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Original Research Article
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EFFECT OF ESSENTIAL OILS ON MULTIDRUG RESISTANT GRAM-NEGATIVE BACTERIA

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Abstract: Obtaining healthy food, free from chemical or synthetic additives, is a major challenge. In this study, we developed a preservation method using essential oils and evaluated their effect on multidrug resistant pathogenic Gram-negative bacteria. Different concentrations (1%,0.1%,0.17% and 0.35%) of Thyme oil and black seed oil were employed in this study against pathogenic *E. coli* and *A. hydrophila* in soft cheese. The used oils at a concentration of 0.1% through dipping method resulted in accepted color and odor, little effect on flavor and normal texture and appearance, while 0.1% during inoculation had the same effect as in dipping method except moderate odor. Thyme oil had the highest reduction rate in case of 1% dipping and 0.17% inoculation on *A. hydrophila,* while against *E. coli* it was found that 0.17% inoculation and 0.35% of the dipping method had the highest reduction effect. Thyme essential oil seems to be a suitable natural food preservative alternative.

Key words: essential oils; thyme oil; black seed oil; cheese; *E. coli*; *A. hydrophila*

Introduction

Annually, many consumers were subjected to foodborne diseases (1), which necessitate the orientation of food safety principles (2). Dairy products represent major sources for those pathogens. Cheese that contains important nutrients but may cause severe health hazards through carrying pathogenic bacteria (3) specially *E. coli* that indicates poor hygienic condition of manufacture processes (4). Multi drug resistant strains of *E. coli* cause severe symptoms start with diarrhea, urinary tract infection (UTI), meningitis and septicemia according to their virulence (5). *Aeromonas hydrophila* (*A. Hydrophila*) is an emerging pathogen that threat public health (6).

Synthetic and food chemical additives that are used to prolong food shelf life may cause intoxications, progressive diseases, that may reach to cancer. To overcome this problem, utilization of natural essential oils (EOs) extracted from aromatic plants to control the growth of pathogenic microorganisms (7, 8) may offer a solution.

EOs are mixtures of various chemical compounds that have been Generally recognized as safe extracts (GRAS) (9). They have antibacterial activities that used in food industry as a bio preservative to prevent spoilage and to extend food shelf life (10) due to presence of phenolic constituents and polypeptides (11) that inhibit spoilage and pathogenic bacteria (12). Additionally, they enhance food nutritive characteristics, sensory properties and shelf life (13).

Nowadays, EOs substitute synthetic flavors (14), but with limited concentrations that have no adverse effect on food due to their strong aroma (15, 16).

Thyme (*Thymus vulgaris* L.) is used in aromatic industrial food additives (17). It acts as an excellent

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natural antimicrobial agent due to presence of thymol which constitutes around 50 % of the components which have potent antimicrobial effect (18). It is considered one of the world's top ten essential oils (19). However, EOs have some selectivity on their action against microbial types (20), Gram-negative bacteria like *E. coli* 0157:H7 is more resistant to EOs (21) due to cell wall structure (22). *N. sativa* seed (Black seed) has pharmacological properties, including antimicrobial activity, so it is used in medicinal and cookery purposes (23).

These natural preservatives should meet the growing consumer demands for clean-label products that are free from chemical or synthetic additives (24). EOs action depends on permeability of cytoplasmic membrane, that cause cytoplasmic contents releasing, (25), or through inhibition of the ATPase enzyme (26).

Food inherent contents of protein and lipid may interfere with EOs antimicrobial action (27). Usage of natural EOs with emergent technologies like high pressure, ultrasound, and electric pulses result in using lower concentrations of EOs, because they damage cell membrane and facilitate cell penetration and antimicrobials action of EOs (28). Although, the sensory attributes of food may be negatively impacted even with low EOs concentration (29). So, we made our effort to obtain healthy natural acceptable product with high value by reducing some of multidrug resistance bacteria with keeping food quality.

Materials and methods

Preparation of Essential Oils:

EOs of Thyme and Black seed with concentrations of 0.1,0.17,0.35 and 1% for each were purchased from the oil extraction unit, National Research Center, Egypt. These oils were stored away from light at 4°C until use.

Preparation of bacterial strains:

Multi drug resistant *E. coli* and *A. hydrophila* strains (which carry virulence and resistance genes) that obtained from our previous study (30) were preserved in Tripticase Soya Broth (TSB, Oxoid) at 4°C. Strain inoculum was incubated in TSB at 37°C for 24 h. then serial dilution was made to provide 10^6 CFU/mL (31).

