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Research article

Assessment of bactericidal activity of some essential oils from medicinal plants and selected food additives on *Salmonella enteritidis, Staphylococcus aureus* and *Escherichia coli*

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Abstract

The aim of the present study was to investigate and compare between the antibacterial properties of some essential oils derived from some medicinal plants and food additives against food borne pathogens *Salmonella enteritidis*, *Staphylococcus aureus* and *E. coli*. The antimicrobial potential was determined performing the disc diffusion assay and also minimal inhibitory (MIC) and bactericidal (MBC) concentrations. The inhibitory ability manifested by the essential oils and food additives against the bacterial strains varied significantly depending fundamentally on concentration, but also on bacterial species. Essential oils were the most bactericidal agents against the tested bacteria. The MIC and MBC values between 0.125 and 1.0 µg/ml for the most active essential oil of *Thymus vulgaris* followed by *Salvia officinalis* and *Achillea santolina* which had MIC and MBC against the tested bacteria ranged from 0.5-4.0µg/ml and 1.5-6.0µg/ml for MIC and MBC respectively. The most active food additives against the tested bacteria were acetic acid and tartrazine while nisin and phloxine B had no effect on gram negative bacteria.

Introduction

Food borne illness resulting from consumption of food contaminated with pathogenic bacteria has been of vital concern to public health. Many naturally occurring compounds found in plants, herbs, and spices have been shown to possess antimicrobial functions and serve as a source of antimicrobial agents against food borne pathogens [1]. Certain plants and their extracts used as flavouring agents are known to possess antimicrobial activity offering a potential alternative to synthetic preservatives. To reduce health hazards and economic losses due to food borne microorganisms, the use of natural products as antibacterial compounds seem to be an interesting way to control the presence of pathogenic bacteria and to extend the shelf life of processed food [2]. Consumer demand for less use of synthetic preservatives has led to research and use of naturally derived antimicrobials, desiring fewer synthetic additives in foods together with their increased safety, quality and shelf-life [3]. Food preservative help to prevent food spoilage by preventing the growth and proliferation of pathogenic microorganisms like *Clostridium* spp., *Bacillus cereus* and *Staphylococcus aureus* [4]. Food preservative is a class of food additive, which are defined as chemical substances deliberately added to foods, directly or indirectly in known quantities for purposes of assisting in the processing of foods; preservation of foods; or in improving the flavour, texture, or appearance of foods [5]. This definition includes any substance used in the production, processing, treatment, packaging, transportation or storage of food [6].

Thyme (*Thymus vulgaris* L.) from family Lamiaceae is a small herbaceous perennial shrub with lots of branches and light purple to pink flowers. It is aromatic and has a pleasant, pungent, clover flavor. This is one of the most widely used genera in folk medicine [7]. Common thyme is native to southern Europe, where it is often cultivated as a culinary herb and used as a spice in many foods. Thyme herb contains tannins, flavonoids, triterpene compounds, and up to 2.5 % of essential oils [2]. The essential oils of *T. vulgaris* are known to have antiseptic, antiviral and antimicrobial activities. *Salvia officinalis* L. is a perennial, evergreen sub shrub, with woody stems, grayish leaves, and blue to purplish flowers. It is a member of the family

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Lamiaceae and is native to the Mediterranean region, though it has naturalized in many places throughout the world [8]. The antimicrobial activity of *S. officinalis* was recognized decades ago and was attributed to the presence of 1,8-cineole, α-thujone and camphor [9]. The genus *Achillea* L. family Asteraceae is commonly found in the Northern Hemisphere, mostly in Europe and Asia [10,11]. *A. santolina* L. belong to the oldest medicinal plants that are used in folk medicine and for pharmaceutical purposes due to the presence of a complex of different pharmacological compounds, such as, alkaloids, bitters, flavonoids, tannins, terpenes etc. [12]. *Achillea* species are used for healing wound, curing stomachache, diarrhea and antiseptic and infection preventing properties, and have also been used as antimicrobial agent [11].

