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Growth inhibition of *Staphylococcus aureus*, *Bacillus cereus*, and *Escherichia coli* assessed in vitro and in food system using thyme and mentha essential oils

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Abstract

This study aimed to assess the antibacterial activity of essential oils (EOs) from *Thymus vulgaris*, *Thymus satureoides*, *Mentha piperita*, and *Mentha spicata* in vitro and in beef minced meat. The inhibitory effect of EOs on *Staphylococcus aureus, Bacillus cereus,* and *Escherichia coli* was evaluated by agar diffusion method and dilution assay. The EOs were tested in in food system calculating CFU in stored (refrigerated) minced beef meat inoculated with pathogens. Results showed the inhibitory effect of thyme and mentha EOs on all bacteria tested in Muller Hinton media. Indeed, *S. aureus* and *B. cereus* were more sensitive to *T. vulgaris* EOs. The addition of EOs to inoculated minced beef resulted in decrease of *S. aureus, B. cereus* and *E. coli* population after 4 days of storage. These findings suggested the possibility of using of thyme and mentha EOs as natural preservatives in meat and meat products preservation.

Key words: Antimicrobials, essential oils, meat, mentha, thyme

Introduction

The control of microbial contamination has been a major concern in the meat industry since pathogenic and spoilage-related bacteria have been found in refrigerated meat and meat products (Dhanze *al.* 2013; Manios et al. 2015). For example, *E. coli* O157:H7 is a common cause of foodborne illness growing in low temperature and acidic conditions, and meat is tested for this pathogen (Torso et al. 2015). *Salmonella typhimurium* was also shown to grow in a broad range of temperatures and to cause systemic diseases that are associated with raw and cooked meats (McConnell and Schaffner 2014; Chen *et al.* 2013; Solarte et al., 2017).

In order to prevent bacterial activity antimicrobial agents are added to the fresh meat and meat products. However, the addition of synthetic chemicals in these foods to extend the refrigerated storage time has raised a safety problem resulting in the preferential use of natural products as biopreservatives (Owen and Palombo 2007).

Indeed, there is a worldwide trend to explore new alternatives to control foodborne diseases, giving priority to methods that reduce disease incidence and avoid negative and side effects on human health (Nedorostova 2009). One of the most promising methods to control the microbial activity in foods seems to be the use of food grade phytochemicals. Indeed, essential oils which often contain major flavouring and bioactive components of herbs may have great potential use as food flavours and preservatives.

Thyme and mentha are aromatic plants which have been long used in foods for culinary purposes. Their essential oils were found to have antioxidant and antimicrobial properties (Rota *et al.* 2008; Desai *et al.* 2012). The antimicrobial activity of essential oils has been extensively studied and demonstrated *in vitro* against a number of microorganisms, usually using a direct-contact antimicrobial assays, such as diffusion or dilution methods. However, there has been very little scientific evidence to support their antibacterial effect in food systems. On the other hand, previous studies on antibacterial activity of plant extract or other antimicrobials have focused on referenced or laboratory-adapted species such as *E. coli* O157:H7, *Staphylococcus aureus, Listeria monocytogenes, Salmonella* Typhimurium DT104, etc or a limited number of pathogenic isolates (Oussalah *et al.* 2006; Shah *et al.* 2013, Rebouças de Araújo et al., 2017).

In addition, the results reported in literature about the susceptibility of bacteria to antimicrobials may not be reproducible in other countries depending on potential differences in bacterial and epidemiologic characteristics (Lamoth *et al.* 2010). Thus, the use of isolates from patients as bacterial model should provide a more accurate account of which features may be associated with colonization and disease associated.

The aim of the current work was, as a first step, to investigate the antimicrobial activity of the essential oils of local thyme and mentha by disc diffusion and dilution methods against several pathogens. In a second step, we examined the antimicrobial activity of two selected samples of essentials oils, which possessed high antibacterial effect and are more available locally, against *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus* in food system using minced meat as food model.

1. Material and methods

1.1 Plant material and essential oils extraction

Four essential oils from *Thymus vulgaris, Thymus satureioides, Mentha spicata,* and *Mentha piperita,* were experimented. Fresh *M. spicata material* was collected from Kabylia area (North Algeria) during the vegetation period, botanically identified and immediately processed. While, *M. piperita* essential oil was provided by SAIDAL laboratory (SAIDAL group, Algiers, Algeria), it was extracted from local mentha by steam distillation. *Thymus vulgaris* was extracted from thymus material purchased from herbs store at Algiers.

