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In Vitro Antibacterial Activity of *Thymus satureioides*, *Mentha pulegium, and Origanum vulgare* Essential Oils Against *Escherichia coli* Isolated from Raw Sheep Milk

Fatima BOUAZZA.
University Mohammed V-Agdal, Faculty of Sciences of Rabat, Departement of Biology. Laboratory of Botany, Mycology and Environement, 4 Avenue Ibn Batouta, B.P. 1014 RP, 10000, Rabat, Morocco.
Email: fatima.fsr@hotmail.fr; Mb.: +212675710522

Rachida HASSIKOU.
University Mohammed V-Agdal, Faculty of Sciences of Rabat, Departement of Biology. Laboratory of Botany, Mycology and Environement, 4 Avenue Ibn Batouta, B.P. 1014 RP, 10000, Rabat, Morocco.

Badr SATRANI
Centre de Recherche forestière. BP 763. Agdal. MA-10050 Rabat. Maroc

Mohamed GHANMI
Centre de Recherche forestière. BP 763. Agdal. MA-10050 Rabat. Maroc

Jihane ENNADIR.
University Mohammed V-Agdal, Faculty of Sciences of Rabat, Departement of Biology. Laboratory of Botany, Mycology and Environement, 4 Avenue Ibn Batouta, B.P. 1014 RP, 10000, Rabat, Morocco.

Youssef EL HANI.
University Mohammed V–Souissi. Faculty of Medecine and Pharmacy of Rabat. Laboratory of Microbiology, Avenue Med Belarbi Alaoui Souissi-Rabat. Morocco.

Khadija KHEDID
National Institute of Hygiene. Laboratory of Medical Bacteriology
27, Avenue Ibn Batouta - Rabat, Morocco.

Abstract **– This study aims to evaluate *in vitro* the antibacterial activity of essential oils extracted from *Thymus satureioides*, *Mentha pulegium* and *Origanum vulgare* against *Eshericha coli* isolated from raw sheep milk of Sardi breed using dilution technique in liquid medium in order to determine MIC and MBC and compared to the effectiveness of those extratcs on *E. coli* isolated from patients and sheep meat.**

The yields of essential oils obtained by steam distillation were determined relatively to the plant dry matter and each oil was analyzed by gas chromatography coupled to mass spectrometry (GC-MS) in order to determine its chemical composition.

Results obtained showed that the most important yield was obtained with *M. pulegium* (3.2%) followed by T. *satureioides* (1.85%) and *O. vulgare* (1,6%).

Essential oils tested proved a very significant antibacterial activity against *Escherichia coli* strains. The great effect was observed with *T. satureioides* followed by *O. vulgare* and *M. pulegium* from low concentrations. The determination of the Minimal Bactericidal Concentrations (MBCs) has shown that plants' extracts studied have a bactericidal effect on all strains and their values in mg/ml are identical to those of the Minimal Inhibitory Concentrations (MICs).

The results of the essential oils' chemical compositions demonstrated that components of these oils are monoterpene oxygenated derivatives, monoterpene hydrocarbons and phenols.

The great antibacterial activity, proved by essential oils tested during this research, opens up an area for the use of medicinal plants as preservatives in dairy industry and for therapeutic purposes.

Keywords **– Sheep Milk, Antibiotic Resistance, Essential Oils, Medicinal Plants, *Escherichia coli.***

I. INTRODUCTION

Food security, in which the microbiological quality of food is an essential component, represents a considerable challenge. Diseases caused by collective food poisoning (TIAC) represent globally a significant number of deaths in developing countries.

In Europe, mortality due to food poisoning is low, but a number of 50000 acute gastroenteritis per million inhabitants per year is commonly cited [1]. In Morocco, the majority of cases of food poisoning have been reported more than 86% of bacterial origin including meat [2] and milk which are the most affected foods [3] – [4].