Nisin (E234) is a group of closely related polypeptides composed of 34 amino acids, and is produced by certain strains of the bacterium as *Lactococcus lactis*s sp. *lactis*. It has a narrow spectrum of activity affecting primarily vegetative cells and spores of Gram-positive bacteria [13]. Bacteria susceptible to nisin include other lactic acid bacteria, *Bacillus*, *Clostridium*, *Listeria*, and *Streptococcus* genera. In the absence of other preservation methods, nisin does not inhibit Gram-negative bacteria, yeasts, or moulds. Consequently nisin is often used in combination with other synergistic preservation methods (known as hurdle technology) such as low pH and high salt concentrations [14]. The colorant phloxine B known as D&C red no. 28 has a bactericidal effect on *S. aureus* and has a potential as a useful antibiotic agent. This colorant is added as an inactive ingredient to drugs like the antibiotic Amoxicillin, has been used as a food colorant, and has been approved as a color additive for cosmetics by the Food and Drug Administration [15]. Acetic acid is written as E260 on products sold in Europe. It is a natural component of vinegar but generally manufactured from wood [16]. It is used as a preservative, acid or colour diluent. Found in pickles, bottled sauces and chutneys. Tartrazine (E102) was one of a mixture of colours which has been deemed safe for use by the European Food Safety Authority which has recommended a safe level of consumption. Tartrazine is a commonly used food colourant which creates a yellow colour and can be seen on many different packaging of foods. Specifically it is found in foods, cosmetics, and beauty products, and in its basic form it is a yellow powder manufactured from coal tar [17]. In the context of the information provided above, the objectives of this study have been undertaken in order to compare the bactericidal effects of some essential oils from medicinal plants and selected food additives on some food-borne pathogenic bacteria.

Experimental

Plant material

Thymus vulgaris (L.), *Achillea santolina* (L.) and *Salvia officinalis* (L.) samples were collected during April 2015. Essential oils from fresh aerial parts were obtained by steam distillation.

Essential oil extraction

Aerial parts of the three plants were subjected to steam distillation for 3.0 - 4.0 h and yielded essential oils from 1.0 % (v/w) of plant. The obtained essential oils were dried after decanting over anhydrous sodium sulfate and then stored in refrigerator at -4°C until needed [18].

Food additives

A number of food additives include; nisin, phloxine B, acetic acid and tartrazine were chosen. Food additives were purchased from Sigma–Aldrich**.**

Microbial strains and culture condition

The essential oils of three medicinal plants and selected food additives were individually tested against the most food poisoning bacterial species *Salmonella enteritidis, Staphylococcus aureus* and *Escherichia coli* which selected for this study. These strains were obtained from Department of Botany, Faculty of Science, Mansoura University. The Bacteriological agar and Luria-Bertani (LB) broth were obtained from Difco.

Antimicrobial Activity test

The antimicrobial activities of essential oils and food additives were measured *in vitro* against the three bacterial cultures by using disc diffusion method [19]. The bacterial suspensions were adjusted with sterile saline to a concentration of 1.0×10^5cfu/ml. The inocula were prepared daily and stored at +4°C until use. Filter paper discs (5 mm diameter) were placed on the pre-inoculated LB agar surface and impregnated with 30 µl essential oil dissolved in Tween 80 or food additive dissolved in distilled water. The plates were incubated at 37°C for 24 h before the diameter of the inhibition zones around each disc were measured in (mm). All the tests were performed in duplicate and repeated twice. Streptomycin (Sigma P 7794) was used as a positive control, and 1 µl was applied to the discs from stock solution (1 mg/ml). While sterile paper discs with distilled water and Tween 80 (which have no anti-bacterial activities) were considered as negative controls. The antibiotic potency of the tested essential oils and food additives were performed by disc diffusion method by using the following concentrations in mg/ml; 0.1, 0.5, 1.0, 5.0, 10, 20, 50, 75,100 and 150.