For comparative investigations of antibacterial activity of essential oils, a commercial essential oil, *Thymus satureioides* (PHYTOSUN aroms, Omega Pharma, France) was purchased from France to be used as reference. Leaves and the flowered celebrities of mentha and the whole plant of thyme were washed with distilled water then dried in the shade during 3 day. *M. spicata* essential oil was isolated by distillation–extraction.

An adequate quantity of air-dried mentha was chopped and placed in a flask where distilled water was also added (approximately 600 ml water in a 1 L flask for a 100 g sample), and subjected to a continuous hydrodistillation in a Clevenger-type apparatus for 3 h, up to the point at which the oil contained in the herbaceous matrix was exhausted.

The obtained extract was mixed with ether and sodium chloride, and then the mix was subjected to decantation for 24 hours at low temperature (approximately 4°C) to separate the essential oil from water. The ether in the extract was evaporated by vacuum rotary evaporator. Collected essential oil was dried over anhydrous sodium sulphate and stored in dark glass bottles at 4 °C until use. *T. vulgaris* and *M. piperita* essential oils were isolated from 5 kg vegetal material by hydrodistillation using a pilot hydrodistillation apparatus.

1.2 Antimicrobial activity assessment

1.2.1 In vitro study

The antimicrobial activity of essential oils was evaluated using three human isolates, *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus.* These isolates were provided by Nedir Mohamed hospital (CHU, Tizi Wezzu, Algeria) where they were obtained from clinical specimens using conservative bacteriological methods.

The preparation of the investigated bacteria suspensions was performed using Brain–Heart Infusion Broth (BHI, OXOID, Basingstoke, UK). After inoculation from the stock culture, the cultures were grown at 37 °C to stationary phase (24 h) in BHI Broth. These cultures were then used to inoculate the final media adjusting the concentration of 10^5 CFU/ml by a standard optical density (620 nm) of 0.5.

Antibacterial activity assays of essential oils were carried out using a laboratory media, Mueller Hinton agar (Becton Dickinson), and a food system, minced meat. Each assay was performed twice in duplicate. Antibacterial activity investigation on Mueller Hinton agar was performed by disc diffusion and MIC (Minimum inhibitory concentration) methods according to the method described by De Billerbeck (2007), but slightly modified. In the disc diffusion method, Mueller-Hinton agar (15 ml), sterilized in a flask and cooled to 45–50 °C, was distributed to sterilized Petri dishes. The media was then inoculated individually by 1 ml of prepared bacterial suspension containing 10^5 /ml.

Filter paper discs (6 mm in diameter, Whatman No. 1) were individually impregnated with 5 µl of the whole essential oil extract, which was subsequently placed aseptically on the surface of the inoculated Petri dishes. The Petri dishes were kept for 2 h for drying, and then incubated at 37° C for 24 h. The diameters of the inhibition zones around the discs were measured in millimetres. Negative control (without EOs) was used.

The sensitivity to the different oils was classified by the diameter of the inhibition halos as: not sensitive for diameters less than 8 mm; sensitive for diameters 9-14 mm; very sensitive for diameters 15-19 mm and extremely sensitive for diameters larger than 20 mm (Ponce *et al.* 2003).

MIC method was performed using standard dilution method. Serial dilutions of the different oils (75-70-60-50-25-20-10-5-3.3-2.5-2-1.6-1.25-1.1-1-0.9%) were used in our experiments. 1 ml aliquot of the serial oil dilutions was mixed with 3 ml melted Mueller-Hinton agar, ant then the mix was poured onto a Petri dish containing 20 ml of the same media and allowed to solidify (approximately 15 min).

The respective Petri dishes containing diluted amounts of essential oil were inoculated with targeted bacteria using three spots of 1 µl of actively dividing bacterial cells (10^5 CFU/ml) for each Petri-dish. The positive control consisted of Muller Hinton Media inoculated with the same amount of cells but without any essential oil, while uninoculated plates containing the essential oil served as negative control. The cultures were incubated for 24 h at 37°C.

Petri-dishes were evaluated for the presence or the absence of colonies after 24 hours of incubation. The MIC was defined as the lowest concentration required to stop the growth of bacteria at the end of 24 h of incubation.

1.2.2 Food model assay

Meat is recognized as one of the most perishable foods supporting pathogen and/or spoilage related bacteria, due to its high water and nutrients content. In order to evaluate the

efficacy of three essential oils: *T. vulgaris*, *T. satureioides*, *M. piperita* in food system, their antibacterial activity against bacteria selected for their high sensitivity to essential oils tested *in vitro*: *E. coli*, *S. aureus*, *B. cereus* was assessed in contaminated minced beef used as food model as described by Oussalah *et al.* (2006), and Solomakos *et al.* (2008), but with some modifications.