Antibiotics are among the most successful drugs used to cure human and animal infections caused by pathogenic bacteria [5] – [6]. Unfortunately, the hopes raised by the discovery of those pharmaceutical products were quickly reconsidered given the ability of bacteria to develop resistance even of optimal use [7]. The prevalence of antibiotic resistance among food borne pathogens has then increased in recent decades [8] because of the increase in the number of antibiotic-resistant Gram-negative bacteria especially *Escherichia coli* [9] – [10] – [11] – [12] – [13].

Recent studies [14] – [8] – [5] have demonstrated that the patterns of antibiotic usage may greatly affect the number of resistant microorganisms which occurs in an environment. An earlier study in Kenya [15] found betalactam antibiotics residues to be prevalent in milk. Evidence has been found which indicates that resistance strains of pathogens can be transmitted to humans through food [16] – [17].

On the other hand, several questions are currently raised about the safety of chemicals used in medicine or food

industry. Many substances produced during the manufacturing process and storage of food under the action of free radicals lead to oxygenated change of taste, odor and color and consequently loss of quality and food safety.

The human use of aromatic and medicinal plants and essential oils is so ancient. They are used traditionally to ward off bad luck, to heal, for relaxation, food flavoring and preserving of food [18] Therefore, many research groups were interested in the functional role plant extracts especially essential oils play. These latters are appreciated for their bioactive efficacy as fungicides [19], bactericides [20], anti inflammatory [21], antiviral [22] and antioxidants [23].

These last years, the use of ethnobotanical information in the search for medicinal plants has greatly attracted attention from the scientific community [24]. The search for biologically active extracts from plants traditionally used is relevant and has been highlighted by Leaman et *al.* (1995) [25] in their study of plants traditionally used in Borneo in the treatment of malaria. This approach has also led to the discovery of rich sources of compounds with antiviral and antibiotic activities [26] – [27] and then molecules witch can be used as food preservatives [28].

In developing countries, such as Morocco and rural societies, the use of medicinal plants is both a necessity and a valuable resource and also provides a real alternative for systems of care and health. Thanks to its special geographical position, Morocco enjoys a bioclimate for a rich and diverse vegetation. More than 4200 species and subspecies have been identified, including a hundred endemic and more than 9% of the total Moroocan flora are used as medicinal plants and aromatic interests.

Our study is part of a plant resource development in Morocco and the improvement of the hygienic quality of milk in order to identify new food preservatives available locally, against pathogens commonly involved in food contamination. It aims to assess the *in vitro* resistance or susceptibility of 10 *Echerishia coli* strains isolated and collected manually from raw sheep milk of Sardi breed in Morocco to *Thymus satureioides*, *Mentha pulegium* and *Origanum vulgare* essential oils ; they were compared to 5 *Escherichia coli* strains isolated from patients and 4 strains from raw sheep meat.

II. Material and Methods

1) Plant material

Harvest of *Thymus satureioides*, *Mentha pulegium* and *Origanum vulgare* samples was made between May and July 2011: *T. satureioides* was harevested in the region of Agadir ; *vulgare* in Masmoda and *M. pulegium,* in Ouazzane (Central Morocco). These species were identified by Dr. Aafi, a botanist at Forest Research Centre of Rabat, Morocco.

2) Extraction of essential oils

The extraction of essential oils from aerial part (stems, leaves and flowers) was performed by steam distillation using a Clevenger-type apparatus [29]. Three distillations were carried out by boiling, for one hour thirty minutes ; 200 g of fresh plant material was placed in 1 L water inside a 2 liter flask**.** The essential oil yield was determined in relation to the dry matter and estimated from three dried samples (30 g) for 48 h in an oven at 60°C. The essential oil was then stored at 4°C in the dark in the presence of anhydrous sodium sulfate [30].

3) Microorganisms tested

10 *Escherichia coli* strains were isolated from the raw sheep milk on Desoxycholate Lactose Agar (DL, Oxoid, England culture medium (selective culture medium to detect bacteria belonging to the family Enterobacteriaceae). The identification of *E.coli* strains was performed by determining the biochemical profile using respectively the kit API 20 E (Biomerieux, France). Seeding and reading these kits were made according to the manufacturer's instructions. The identification of each isolate was obtained using the Api Plus software [31] – [32] – [33] – [34] – [35].