MIC and MBC Determinations

The minimum inhibitory concentration (MIC) was defined as the lowest concentration that prevents visible growth of the bacteria [20]. The minimum bactericidal concentration (MBC) was determined as a concentration where 99.9% or more of the initial inoculum is killed [21]. The essential oils were dissolved in Tween-80 (0.05%) and food additives

dissolved in distilled water(100µg/ml).The MIC and MBC concentrations were determined by serial dilution with LB broth. The bacterial cell number was adjusted at 1.0×10^5 cfu/ml for the all strains tested. All samples were incubated at 37°C during 18-24 h. To confirm results of MBC, the experimental suspensions were sub-cultured in nutrient agar plates according to the method described by Ronda [22] and were incubated at 37°C during 18-24 h. We have used this method because it is more sensitive than the agar dilution.

Statistical analysis

The Student t-test was employed for statistical analysis. Significance was tested at the $P < 0.05$ level. Experiments were repeated twice.

Results and Discussion

Because of *Salmonella enteritidis, Staphylococcus aureus* and *Escherichia coli* are important bacteria among food borne pathogens, they were selected for the comparison between antibacterial assay of the essential oils and food additives in this study. The results in Table (1) showed variable degrees of antibacterial activity of the investigated essential oils and food additives against the tested organisms. *T. vulgaris* and *S. officinalis* exhibited highest antibacterial activity against *S. enteritidis, St. aureus* and *E. coli* (ranging from 26-40mm) followed by *A. santolina* (ranging from 18-28mm) while acetic acid and tartrazine gave moderate activities (ranging from 14-22mm). Nisin and phloxine B showed weak activities against *S. enteritidis* and *E. coli* ranged from 8-10mm, but showed moderate activity against *St. aureus* not exceed 18mm.

Table (2) showed the antimicrobial potentiality of different concentrations of essential oils (EOs) and food additives on *S. enteritidis*. The results were indicated that the smallest concentrations of both essential oils of *T. vulgaris* and *S. officinalis* were affected on *S. enteritidis* while this concentration of *A. santolina* essential oil and other food additives had noeffect on it. The concentrations more than 5.0 mg/ml up to 150 mg/ml of all tested oils and food additives were affected on *S. enteritidis*. All concentrations of nisin and phloxine B had no effect on the tested organism.

Table 1. Inhibition zone (mm) of essential oils and food additives by disc diffusion method (mean of duplicate tests)

EOs and food additives	*S. enteritidis*	*St. aureus*	*E. coli*
T. vulgaris	24	40	27
A.santolina	22	28	18
S.officinalis	30	40	26
Nisin	8	16	8
phloxine B	10	18	9
acetic acid	18	20	18
Tartrazine	18	22	14
Streptomycin	10	16	12

Results present in Table (3) showed the effect of different concentrations of essential oils and food additives on *St. aureus*. The low concentrations of essential oils had moderate effect on *St. aureus* (10-18mm) while the same concentrations of food additives had no effect on studied bacteria. The inhibitory effect of all essential oil and food additives at concentrations from 10 to 150 mg/ml were very high (18-25mm).

The effect of essential oils and food additives at different concentrations on *E. coli* were illustrated in Table (4). It was observed that the concentration of (1.0 mg/ml) of the three essential oils gave low effect on *E. coli* (≤10mm) while the concentration of (5.0 mg/ml) for the essential oils had moderate effects (11-18mm). The concentrations from 5.0-50 mg/ml of acetic acid and tartrazine had the same effects. The concentrations from 10- 150 mg/ml of all essential oils were showed very high inhibitory effect while the same effect were observed at concentrations of 75-150 mg/ml of acetic acid and tartrazine. All concentrations of nisin and phloxine B had no inhibitory effect on the tested organism except concentrations of 100 and 150 mg/ml which had low effects.