Major muscles were obtained from fresh beef carcasses in a local abattoir, and transported to the laboratory under refrigerated conditions within 30 min. The meat was minced using a steel meat grinder, and minced meat was weighed and divided manually using gloves into individual samples (150g). The samples were inoculated by a targeted pathogen grown overnight at 37°C (with adjusted concentration of 10^5 CFU/ml) and added by an essential oil at a final concentration of 40 µl/100 g.

Treatment of the samples with essential oils at this concentration was examined for its effect on organoleptic proprieties of treated meat, because preliminary experiments showed that use of high concentration of essential oils provided unacceptable organoleptic properties in minced meat. To ensure proper distribution of the pathogen and essential oil, the treated samples were further homogenized mixing them manually for few minutes in aseptic conditions. Inoculated minced meat without essential oil was used as control.

Samples from all treatments and controls were wrapped and stored under aerobic conditions at 4±2 °C for seven days. Enumeration of bacterial population of each sample was performed at day 1, 4 and 7 of refrigerated storage. Microbiological analysis was performed in duplicate and or triplicate.

1.3 Statistical analysis

The statistical package StatBox for Windows was used to explore the statistical significance of the results obtained. Analysis of variance was carried out on the results of microbiological analyses with the aim to verify the existence of statistically significant difference between the different treated minced meats throughout the storage period. A probability level of $P < 0.05$ was used in testing the statistical significance of experimental data.

2. Results

2.1 In vitro antimicrobial activity

In this study, pathogenic Gram positive and Gram negative bacteria were firstly challenged *in vitro* with mentha and thyme essential oils to evaluate their antibacterial properties The zone inhibition diameters obtained with tested essential oils are listed in Table 1.

Table 1. Antimicrobial activities (mm of zone of inhibition) of the T. vulgaris, T. satureioides, M. piperita, and M. spicata essential oils. Ø, diameter of zone of inhibition. Ø<8 mm, resistant; 9<Ø<14 mm, sensitive; 15<Ø<19 mm, highly sensitive; Ø>20 mm, extremely sensitive (Ponce et al, 2003).

	Essential oil			
	T. vulgaris	*T. satureioides*	*M. piperita*	*M. spicata*
S. aureus	42	16	24,74	11
B. cereus	40,7	19	9,43	10,5
E. coli	21	14,25	9,88	9,05

All pathogens tested were sensitive (diameter of inhibition zone superior to 8 mm) to both thyme and mentha essential oils. The sensitivity to *T. vulgaris* was in the following order: *S. aureus* > *B. cereus* > *E. coli*, while the sensitivity order to *M. piperita* was *S. aureus* > *E. coli* > *B. cereus*. However, the bacterial species tested were found to be more sensitive to thyme than mentha essential oil. Whatever the essential oil used the Gram positive bacteria *S. aureus* and *B. cereus* appeared to be more sensitive to essential oils than Gram negative bacteria *E. coli.* Indeed, high growth inhibition was obtained by *T. vulgaris* tested against *S. aureus* (diameter: 42 mm) and *B. cereus* (diameter: 40,7 mm).

In addition, essential oils from *T. vulgaris* showed low MICs (Table 2) compared to those from *M. spicata*, respectively 1% and 5 % (v/v) against *S. aureus*, *B. cereus*, *E. coli*.

Table 2. The values of minimal inhibitory concentrations (MIC) of T. vulgaris, T. satureioides, M. piperita, and M. spicata oils.

	MIC (%)			
Organism	*T. vulgaris*	*T. satureioides*	*M. piperita*	*M. spicata*
S. aureus	1	1,1	2,5	5
B. cereus	1	1,1	ND	5
E. coli	1	1,25	ND	5

2.2 Antimicrobial activity in food system

The results of *S. aureus* growth evaluation in minced meat treated with essential oils are shown in Figure 1.

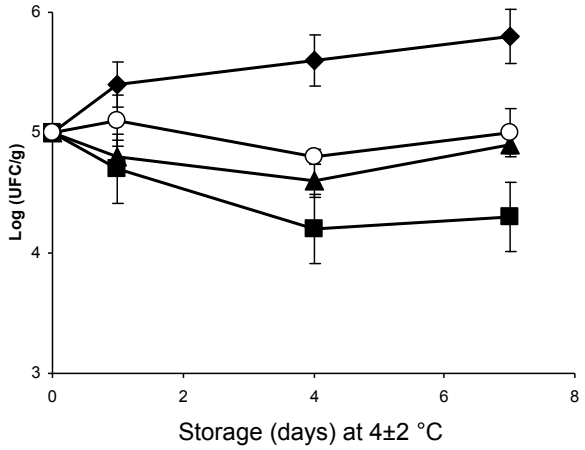

Figure 1. Inhibition of S. aureus growth in minced beef treated by (■) T. vulgaris, (▲) T. satureioides, and (○) M. piperita essential oils during refrigerated storage. Controls (♦)

The results showed that the addition of *T. vulgaris*, *T. satureioides*, and *M. piperita* in minced beef samples resulted in populations of *S. aureus* significantly lower ($P < 0.05$) than in the control, throughout four days storage at 4±2 °C.