Escherichia coli 1, *Escherichia coli ATCCS, Escherichia coli 2, Enterohemorragic Escherichia coli (EHEC) O157* and *Enteropathohenic Escherichia coli (EP)* isolated from humans and *Enterotoxinogenic Escherichia coli (ETEC)*, *Enteropathogenic Escherchia coli (EPEC*), *Enteroagregatif Escherichia coli (EAggec)* and *Enteroinvasive Escherichia coli* isolated from raw sheep meat; in the Bacteriology Laboratory of the National Institute of Hygiene of Rabat were also tested.

4) Evaluation of the antibacterial activity of essential oils

With the evaluation of antimicrobial activity, we aim to determine the antibacterial parameters (minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)) of different essential oils studied. To do this, we used the method of broth microdilution coupled with the spread on solid medium as described by Chabbert and Daguet [36].

Determination of MIC essential oils against bacterial strains was done by microtiter technique with flat bottom sterile plates (Bio-Rad), as described by Eloff (1998) [37], ; tetrazolium (MTT: 3 - (4,5 - dimethylthiazol-2-yl) - 2,5 diphenyltetrazolium bromide (Sigma, St. Louis, MO) was used as an indicator of viability.

100 µl of BHI (Brain Heart Infusion) was deposited at each well, and 90 µl of the essential oil mixed with Tween 80 or successive dilution of ½ was then added to each well. The latter was then inoculated with 10 µl of a microbial suspension (equivalent to 10^6 cells / ml).

After incubation at 37°C for 24 h, 10 µl of the MTT, freshly prepared with 0.4 mg / ml of sterile saline, was added to each well. The plate was incubated again for 10 to 30 min at 37°C. Wells, where growth occurred, showed a blue-violet color. Negative controls were prepared in isolated wells by adding strains tested in only the adequate culture medium without extract.

In addition, the determination of the MBC requires streaking 100 µl of the wells contents at a concentration greater than or equal to the MIC in the dilution series previously established on nutrient agar. Thus, the MBC was determined after incubation for 24 h at 37°C. This was the lowest concentration that completely inhibited growth.

5) Chromatographic analyses

Chromatographic analysis was performed on a gas chromatography with electronic pressure control type Hewlett Packard (HP 6890 series) equipped with a capillary column HP-5 (30 m x 0.25 mm), having a film thickness of 0.25 microns, an FID detector set at 260°C and fed with a mixture of gases and an H2/Air split-splitless injector set at 275°C. The injection mode was split (split ratio: 1/50). The gas used was nitrogen with a flow rate of 1.7 ml min^{-1}. The column temperature was programmed from 50 to 250°C at 4°C min^{-1}. The device is controlled by a computer system type "HP ChemStation" that manages the operation of the device and monitors the chromatographic analysis. The identification of the components was carried out on the basis of Kováts indices (IK) and the gas chromatography coupled to mass spectrometer (GC-MS). The latter is performed on a gas chromatography type Hewlett Packard (HP 6890 series) coupled to a mass spectrometer (HP 5973 series). Fragmentation is performed by electron impact at 70 eV. The column used was a HP-5MS capillary column (30 m x 0.25 mm), with film thickness of 0.25 microns. The column temperature was programmed from 50 to 250°C at 4°C min^{-1}. The carrier gas was helium with a flow rate of 1.5 ml min^{-1}. The injection mode was split mode (split ratio 1/70). The device was connected to a computer system that manages a library of mass spectra NIST 98 [37].

III. Results

1. Essential oils yields

Average yields of essential oils were calculated in milliliters relative to 100 g of dry plant material. These rates are 1.85% for *Thymus satureioides,* 3.2% for *Mentha pulegium* and 1.6% for *Origanum vulgare*. These rates indicate that the performance of *M. pulegium* is the highest. As a result, it should be interesting to consider its use subject to good performance of the antibacterial activity.