The use of plant essential oils as antimicrobial agents can be of great significance in food preservation techniques. In the last few years, a number of studies have been conducted in different countries to prove such efficiency [23]. Many plants have been used as food spices because of their antimicrobial agents, which are dueto compounds synthesized in the secondary metabolism of the plant. Several studies indicate that the essential oils of the herbal belonging to *Lamiaceae* family possess biological activity against several bacteria and yeast [24,25]. The tested essential oils were performed best antimicrobial activities. Fournomiti [26] demonstrated that thyme and sage essential oils are promising natural components suitable for use as antimicrobial agents. The antimicrobial activities of EOs derived from those plants have captured the attention of scientists as they could be used as alternatives to the increasing resistance of traditional antibiotics against pathogen infections. On the other hand, Also haili and Al-fawwaz [27] said that *Achillea fragrantissima* essential oil showed significant high antimicrobial activity against all tested microorganisms.

The results indicated that nisin has a narrow spectrum of activity against gram negative bacteria. This was agreed with the results of Bauer and Dicks [14] who demonstrated that nisin had affected vegetative cells and spores of Gram-positive bacteria but had a weak effect activity against gram negative bacteria. In the absence of other preservation methods, nisin alone generally does not inhibit Gram-negative bacteria, yeasts, or moulds. Consequently, to widen its efficacy, nisin may be used in hurdle technology, in which synergistic preservation methods such as low pH and high salt concentrations are combined [28].

Table 2. Effect of different concentrations of essential oils (EOs) and food additives on *S. enteritidis*

EOs and food additives	Concentrations (mg/ml)									
	0.1	0.5	1.0	5.0	10	20	50	75	100	150
T. v.	-	+	+	++	+++	+++	+++	+++	+++	+++
A. s.	-	-	-	++	++	+++	+++	+++	+++	+++
S. o.	-	+	++	++	++	+++	+++	+++	+++	+++
nisin	-	-	-	-	-	-	-	-	-	-
phloxine B	-	-	-	-	-	-	-	-	-	-
acetic acid	-	-	-	++	++	+++	+++	+++	+++	+++
Tartrazine	-	-	-	+	+	++	++	+++	+++	+++

Where *T. v.= Thymus vulgaris; A. s.= Achillea santolina* and *S. o.= Salvia officinalis*, inhibition zone - (no effect), + (≤10), ++ (11-18), +++ (18-25mm).

Table 3. Effect of different concentrations of essential oils (EOs) and food additives on *St. aureus.*

EOs and food additives	Concentrations (mg/ml)									
	0.1	0.5	1.0	5.0	10	20	50	75	100	150
T. v.	++	++	++	++	+++	+++	+++	+++	+++	+++
A. s.	++	++	++	+++	+++	+++	+++	+++	+++	+++
S. o.	+	++	++	++	+++	+++	+++	+++	+++	+++
nisin	-	-	+	+	+++	+++	+++	+++	+++	+++
phloxine B	-	-	-	+	+++	+++	+++	+++	+++	+++
acetic acid	-	-	++	++	+++	+++	+++	+++	+++	+++
Tartrazine	-	-	++	++	+++	+++	+++	+++	+++	+++

Where *T. v.= Thymus vulgaris; A. s.= Achillea santolina* and *S. o.= Salvia officinalis*, inhibition zone - (no effect), + (≤10), ++ (11-18), +++ (18-25mm).

Table 4. Effect of different concentrations of essential oils (EOs) and food additives on *E. coli.*

EOs and food additives	Concentrations (mg/ml)									
	0.1	0.5	1.0	5.0	10	20	50	75	100	150
T. v.	-	-	+	++	+++	+++	+++	+++	+++	+++
A. s.	-	-	+	++	++	+++	+++	+++	+++	+++
S. o.	-	-	+	++	+++	+++	+++	+++	+++	+++
Nicin	-	-	-	-	-	-	-	-	+	+
phloxine B	-	-	-	-	-	-	-	-	+	+
acetic acid	-	-	-	++	++	++	++	+++	+++	+++
Tartrazine	-	-	-	++	++	++	++	+++	+++	+++

Where *T. v.= Thymus vulgaris; A. s.= Achillea santolina* and *S. o.= Salvia officinalis*, inhibition zone - (no effect), + (≤10), ++ (11-18), +++ (18-25mm).