However, the populations of the pathogen, after an initial decrease (storage day 4), from 5,6±0,212 (control) to 4,2±0,3 (*T. vulgaris),* 4,6±0,1 (*T. satureioides*) or 4,8±0,1 log CFU/ml (*M. piperita*) were slightly increased ($P < 0.05$) at the seven day of refrigerated storage. In addition, the activity of *T. vulgaris* essential oil seemed to be more efficient

against *S. aureus* as compared to those obtained by *M. piperita*, *T. satureioides*. Same inhibitory effects were observed with thyme essentials oils against *B. cereus* (Figure 2).

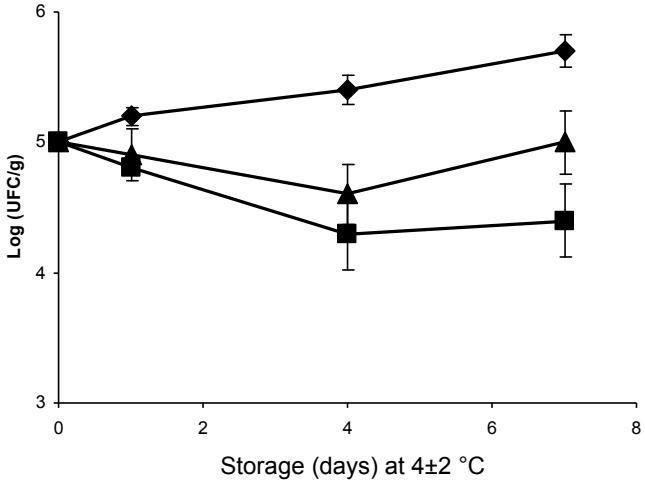

Figure 2. Inhibition of B. cereus growth in minced beef treated by (■) T. vulgaris, and (▲) T. satureioides essential oils. Controls (♦)

Indeed, final populations of *B. cereus* in minced meat were significantly ($P < 0.05$) lower in minced meat treated with *T. vulgaris* (4,4±0,3) and *T. satureioides* (5±0,2) than those of the control (5,7±0,1 CFU/g) after seven days of refrigerated storage. *T. vulgaris* was more active against *B. cereus* as well against *S. aureus*.

Regarding to the inhibitory effects of essential oils against the Gram negative bacterium *E. coli*, the results (Figure 3) demonstrated that the bacterial growth in minced meat treated with both *T. vulgaris* and *T. satureioides* was significantly inhibited ($P < 0.05$), resulting respectively in final *E. coli* population of 4,5±0,1 and 5±0,2 as compared to the control (5,7±0,071 log /ml) after seven days of storage at 4±2 °C. The *T. vulgaris* essential oils were shown significantly ($P < 0.05$) more inhibitory against *E. coli* than those of *T. satureioides*.

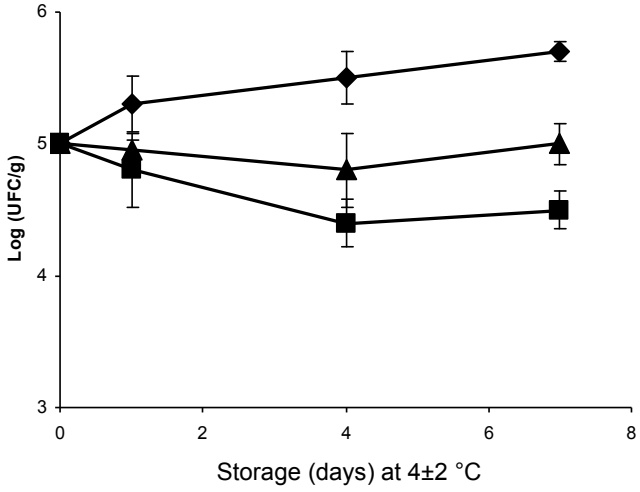

Figure 3. Inhibition of E. coli growth in minced beef treated by (■) T. vulgaris, and (▲) T. satureioides essential oils. Controls (♦.

