysis (PCA) (Figure 4). Thus, the projections of PCA variables on a factorial graph was obtained, with a graphical representation of the active species. In this projection, we noted the presence of two groups: Gram - were correlated on axis 1, whereas Gram + were correlated on axis 2.

Concerning the distribution of plants, it was found that *R. officinalis* and *C. officinalis* were negatively correlated with Gram +. While, *O. majorana*, *T. satureioides*, *S. officinalis*, *M. pulegium* and *M. rotundifolia* were negatively correlated with Gram -, which means that when the concentration of plant extract increased, the percentage of survival decreased. *Origanum compactum* and *L. dentata* had the least efficient antibacterial effect against all strains studied.

The method used to evaluate the antibacterial activity also affected the results. Fazeli *et al.*, (2007) [47] founded that the diffusion method from wells on agar was more suitable for studying the activity of aqueous, organic and hydro-ethanolic plant extracts than the agar diffusion method. In addition, the effect of a plant substance depends on several factors including the mode of extraction and concentration of active compound.[47] According to the study of Bagre, 2007,[48] some chemical groups are eliminated and others are concentrated during the liquid-liquid extraction. Thus, the alkaloids and sterols that were less concentrated in the aqueous total extract were strongly present in the acetate extract while the concentration of flavonoids remained regardless of the solvent used.

Polyphenols, such as tannins and flavonoids as epigallocatechin, catechin, myricetin, quercetin[49] and luteolin[22] are highly active antimicrobial agents. Another study on 10 methanolic extracts from the *Lamiaceae* family showed that the rosmarinic acid turned out to be the most predominant phenolic compound in all the examined plant organs, and may be considered as the chemotaxonomic marker of this family. This compound is known as an antiviral, antibacterial, antioxidant agent.[50] Extracts of *L. dentata*, *M. pulegium* and *M. rotundifolia* contained an appreciable amount of polyphenols but they showed a weak inhibitory activity against the different studied strains probably because their activity is concealed by the presence of sugars.[51,52]

For the six tested strains, there was no correlation between the phenolic and flavonoid contents of the nine plants and their antibacterial activities ($0.0014 \geq R2 \geq 0.134$), indicating that these compounds were not responsible for the observed antibacterial activity. Similar results (R2 = 0.00) were obtained by Turkmen *et al.*, (2007)[53] in the evaluation of the antibacterial activity of tea extracts. The same result was found for the EBr of cumin with R2 = 0.323.[54] Several studies have shown that a relationship exists between the antioxidant activity and the antibacterial extracts of MAPs,[55] which may in part be responsible for the activity obtained in this study. However, the antibacterial activity of extracts with low antioxidant activity and low levels of polyphenols and flavonoids was probably related to other bioactive compounds. A phytochemical study on essential oils of *O. compactum* and *R. officinalis*, showed that p-Thymol, Eucalyptol and Citronellal were respectively the major components in those investigated plants.[56] These compounds are considered to have a high antibacterial action.[57] Moreover, the total antimicrobial activity of an extract is not always attributed to the major compounds, but can be due to synergistic[57] or antagonistic interactions[58] with other minority compounds.

Recent results have shown that saponins were the compounds with the highest antibacterial effect compared to polyphenols and flavonoids.[59]

The alkaloids, in turn, are known for their high antibacterial power.[17] These alkaloids concentrated in our ethanol extracts could be partly responsible for the achieved antibacterial activity. Oxygenated terpenes and especially terpene alcohols are also highly active antimicrobial agents.[60]

CONCLUSION

Results of this study showed that all the investigated ethanol extracts present antibacterial properties. *Bacillus cereus* and *L. monocytogenes* were the most sensitive to the different extracts, with a dose-response relationship. However, the highest activity was recorded in *O. majorana* followed by *T. satureioides*. These results were directly related to the quantitative and/or qualitative diversity of the extracts compounds. Generally, the total activity is not only attributed to the major compounds, because the interactions between different compounds can exist synergistically or antagonistically [57, 58]. This allows understanding and determining the appropriate chemical formulation for the active compound once identified.

This study could contribute to the knowledge of *in vitro* antimicrobial potential of some plants from the *Lamiaceae* family, which makes these plants and generally MAPs, an ethnobotanical heritage to be preserved and promoted in order to discover new bioactive molecules. Thus, the results of this work seem to be important from the point of view of additional pharmacological applications of medicinal plants belonging to the *Lamiaceae* family.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATION USED

HAE: Hot aqueous extract; **CAE:** Cold aqueous extract; **EE:** Ethanolic extract.

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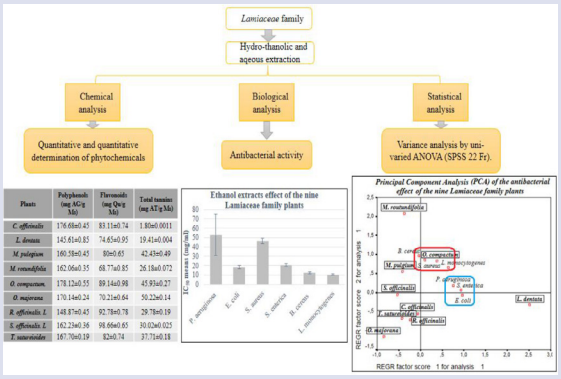
GRAPHICAL ABSTRACT



SUMMARY

- Ethanol extracts of the Lamiaceae family plants were rich in polyphenols, with middling values of flavonoids, and relatively poor in condensed tannins, with the detection of saponins, essential oils, irridoids, alkaloids, anthocyanins, and aldehydes.
- The extracts action was directly bactericidal for B. cereus and L. monocytogenes. For P. aeruginosa, the MBC value was twice that MIC.
- The ethanol extract of O. majorana and T. satureioides showed the highest antibacterial activity against the six strains, with a bactericidal effect.
- The principal component analysis revealed that R. officinalis and C. officinalis were negatively correlated with Gram positive strains. While, O. majorana, T. satureioides, S. officinalis, M. pulegium and M. rotundifolia were negatively correlated with Gram negative stains.

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