2. Antibacterial activity of essential oils tested

The results of the antibacterial effect test of different essential oils used are summarized in Figure 1. Note that all essential oils have an inhibitory activity against the bacteria tested.

Moreover, the MICs determination of these natural extracts showed varying levels of action that could be mainly due to the chemical composition of each essential oil.

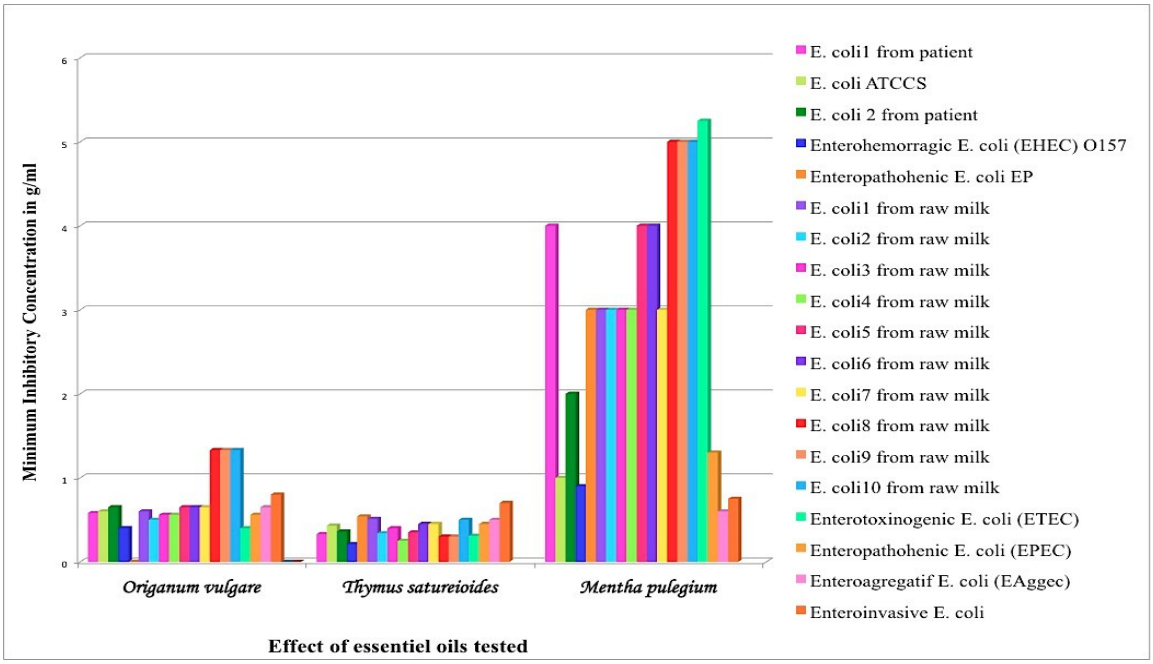

Fig.1. Minimum inhibitory concentrations of essential oils tested on the growth of Escherichia coli strains

The inhibitory effect of these extracts also proved to be bactericidal against all species studied (Table I). Analysis of the antibacterial test results obtained *in vitro* with the four essential oils shows a remarkable efficiency of these natural products against pathogenic strains studied with difference in efficacy depending on the species and essential oil dilutions.

In Figure 1, *Thymus satureioides* essential oil showed the greatest antibacterial effect against all strains studied. Indeed, *Escherichia coli (EHEC) O157* was the most sensitive strain with a MIC of about 0.21 mg/ml followed by *E. Coli* isolated from raw sheep milk whose MICs range between 0.25 and 0.51 mg/ml. By cons,

Enteroinvasive E. coli has been resistant with a MIC of about 0.7 mg/ml.