Table 5. Antibacterial activity of the essential oils (MIC and MBC - µg/ml)

EOs and food additives	*Salmonella enteritidis*		*Staphylococcus aureus*		*E. coli*	
	MIC	MBC	MIC	MBC	MIC	MBC
T. v.	0.125	1.0	0.5	1.0	1.0	1.5
A. s.	4.0	4.5	5.0	6.0	4.0	5.0
S. o.	1.0	2.0	0.5	1.5	2.0	2.0
Nisin	0.0	0.0	16	32	0.0	0.0
phloxine B	0.0	0.0	8.0	32	0.0	0.0
acetic acid	8.0	16	4.0	5.0	8.0	16
tartrazine	16	64	32	64	8.0	18
Streptomycin	1.5	2.0	1.0	1.5	2.0	3.0

Where *T. v.= Thymus vulgaris; A. s.= Achillea santolina* and *S. o.= Salvia officinalis*

Our results showed that phloxine B has very low activity against gram-negative bacteria as showed the results of Rasooly [29] who said that phloxine B has no activity against gram-negative bacteria and the effective usage of the

dye for gram-negative bacteria may require the use of other agents to increase the permeability of the cell outer membrane. Acetic acid and tartrazine from our results were showed moderate activities against the tested three organisms. These results were confirmed the previous findings of Etsuzo [16] who said that the effect of vinegar on growth of food-borne pathogenic bacteria including *E. coli* (EHEC) O157:H7 was inhibited with a 0.1% concentration of acetic acid. This inhibition was generally increased in the presence of sodium chloride or glucose. Tartarzine, which is used as a colouring agent, had bactericidal activity against gram-positive and, at the higher concentrations, against some Gram-negative bacteria. Inetianbor [4] said that colouring agent had variable antibacterial, antifungal activities, and were effective against some viruses.

Essential oils of *T. vulgaris*, *A. santolina* and *S. officinalis* showed strong antimicro bial activity (both MIC and MBC) against the food borne pathogens tested (Table 5) at concentrations that ranged from 0.125 -4.0µg/ml for MIC and 1.5-6.0µg/ml for MBC. *T. vulgaris* essential oil showed significant bactericidal effects against the bacterial strains tested, with minimal bactericidal concentration 1.0µg/ml. *S. officinalis* and *A. santolina* essential oils also displayed an antimicrobial activity, however, less efficient comparing to *T. vulgaris* essential oil as MIC and MBC against the tested bacteria ranged from 0.5- 4.0µg/ml and 1.5-6.0 µg/ml for MIC and MBC respectively. From the results *S. enteritidis* and *E. coli* were resistant to nisin and phloxine B i.e. these additives had no effect as food preservatives on gram negative bacteria. On the other hand, they gave bactericidal concentration of 32µg/ml on *St. aureus*. Acetic acid and tartrazine had a similar intermediate effect on all studied bacterial species. MIC and MBC values of acetic acid were ranged between 4.0-8.0µg/ml and 5.0-16µg/ml on tested bacteria while tartrazine had MIC and MBC values ranged from 8.0-32µg/ml and 18-64µg/ml respectively.

The essential oils are promising natural component suitable for use as an antimicrobial agent with a particular interest for the pharmaceutical industry as it represents an inexpensive compound.

Moreover, a focus must be upon the bactericidal or bacteriostatic activity of the EOs which is tightly dependent on the concentration used [30,31].The effects of food additives may be immediate or may be harmful in the long run if one has constant exposure or accumulations. Immediate effects may include headaches, change in energy level, and alterations in mental concentration, behavior, or immune response [32]. Long-term effects may increase one's risk of cancer, cardiovascular disease and other degenerative conditions. Food that has no additives at all is to be preferred, most especially if it is to be used to feed children. Many foods available in the market contain different types of preservatives. These chemicals can give rise to certain health problems [5].

Conclusion

Food borne illness and diseases caused by food borne pathogenic bacteria is continuing to increase every day and it has become an important topic of concern among various food industries. Many types of synthetic antibacterial agents have been used in food processing and food preservation; however, they are not safe and have resulted in various health-related issues.

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