

Application of *Mentha suaveolens* essential oil as an antimicrobial agent in fresh turkey sausages

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ABSTRACT

The aim of this study is to evaluate the antimicrobial effect of *Mentha suaveolens* essential oil against pathogenic bacteria in fresh turkey sausages. The essential oil was extracted by hydrodistillation. The antibacterial activity was carried out by agar diffusion and microplates methods against *Escherichia coli*, *Salmonella*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, and *Streptococcus faecalis*. The antioxidant activity was carried out by ferric reducing antioxidant power and free radical scavenging activities against 2,2-diphenyl-1-picrylhydrazyl. The antimicrobial effect on sausages was conducted by the enumeration of *S. aureus* and *E. coli* during the storage period of fresh sausages manufactured with different concentrations of essential oil. The results showed that the essential oil of *M. suaveolens* has an antibacterial effect against Gram-negative and Gram-positive bacteria in addition to its antioxidant activity ($EC_{50} = 3.95 \pm 0.03$ mg/mL and $IC_{50} = 3.11 \pm 0.02$ mg/mL). Moreover, the addition of essential oil to fresh sausages has a significant effect against the tested pathogenic bacteria. The present data clearly demonstrate that the essential oil of *M. suaveolens* has a remarkable antimicrobial and antioxidant activities and can be used as a food additive to extend the shelf life of food products.

1. INTRODUCTION

Sausage is a processed meat product, consists of chopped meat, water, binders, and seasonings, stuffed into natural or artificial casings. Its manufacture involves a number of handling steps, which increase the chances of its contamination by pathogenic bacteria (*Escherichia coli* O157:H7, *Staphylococcus aureus, Salmonella...*) through the contaminated raw meat, ingredients, processing equipment, and from postprocessing contamination [1]. These pathogens have also been detected in raw meat and have been shown to survive certain sausage manufacturing processes [2-4]; also, they are implicated in a large number of foodborne diseases outbreaks [5,6]. Moreover, the fresh sausage does not undergo heat treatment and has a high water activity giving it a short shelf life and subjecting it directly to the action of the microorganisms.

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The manufacturers of sausages use the synthetic food conservatives to extend its shelf life, but these latter are limited in several countries because of their undesirable effects on health. For this reason, the use of natural additives is a primordial perspective to improve the bacteriological and organoleptic quality of food. Furthermore, the use of aromatic plants is a best choice with double effects; it contributes to the valorization of plant heritage of the regions threatened by poverty and provides the biological substances capable of replacing artificial ones. Some studies have shown that the treatment of sausages with essential oils makes it possible to inhibit the proliferation of pathogenic bacteria and to extend the shelf life of this product [7-9].

In Morocco, the Aromatic and Medicinal Plants sector records a sharp socioeconomic growth in recent years. It is one of the richest phytobiological fields in the world due to its diversity: 4200 species of plants including 800 endemics among them 382 species are known for their medicinal and/or aromatic use [10,11]. The genus *Mentha* is one of the important elements of Lamiaceae family; it is represented by 19 species and 13 natural hybrids [12]. Among its species include *Mentha suaveolens* which located in North Africa, Europe, America, and Japan [13]. This plant has a wide range of benefits: Analgesic, stomachic, choleretic, tonic, carminative action, antispasmodic, hypotensive, sedative, insecticide, anti-inflammatory, hepatoprotective, antimicrobials, acetylcholinesterase, and monoamine

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oxidase inhibitors, it is applied also in the treatment of digestion problems, influenza, respiratory diseases, rheumatisms, skin diseases, irritation, nausea, anorexia, and bronchitis [14-17].

Essential oils are complex natural mixtures of volatile secondary metabolites isolated from plants by hydrodistillation. Its chemical composition depends on the region and season and consists mainly of terpenoids including monoterpenes, sesquiterpenes, and their oxygen derivatives [13,17-19]. Formerly, essential oils of mint were used by the Egyptians, Romans, and Greeks populations as fragrances, food flavors, deodorizers, and pharmaceuticals products [20]. In recent years, some studies have indicated that the essential oils of M. suaveolens have an inhibitory effect on the proliferation of pathogenic bacteria [21]. Moreover, it has been reported that these essential oils possess also the antioxidant properties [22]. Thereby, in this study, we proceeded to (i) extract the essential oils of *M. suaveolens* harvested from Meknes Region in Morocco and (ii) evaluate its antibacterial and antioxidant activities, in addition to its effect in the survival of E. coli and S. aureus in fresh sausages. Moreover, it is important to note that the particularity of this work lies in the lack of preestablished research work on the effect of M. suaveolens essential oil on food conservation.