Origanum vulgare essential oil showed also a great antibacterial action against the strains studied. Thus, it was more active on *E. coli (EHEC) O 157* isolated from patients and E. *coli (ETEC)* isolated from raw sheep meat (MIC = 0.40 mg/ml), which is the most sensitive. *E. coli* from raw sheep milk was totally inhibited from 0.50, whose *E coli 8, E coli 9 and E coli 10* were inhibited at

high concentration of 1.33 mg/ml. Whereas, the rest bacteria were inhibited from MIC of 0.65 to 0.80 mg/ml.

The determination of MICs for *Mentha pulegium* essential oil showed antibacterial activity levels that varied based on the strains tested. The essential oil of this plant could completely inhibit bacterial growth of all *E. coli* tested with MICs that fluctuate between 0.6 and 5.25 mg / ml. In the presence of the latter extract, *E. coli (EAggec)* was the most sensitive while *E. coli (ETEC)* was more resistant. *E. coli* from raw sheep milk was completely inhibited from 3 to 5 mg/ml.

Based on the MIC, we note that essential oils used have different activities against strains of *Escherichia coli*. The activity of *Thymus satureioides* had the best performance followed by the essential oil of *Origanum vulgare* and *Mentha pulegium,* respectively.

To characterize the antibacterial activity of these essential oils, cultures of each strain that have not started growth in the presence of the plant extract were transferred to a neutral culture medium without extract. After the incubation time, we note that the antibacterial activity of all the plants' extracts is bactericidal against all strains of *Escherichia coli* studied. Indeed, no resumption of growth was observed, which proves the bactericidal effect of these extracts (Table I).

Table I : Minimum bactericidal concentration of essential oils tested on the growth of E. coli strains Minimum bactéricidal concentrations in mg /ml

Bacteria tested	***O. vulgare***		***T. Satureioides***		***M. pulegium***	
E. coli1	0.58	B	0.33	B	4	B
E. coli ATCCS	0.6	B	0.43	B	1	B
E. coli 2	0.65	B	0.36	B	2	B
E. coli (EHEC) O157	0.4	B	0.21	B	0.9	B
E. coli EP	0.6	B	0.54	B	3	B
Escherichia coli1	0.5	B	0.51	B	3	B
Escherichia coli2	0.5	B	0.34	B	3	B
Escherichia coli3	0.56	B	0.4	B	3	B
Escherichia coli4	0.56	B	0.25	B	3	B
Escherichia coli5	0.56	B	0.35	B	4	B
Escherichia coli6	0.65	B	0.45	B	4	B
Escherichia coli7	0.65	B	0.45	B	3	B
Escherichia coli8	1.33	B	0.3	B	5	B
Escherichia coli9	1.33	B	0.3	B	5	B
Escherichia coli10	1.33	B	0.5	B	5	B
E. coli (ETEC)	0.4	B	0.31	B	5.25	B
E. coli (EPEC)	0.56	B	0.45	B	1.3	B
E. coli (EAggec)	0.65	B	0.5	B	0.6	B
Enteroinvasive E. coli	0.8	B	0.7	B	0.75	B

B : Bactericidal effect

3. Chemical compositions

The chromatographic analysis of plants' essential oils tested revealed the presence of volatile compounds: 24 for *T. satureioides,* 23 for *M. pulegium* and 18 for *O. vulgare,* respectively. These components represent : 99.55 %, 99 .7% and 97.64% of each essential oil cited, respectively. The combination of volatile compounds of these species varies in terms of diversity and concentration (Table II).

Table II: Chemical compositions of essential oils tested

N°	Ik	Component	% of components		
			Thymus satureioides	***Origanum vulgare***	***Mentha pulegium***
1	919	Tricyclene	0.43	-	-
2	923	α-thujene	1.18	-	-
3	930	α-pinène	6.04	0.9	0. 39
4	945	Camphene	10.32	9.7	
5	952	Cyclohexanone-3-méthyl	-	-	0.28
6	959	1-octen-3-ol	-	0.9	-
7	970	Sabinene	0.20	0.5	0.45
8	973	β-pinène	1.48	0.4	0.16
9	988	Myrcène	1.14	1.4	0.99