Preparation of white soft cheese:

Milk was pasteurized at 75°C for 15 seconds, then cooled to 43°C, salted and divided into 2 main groups (1st for inoculation, 2nd for dipping). For inoculation group, EOs (with 0.5 tween 80) with different concentrations and bacterial strain (mentioned later) were added. Following thorough stirring, milk was renneted (32) and left to curdled, cut and whey drained. Cheese was preserved at 4 ±1°C for 10 days (33). Samples were examined for sensorial evaluation and bacterial counts at 2 days interval (34). All tests were accomplished in three replicates and the mean values were then measured.

Preparation of treated samples (dipping):

Prepared white soft cheese samples were grouped into the following: control groups dipped in tested pathogens suspension only, and other groups dipped in the tested pathogens suspension *supplied with* 0.1, 0.17, 0.35 and 1% of thyme oil and Black seed oil separately. Dipping was done for 30 second (35).

Sensory evaluation of treated samples:

Cheese sensorial evaluation was done by nine-point hedonic scale and was performed by ten trained members of Animal Health Research Institute, where 1 unacceptable and 9 very acceptable, while the limit of acceptability was 5 (36). Cheeses were evaluated for their appearance and color, body and texture, odor and flavor. Cheese samples were cut into cubes (1.5 x 1.5 x 1.5 cm) and covered with plastic wrap to prevent dehydration. Each member was given three cubes of each sample. The maximum acceptable concentration of the studied essential oils in soft cheese samples was determined through sensory evaluation tests. For each of the examined essential oils (thyme and black seed), different soft cheese samples were made by adding different concentrations (0.1, 0.17, 0.35 and 1%) for each essential oil (by inoculation and dipping methods.

E. coli count:

Counting was done through a direct method by the spread surface technique on selective agar

media, eosin methylene blue (EMB) agar, and incubation for 24 hours at 37°C (37).

A. hydrophila count:

Aeromonas isolation medium base with the addition of Aeromonas Selective Supplement (FD039) was used (38). Cultured plates were incubated at 35-37°C for 18-24 hrs. Dark green and opaque with a dark center were considered *A. hydrophila.*

Statistical analysis:

Reduction rate was calculated as [reduced number-main number/main number] x 100. Results were statistically evaluated by application of analysis of variance (ANOVA) test (39).

Results and discussion:

Dairy products are suitable environment for bacterial contamination ruining products quality and making them unfit for human consumption which may lead to public health hazard (40). So, it is necessary to decrease bacterial count and improve product quality to obtain healthy food. One of preservative methods of food is using natural additives like EOs.

EOs had the ability to damage cell membrane of bacterial cell and increases its permeability and elimination of protein and ions (41), disturb bacterial cell membrane and decrease the intracellular ATP pool of the bacterial cell, causing loss of cytoplasmic membrane integrity and cell death that result in bactericidal effect (42). Difference in the structure of bioactive compounds may be affected by time of the part of harvesting and method of extraction that made variation in EOs inhibitory effect (43). Pathogenic *E. coli* is more resistant to EOs than other types (44). There is restriction in EOs usage as preservatives like negative effect on flavor meanwhile high concentrations are prerequisite to reach to satisfactory antimicrobial activity (45).

Most recently emerging foodborne pathogens are *A. hydrophila*, and *E. coli* (46). In this study, we used thyme and black seed oils to overcome harmful effect of multidrug resistant Gram-negative bacteria like *E. coli* and *A. hydrophila.* Thyme oil is used as a flavoring agent in the food industry (47).

Effect of essential oils on sensory evaluation:

In this study, thyme and black seed oils were added to manufactured cheese through inoculation and dipping. During dipping method 1% concentration of the 2 types was not accepted due to their very strong aroma, bitter taste with no clear effect on color and little effect on texture and appearance. While the same concentration through inoculation results in rejected cheese due to unpleasant color, odor, taste and unaccepted texture and appearance. On other hand, 0.1% concentration of both oils during dipping method was nearly accepted for color, odor and texture and appearance with little effect on flavor, while the same concentration during inoculation was accepted for color, appearance and texture with moderate effect on both odor and flavor. The most concentration was acceptable was 0.17% during dipping method, while the same concentration in inoculation method was acceptable except moderate effect on odor and flavor at 1st days that become normal with time. The concentration of 0.35% during dipping has the same effect as in case of 0.17% but 0.35% during inoculation was not accepted (table 1).