2. MATERIALS AND METHODS

2.1. Plant Material and Essential Oil Extraction

Samples were collected during March 2015. Extraction of essential oil was carried out by hydrodistillation for 3 h using a Clevenger-type apparatus. The plant material (about 100 g) was placed in a flask (1 L) together with double distilled water (500 mL). The mixture was boiled for 3 h and the collected essential oils were dried over anhydrous sodium sulfate and stored in sealed glass vials at 4°C in the dark until used.

2.2. Antibacterial Activity

The essential oils are insoluble in water and to allow its diffusion into the culture medium, it was solubilized in dimethyl sulfoxide (DMSO, Sigma-Aldrich). The antibacterial activity was performed on six strains belonging to Gram-negative and Gram-positive bacteria. *Salmonella* spp. and *S. aureus* were isolated from food in Laboratory of Microbiology and health at the Faculty of Sciences, Meknes. However, others pathogenic bacteria were collected from the Regional Hospital of Meknes (Mohammed V Hospital). Furthermore, *E. coli* (ATCC 25922) was used as a reference strain of this study. These strains are resistant to multiple antibiotics and their profile was determined according to the agar disk diffusion standard method [23] [Table 1].

2.2.1. Disc diffusion assay

In vitro antibacterial activity of *M. suaveolens* essential oil was evaluated against six bacterial strains by the disc diffusion method. A bacterial suspension equivalent to 0.5 McFarland (10^8 cfu/mL) was prepared and inoculated by swabbing on a Petri dish containing Mueller-Hinton agar (Biokar). On the surface of each Petri dish, 10 µL of essential oil was dropped on 6 mm diameter filter paper discs (Whatman No. 4). A disc soaked in 10 µL of DMSO was used as a negative control. Chloramphenicol (30μ g) was used as a positive control for *Streptococcus faecalis*, imipenem (10μ g) for *Pseudomonas aeruginosa* and gentamicin (10μ g) to other strains. The used Petri dishes were incubated at 37° C for 18-24 h. After incubation, the inhibition diameter was measured in millimeters (disk included). The strain will be nonsensitive if the diameter is less than 8 mm, sensitive

if the diameter varies between 9 and 14 mm, very sensitive if the diameter varies between 15 and 19 mm and extremely sensitive if it's >20 mm [24].

2.2.2. Determination of minimum inhibitory concentration (MIC) and minimum bactericide concentration (MBC)

MIC and MBC were performed according to the microplates method [25-27]. This method consists in inoculating a decreasing concentration of essential oil by a bacterial inoculum. After incubation, the range of bacterial growth indicates the MIC and MBC. The MIC is defined by the lowest concentration of essential oil capable of inhibiting the growth of 90% of the bacterial population. However, the MBC is the lowest concentration of essential oil capable of killing more than 99.9% of initial microbial inoculum.

2.3. Antioxidant Activity

2.3.1. Evaluation of antioxidant activity by Fe (III) to Fe (II) reduction capacity

The reduction of ferric to ferrous ion was used to determine the reductive capacity of essential oils [28]. Briefly, 1 mL of each concentration was mixed with 2.5 mL of potassium hexacyanoferrate $K_3Fe(CN)_6$ solution and 2.5 mL of phosphate buffer (0.2 mol/L, pH 7.0) and incubated at 50°C for 30 min. After, we added 2.5 mL of trichloroacetic acid (10%) to the mixture. Then, 2.5 mL of this solution was homogenized with distilled water (2.5 mL) and FeCl₃ (0.5 mL, 0.1%). The absorbance was measured at 700 nm and the concentration of the samples at which the absorbance of 0.5 (EC₅₀) was determined. Ascorbic acid was used as positive control for comparison.

2.3.2. Evaluation of antioxidant activity by 2,2-diphenyl-1picrylhydrazyl (DPPH) radical scavenging method

The purple-colored methanol solution of DPPH was used to measure the electron donation ability of the plant essential oils and some pure compounds [29]. In this study, 2 mL of different essential oils concentrations were homogenized with 1 mL of 0.2 mM solution of DPPH and incubated in the dark for 30 min. After, the absorbance (A) of the resulting solution was measured at 517 nm [30]. The inhibition percentage was calculated as follows:

% Inhibition =
$$\frac{(A \text{ blank} - A \text{ sample})}{A \text{ blank}} \times 100$$

The concentration providing 50% inhibition (IC₅₀) was calculated from the graph and ascorbic acid was used as positive control.