10	1001	δ-2-carène	-	-	0.16
11	1018	α -terpinène	1.02	0.6	-
12	1019	limonene	-	0.9	1.84
13	1022	β -phyllandrene	-	0.8	-
14	1025	o-cymène	4.30	0.5	-
15	1029	p-cymène	1.24	25.9	-
16	1034	E- β -cyméne	-	0.5	-
17	1054	Menthone		-	0 .39
18	1057	γ-terpinene	5.78	2.8	-
19	1072	p-mentha-3,8-diéne	-	-	1.23
20	1084	Terpinolène	0.23	-	-
21	1096	Linalool	2.83	1.9	-
22	1098	α -thujone	-	0.9	-
23	1160	p-cymen-8-ol	-	0.8	-
24	1164	Bornéol	**25.98**	-	-
25	1065	Terpen-4-ol	-	0.3	-
26	-	p-menthéne-5-one	-	_	1.37
27	1173	Menthol	-	-	0.74
28	1175	terpin-1-ol	1.88	-	-
29	1189	α -terpineol	8.75	0.9	-
30	1194	dihydrocarvone	-	-	2.57
31	1195	Verbanol	0.46	-	-
32	1226	Cis carveol	0.48	-	-
33	1238	R(+)-ulègone	-	-	**80.33**
34	1240	Carvone	0.62	-	-
35	-	Eucarvone	-	-	3.75
36	1252	pépritone	-	-	0.97
37	1283	α -Terpin-7-al	0.89	-	-
38	1288	Thymol	0.23	-	-
39	1298	Carvacrol	**17.54**	**31.5**	-
40	1365	myrtenyl acetate	-	**16.4**	-
41	1410	β-caryophylene	-	0.5	-
42	1415	Caryophellene	5.98	0.5	-
43	1419	Caryophylléne	-	-	0.95
44	1453	NI	0.55	-	-
45	1509	δ-cadinene	-	0.2	-
46	1630	γ-eudesmol	-	-	0.48
47	1649	α-eudemol	-	-	0 .59

IV. DISCUSSION

The use of medicinal plants with antimicrobial properties constitutes one of the most interesting avenues to explore [38]. It is in this context that we are interested in the study of the antimicrobial activity of *Thymus satureoides*, *Mentha pulegium* and *Origanum vulgare* extracts known for their use in traditional medicine and their diverse therapeutic (anti-inflammatory, antispasmodic, sedative, antibacterial and antioxidant) activities [39].

Essential oil yields of *T. satureioides, M. pulegium, O. vulgare and A. triphylla* are : 1.85%, 3.2% and 1.6%, respectively. These yields are relatively average compared to some plants that are used industrially as a source of essential oil. Our results are comparable to those obtained by El Ouali Lalami and *al* (2013) [40], who had obtained a yield of 1.1% for *T. satureioides* essential oil, then to those obtained by [41] who had a yield of 2.3% for *Mentha pulegium* essential oil collected from the same region, and to Souza and *al.* (2007) [42] concerning *O. vulgare* essential oil. In fact, many factors influence the performance, content, features, physico-chemical and chemical composition of essential oils as: species, environmental conditions, technical extraction, drying, time and place of harvest, tillage practices and the age of the plant [43] – [44] – [45].

Escherichia coli strains, food contamination bacteria commonly found in the digestive tract of humans and animals were tested in this study. In fact, it is known that most of *E. coli* strains are harmless. Some, however, such as *Enterohemorrhagic E. coli (EHEC)* can cause severe foodborne illness. Transmission to humans occurs mainly through consumption of contaminated foods, such as raw

ground or undercooked meat, raw milk and raw vegetables [3] – [2] – [4].

Esherichia coli isolated from raw sheep milk of Sardi breed was determined through the essenial oil susceptibility on broth microdilution coupled with the spread on solid medium method. The results showed that essential oils used have, to varying degrees, antibacterial activities and inhibitory effects on *in vitro* growth of these bacterial strains.