3. Discussion

According to the literature, the antimicrobial activities of natural compounds in laboratory media are relatively different from those observed in food system because food ingredients can have an influence on the in situ activities of antimicrobials (Pol *et al.* 2001). Results obtained are in accordance with those reported by Nedorostova *et al.* (2009) demonstrating that *T. vulgaris* essential oils were active against several Gram positive and Gram negative bacteria except *P. aeruginosa.*

It has been suggested that the lipopolysaccharide covering of the outer membrane may restrict diffusion of hydrophobic compounds through the cell wall of Gram-negative bacteria (Davidson and Branen 2005). Mahboubi and Haghi (2008) mentioned that a significant activity against Gram-positive bacteria especially *S. aureus* was obtained by *Mentha pulegium* L. essential oil, whereas the least susceptible were Gram-negative bacteria especially *Escherichia coli*.

The data showed that Gram-positive bacteria were more sensitive than Gram negative ones to essential oils tested. These results could be explained by the difference in chemical composition (percentage of bioactive component) between the two essential oils. Previous antimicrobial activity studies on various thyme and mentha species from different localities showed inhibitory activity against a human, animal and plant pathogens, including food poisoning bacteria (Solomakos *et al.* 2008; Nedorostova *et al.* 2009, Djenane *et al.* 2012).

The essential oils were reported to be hydrophobic components that accumulate in the lipid bilayer (cytoplasmic membrane) resulting in disruption of the bacterial membrane structure and function. The membrane functions as a barrier and as a matrix for enzymes and as an energy transducer are then compromised (Sikkema *et al.* 1994; Cristiani *et al.* 2007). Loss of integrity and leakage of cellular material such as ions, ATP and nucleic acid were reported as result of the extent of membrane damage induced by bioactive components of essential oils (Trombetta *et al.* 2005).

Additionally, our results were in agreement with those reported by Mahboubi and Haghi (2008) who showed that *Mentha pulegium* L. essential oil had inhibitory or germicidal effect depending on the type of organisms and revealed significant antimicrobial activity against Gram-positive bacteria. The antibacterial efficacy of thyme and mentha in minced beef meat was examined, since pertinent results in Muller Hinton media showed a strong antibacterial activity against the examined pathogen. Oils from *T. vulgaris*, *T. satureioides*, and *M. piperita* were tested against sensitive bacteria.

Before EOs antimicrobial activity investigation, the minced meat was subjected to sensory test to evaluate the sensory color, odor and overall acceptability attributes of minced beef meat treated with essential oils. The optimal concentration of essential oils that did not affect the organoletic proprieties of the product was 40 µl/100 g (other data not shown). The efficacy of essential oils in minced beef was different (less efficient) compared to laboratory media model due to the impact of food system composition on antimicrobial activity.

The antibacterial properties of the essential oils are generally weakened when added to foods due to reaction of their bioactive molecules with food components (Helander *et al.* 1998; Shah *et al.* 2013). These results revealed probably the difference in antibacterial activity of oil components such as carvacrol and thymol reported to be major and active components of thyme (Cristiani *et al.* 2007).

Some previous studies performed on the antimicrobial activity of carvacrol have shown that it has a broad spectrum of antimicrobial activity against both Gram-positive and Gram negative bacteria (Friedman and Mandrell 2002). Carvacrol (phenolic compound) was found to inhibit potentially the bacterial growth, owing to its high abundance in some oils,

and high specific activity as compared to other essentials oils components (Dorman and Deans 2000).

According to some authors, the antimicrobial activity of thyme essential oils against foodborne pathogens such as *E. coli* O157:H7 oils could be enhanced in vitro (Burt and Reinders 2003) or in foods (Hao *et al.* 1998) at temperatures higher than 4°C. Recently, a study by Solomakos *et al.* (2008) demonstrated that treatment of beef meat with thyme essential oils at 0.6% had an inhibitory activity against *E. coli* O157:H7 at 10 °C.

Conclusions

The findings of the current study highlight the promising antibacterial activity of thyme and mentha essential oils as natural antibacterial agents. Both essential oils exerted in vitro inhibitory effects on all pathogens tested. Gram-positive bacteria were more sensitive than Gram negative ones to essential oils tested.

However the efficacy of essential oils in minced beef meat was different compared to laboratory media model due to the impact of food system composition on antimicrobial activity. The results suggested that addition of essential oils at the level no affecting the organoleptic proprieties of the product exerted an inhibitory effect on microbial growth of minced meat during refrigerated storage. *T. vulgaris* was shown to have the best protective effect, keeping the microbial load change to acceptable levels in the first 7 days of refrigerated storage. Considering the increasing consumer demand in food added with natural agents these findings suggest the use of thyme or mentha oil as antibacterial agent in meat preservation.

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