2.4. Antimicrobial Activity in a Food System

To evaluate the antimicrobial activity of *M. suaveolens* essential oil in a food system, a sufficient amount of fresh sausage was prepared according to the method described previously [31] and mixed with three distinct oil concentrations (2 mg/g, 5 mg/g, and 10 mg/g). In addition to these tests, a negative control containing no oil was also used. Three batches were prepared for each concentration. Then, they were inoculated with *S. aureus* and *E. coli* at a concentration of 10° cfu/g and stored at 4°C for 48 h. The microbiological analysis was carried out during 0, 6, 24, and 48 h. Briefly, a sausage samples (10 g) of each batch were mixed with 90 mL of sterile peptone water (Biokar), the decimal dilutions were prepared. *S. aureus* was enumerated on Baird-Parker agar (Biokar) with egg yolk tellurite emulsion and incubated at 37°C for 48 h. *E. coli* was enumerated on Rapid *E. coli* 2 Agar (Bio-Rad) after 24 h of incubation at 44°C.

2.5. Statistical Analyses

Measurements were carried out in triplicate. The data obtained were presented as means \pm standard error and the significance of difference between test and control groups was statistically analyzed using Fisher least significant difference test. A probability level of P < 0.05 was used in testing the statistical significance of all experimental data. All the statistical analyses were achieved using the Microsoft Excel and XLSTAT.

3. RESULTS

3.1. Antibacterial Activity

The results show an inhibition of bacterial growth proportional to the diameter zone [Figure 1]. Furthermore, the inhibition diameters produced by essential oil vary according to the bacterial strain. The highest inhibition zone is observed in *S. aureus* (21 ± 1 mm), followed by *E. coli* (17 ± 0.5 mm), *Klebsiella pneumonia* (16 ± 0.5 mm), *Streptococcus faecalis* (14 ± 0.5 mm), *Salmonella* spp. (14 ± 0.4 mm), and *P. aeruginosa* ($13 \text{ mm} \pm 0.3$). The MIC varies between a minimum of 0.48 mg/mL detected in *S. aureus* and a maximum of 7.81 mg/mL detected in *Salmonella* spp. and *P. aeruginosa*. Furthermore, the MBC varies between a minimum of 0.48 mg/mL detected in *S. aureus* and 15.62 mg/mL detected in *P. aeruginosa*. The ratio of MBC/MIC fluctuates between 1 and 2 [Table 2].

3.2. Antioxidant Activity

The reducing ability of a compound can serve as a significant indicator of its potential antioxidant. In the present study, the ferric reducing antioxidant power (FRAP) method was used to test the antioxidant capacity of M. suaveolens essential oil by reducing the ferric ion Fe³⁺ to the ferrous ion Fe²⁺. The results obtained show that the iron reduction capacity was proportional to the concentration of essential oil. Furthermore, these results show that the ability of this essential oil to reduce the iron was less than that of ascorbic acid and reached the maximum at a concentration of 12.5 mg/mL. However, the ascorbic acid was reached a total reduction at a concentration of 0.75 mg/mL [Figure 2]. The EC₅₀ of essential oil and ascorbic acid was $3.95 \pm$ 0.03 mg/mL and 0.11 ± 0.02 mg/mL, respectively, [Table 3]. In the other hand, the reduction of DPPH is accompanied by its passage from the violet color characteristic of DPPH solution to the yellow color measurable at 514-518 nm. In this study, the absorbance (OD) was measured by spectrophotometry at 517 nm, and the percentage of inhibition was calculated using the formula given above. The concentration necessary to reduce 50% of the DPPH radical (IC₅₀) was 3.11 ± 0.02 mg/mL and 0.25 ± 0.01 mg/mL for essential oil and ascorbic acid, respectively [Table 3].

3.3. Antimicrobial Activity in a Food System

The effect of *M. suaveolens* essential oil was evaluated by the enumeration of *E. coli* and *S. aureus* during the storage period of fresh sausages manufactured by different concentrations of this essential oil. The results show that the survival of *E. coli* and *S. aureus* in fresh turkey sausages depends significantly on the essential oil concentration and storage duration [Table 4].