The essential oils of *Thymus satureioïdes* showed an interesting antibacterial activity at the very low concentrations used (from 0.2 to 0.7 mg/ml) especially against *E. coli* (EHEC) O157. These results confirm those obtained by El Ouali Lalami and *al* (2013) [40], which demonstrated the effectiveness of *T. satureioides* essential oil on the growth of *E. coli* at MICs of 1.5 mg/ml. Indeed, Amarti et al. (2009) [46] reported that the extract of different species of thyme has a significant antimicrobial activity against Gram positive and Gram negative bacteria at low concentrations.

O. vulgare essential oil has completely inhibited the growth of *E. coli* at low concentrations ranging from 0.4 to 1.33 mg / ml. Our results are similar to other researchs that have proved that this plant extract have a strong antibacterial activity, especially against multi-resistant bacteria as *E. coli* [47] – [44] – [41] – [48].

Analysis of *Mentha pulegium* oil components coupled with their MICs on all strains has been an interesting screening since it allows the selection of components that will be further tested separately. Then, our results showed that essential oil from this plant has an inhibitory action against bacteria studied with an antibacterial potential remarked at relatively high concentrations (0.6 to 5.25 mg/ml) compared to the previous two extracts reported. These results are consistent with the work of several researchers who have demonstrated that *M. pulegium* has an inhibitory effect against a wide range of species of bacteria involved in food intoxications [49] – [50] – [51]. According to Hmiri et *al*. (2011) [41], the main biologically active constituents of *M. pulegium* are monoterpens. Those compounds could be responsible for the observed activity [51].

The *in vitro* antibacterial activities of essential oils used may be due to a synergitic effect of some components. The major essentiel oils'constituents are borneol (26,28%) and carvacrol (17.24%) for *T. satureioides* while the essential oil of *M. pulegium* is characterized by the presence of pulegone as the main component with a content of 80.33 %. These results are similar to most of studies already carried out in Morocco [52] – [53] – [41].

The use of medicinal plants with antimicrobial properties is then a more interesting avenues to explore. It is in this context that we are interested to study also the antibacterial activity of *O. vulgare* essential, plant known for its use in morrocan traditional medicine, specially with milk and its derivatives.The main components of *O. vulgare* essantial are carvacrol and Myrtenyl. The first one presents the highest percentage with an average rate of approximately 31.5%, followed by Myrtenyl acetate with a rate of 16.5%. The high rate of carvacrol recorded, gives this essential oil the powerful antibiotic, antiseptic and antifungal properties [54]. We understand why this natural molecule is highly sought after in the field of pharmacy and of aromatherapy.

V. Conclusion

Preliminary results of antibacterial study of *Thymus satureioides*, *Mentha pulegium* and *Origanum vulgare* essential oils showed that the four essential oils tested proved to have important antibacterial activities *in vitro* against highly pathogenic bacteria responsible for food poisoning. The higher antibacterial effect was observed in the case of *T.satureioides* while the lowest was observed with *M. pulegium.* These actions may be related to the presence of major constituents and a synergitic effect of some components, including terpenes and phenols.

This is because natural products tested are most effective, cheap to produce and could be analyzed easily as preservative in the food industry, and they have no side effects on the well being of humans or animals. It is advisable to conduct a thorough analysis of the action mechanisms of these essential oil compounds and a more advanced research on the synergy of basic compounds and the combination of essential oil extracts in food products. Other studies are needed to estimate the toxicity of these plants' extracts and assess their potential *in vivo ;* to seek strategies for potential application of these in the conservation of milk and its derivatives and the fight against microorganisms responsible for food poisoning.

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AUTHOR'S PROFILE

Fatima BOUAZZA

Ph.D. student in Rabat Morocco, Departement of biology. University Mohammed V-Agdal, Faculty of Sciences of Rabat, Laboratory of Botany, Mycology and Environement, 4 Avenue Ibn Batouta, B.P. 1014 RP, 10000, Rabat, Morocco.
Email: fatima.fsr@hotmail.fr; Mb.: +212675710522