4. DISCUSSION

The antibacterial activity of *M. suaveolens* essential oil shows that *S. aureus* is extremely sensitive with an inhibition zone of 21 ± 1 mm and a MIC of 0.48 mg/mL. However, the others bacteria tested have an inhibition zone between 13 ± 0.3 and 17 ± 0.5 mm and a MIC between

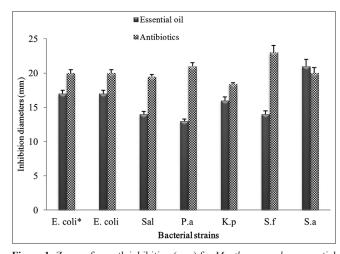


Figure 1: Zones of growth inhibition (mm) for Mentha suaveolens essential oil against pathogenic bacteria. Disc diameter 6.0 mm; E. coli*: Escherichia coli ATCC 25922; E. coli: Escherichia coli; Sal: Salmonella spp.; P.a Pseudomonas aeruginosa; K.p: Klebsiella pneumonia; S.f: Streptococcus faecalis; S.a: Staphylococcus aureus

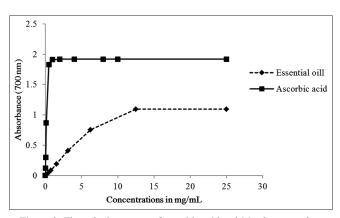


Figure 2: The reducing power of ascorbic acid and *Mentha suaveolens* essential oil

1.95 and 7.81 mg/mL. The ratio of CMB/CMI is <4 for all the bacteria tested which shows that this essential oil has a bactericidal effect [32].

The antimicrobial activity of essential oils depends on their hydrophilic or lipophilic characteristics. However, the mechanism of essential oils action on microorganisms is complex and related with the ability to cause injury to the phospholipids present in cell membranes, resulting in higher permeability and leakage of the cytoplasm, or by its interaction with other enzymes present in the cell wall [33]. The high susceptibility of Gram-positive bacteria to essentials oils in comparing with Gramnegative bacteria might be explained by the higher susceptibility of its cell wall to the lipophilic components of essential oils. Moreover, the lower sensibility of Gram-negative bacteria can be explained by the difficulty of essential oils to diffuse through the hydrophilic barrier of the cell wall [34]. Other studies carried out previously showed that the essential oil of *M. suaveolens* had a significant effect on the eradication of Gram-positive bacteria [35,36].

The antioxidant activity of a compound corresponds to its capacity to resist oxidation. Many methods are currently used to evaluate this activity. In this study, the reducing capacity of iron (FRAP) and the DPPH radical scavenging activity methods are used. The results

Table 1: Bacteria tested	with their antibiotic	resistance profile.
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Antibiotics	E. coli	Sal	S.a	P.a	К.р	S.f
Cefotaxime (30 µg)	S	S	NT	R	R	S
Ciprofloxacin (5 µg)	S	S	S	R	R	R
Chloramphenicol (30 µg)	S	S	S	NT	R	S
Ceftriaxone (30 µg)	S	S	NT	R	R	S
Ceftazidime (30 µg)	S	S	NT	R	NT	NT
Trimethoprim-sulfamethoxazole (1.25/23.75 μg)	R	S	S	NT	R	NT
Oxacillin (5 µg)	NT	NT	S	NT	NT	R
Ampicillin (10 µg)	R	R	R	NT	R	R
Amoxicillin-clavulanic acid (20/10 μg)	R	S	NT	R	R	R
Tetracycline (30 µg)	R	S	R	NT	NT	R
Streptomycin (10 µg)	R	R	NT	NT	R	NT
Gentamicin (30 µg)	S	S	S	NT	S	NT
Amikacin (30 µg)	S	S	NT	S	S	NT
Vancomycin (30 µg)	NT	NT	S	NT	NT	S
Penicillin (10 UI)	NT	NT	R	NT	NT	R
Erythromycin (15 μg)	NT	NT	R	NT	NT	NT
Imipenem (10 µg)	NT	NT	NT	S	NT	NT
Ticarcillin (75 μg)	NT	NT	NT	R	NT	NT
Tobramycin (30 μg)	NT	NT	NT	R	NT	NT
Piperacillin (100 µg)	NT	NT	NT	R	NT	NT

R: Resistant, S: Sensitive, NT: The antibiotic is not tested, E. coli: Escherichia coli, Sal: Salmonella spp., P.a: Pseudomonas aeruginosa, K.p: Klebsiella pneumonia, S.f: Streptococcus faecalis, S.a: Staphylococcus aureus

Table 2: Results for	r MIC and MBC fo	r Mentha suaveolens	essential oil.
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Bacteria	Gram	MIC (mg/mL)	MBC (mg/mL)	MBC/MIC
E. coli ATCC 25922	-	1.95	1.95	1
Escherichia coli	-	1.95	1.95	1
Salmonella spp.	-	7.81	7.81	1
Pseudomonas aeruginosa	-	7.81	15.62	2
Klebsiella pneumonia	-	1.95	1.95	1
Streptococcus faecalis	+	7.81	7.81	1
Staphylococcus aureus	+	0.48	0.48	1

MBC: Minimum bactericide concentration, MIC: Minimum inhibitory concentration

Table 3: The IC₅₀ values (mg/ml) of *Mentha suaveolens* essential oil and ascorbic acid.

Antioxidant Activity	Mentha suaveolens essential oil	Ascorbic acid
IC ₅₀ (mg/mL)	3.11±0.02	0.25±0.01
EC ₅₀ (mg/mL)	3.95±0.03	0.11±0.02

show that the essential oils of *M. suaveolens* have a remarkable antioxidant activity but lower than that of ascorbic acid. This may be due to the limitation of these essential oils in substances that have powerful antioxidant capabilities such as flavonoids [37,38]. However, it exhibits this activity at concentrations compatible with their bactericidal effect. On the other hand, it has been reported in the literature that *M. suaveolens* is a plant rich in essential oil and endowed with a potent antibacterial and antioxidant activity [21,22].

The plant essential oils have attracted interest as potential preservatives because they are generally recognized as safe and have a wide acceptance from consumers. Indeed, the antimicrobial activity of various plant species has been known for a long time and used to extend the shelf life of food products [39]. Moreover, the species those belonging to Lamiaceae family have been used for a long time for improving the taste and organoleptic properties of different foods [40]. This effect is due to the essential oil fraction contained in the plant's species which inhibits the growth of pathogenic bacteria, fungi, and yeasts [26,41,42]. Furthermore, several studies have demonstrated the activity of essential oils against pathogens in food systems [7-9]. In the same way, the present study shows that under the effect of M. *suaveolens* essential oil; there is an important abatement of *S. aureus* and *E. coli* during the storage period, these pathogens have been cited among the most incriminated in food poisoning [43-46]. In addition, the use of essential oils in food preservation makes it possible to improve the taste quality and to give a taste appreciated for the consumer [7].

5. CONCLUSION

The essential oil of *M. suaveolens* has an important antibacterial activity against several multiresistant pathogenic bacteria, in addition

Table 4: Level variations of Escherichia coli and Staphy	lococcus aureus during the storage of sausag	ges supplemented with Mentha suaveolens essential oil

Bacteria	Time		Concentrations of essential oil			
		Blank	2 mg/g	5 mg/g	10 mg/g	
Escherichia coli (Log cfu/g)	0 h	6.3±0.2 ^B	6.3±0.2 ^B	6.3±0.2 ^B	6.3±0.2 ^B	
	6 h	6.4±0.1 ^B	6.23±0.25 ^B	6±0.3 ^c	5.95 ± 0.1^{CD}	
	24 h	6.67±0.1 ^A	6.03±0.1 ^c	5.69±0.2 ^E	5.37 ± 0.2^{F}	
	48 h	6.84±0.1 ^A	5.77 ± 0.1^{DE}	5.14±0.1 ^G	4.63 ± 0.3^{H}	
Staphylococcus aureus (Log cfu/g)	0 h	6.3±0.3 ^{BC}	6.3±0.3 ^{BC}	6.3±0.3 ^{BC}	6.3±0.3 ^{BC}	
	6 h	6.3±0.2 ^{BC}	6.1±0.15 ^{CD}	5.89±0.2 ^D	5.81 ± 0.1^{DE}	
	24 h	6.59±0.1 ^{AB}	5.86 ± 0.1^{DE}	4.97 ± 0.15^{F}	4.44±0.1 ^G	
	48 h	6.8±0.2 ^A	5.51 ± 0.2^{E}	4.34±0.1 ^G	3.63 ± 0.2^{H}	

Data are averages (±standard errors). Different letters stand for statistically significant differences at P<0.05 (Fisher's LSD). LSD: Least significant difference

to its antioxidant activity. Furthermore, the results of this study showed that this essential oil can be used as a natural food additive for the conservation and improvement of the hygienic quality of food products.

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