EL- Kholy et al. (48) reported that 0.1% of thyme oil was the most acceptable concentration according to sensory evaluation.

Effect of essential oils on bacterial count:

A. hydrophila:

A. hydrophila is an aquatic organism occurring naturally in freshwater like rivers and lakes (49). It is transmitted to man through the consumption of contaminated food or water (50). It is responsible for *Aeromonas* septicemia (MAS) epidemic outbreak (51).

In this study, the most reduction rate was resulted from adding thyme oil with 1% during dipping method and reached 100% from 2nd day and still along 10 days at refrigerator without deterioration in case of *A. hydrophila*. While, the same concentration of black seed oil had a moderate reduction effect on *A. hydrophila* not as in case of thyme oil, it was 14.29% at 2nd day, 32.86% at 4th day, gradually increased with time 38.57% at 6th day, 57.14% at 8th day, 61.43% at 10th day (table 2).

Table 1: Sensory evaluation of manufactured soft cheese treated with essential oils (Thyme oil +Black seed oil).

EO	Method	Color	Odor	Flavor	Appearance and texture
Thyme oil 1%	**Dipping**	No effect on color	Very strong aromatic odor	Not palatable (better taste)	Little effect on appearance and texture
Black seed oil 1%	**Dipping**	No effect on color	Very strong aromatic odor	Not palatable (better taste)	Little effect on appearance and texture
Thyme oil 1%	**(Inoculation)**	Not accepted (strong effect on color)	Very strong aromatic odor	Not palatable (Better taste).	Affect texture (No curd formation).
Black seed oil 1%	**(Inoculation)**	Not accepted (strong effect on color)	Very strong aromatic odor	Not palatable (better taste)	Affect texture(No curd formation)
Thyme oil 0.1%	**Dipping**	Accepted	Accepted	Little effect on Flavor(Slightly accepted)	No effect on appearance and texture
Black seed oil 0.1%	**Dipping**	Accepted	Accepted	Little effect on Flavor(Slightly accepted)	No effect on appearance and texture
Thyme oil 0.1%	**(Inoculation)**	Accepted	Moderate odor	Little effect on Flavor(Slightly accepted)	No effect on appearance and texture
Black seed oil 0.1%	**(Inoculation)**	Accepted	Moderate odor	Little effect on Flavor (Slightly accepted)	No effect on appearance and texture
Thyme oil (0.17%)	**Dipping**	Accepted	Accepted	Accepted	No effect on appearance and texture
Black seed oil (0.17%)	**Dipping**	Accepted	Accepted	Accepted	No effect on appearance and texture
Thyme oil (0.17%)	**(Inoculation)**	Accepted	Moderate effect	Moderate effect	No effect on appearance and texture
Black seed oil (0.17%)	**(Inoculation)**	Accepted	Moderate effect	Moderate effect	No effect on appearance and texture
Thyme oil (0.35%)	**Dipping**	Accepted	Moderate effect but decreased with time	Moderate effect but decreased with time until become accepted.	No effect on appearance and texture
Black seed oil (0.35%)	**Dipping**	Accepted	Moderate effect but decreased with time	Moderate effect but decreased with time until become accepted.	No effect on appearance and texture
Thyme oil (0.35%)	**(Inoculation)**	Little effect on color	Very strong aromatic odor	Not palatable (better taste)	No effect on appearance and texture
Black seed oil (0.35%)	**(Inoculation)**	Little effect on color	Very strong aromatic odor	Not palatable (better taste)	No effect on appearance and texture

Table 2: Reduction rate of *A. hydrophil*a with the addition of 1% EOs through dipping.

	2nd day	4th day	6th day	8th day	10 th day
Control	13.5%	-59.6%	-92.3%	-342.3%	-145.2%
***Aeromonas hydrophila* + Thyme oil**	100%	100%	100%	100%	100%
***Aeromonas hydrophila* + Black seed oil**	14.29%	32.86%	38.57%	57.14%	61.43%

Table 3: Reduction rate of *A. hydrophila* with the addition of 0.1% EOs through inoculation.

	2nd day	**4th day**	**6th day**	**8th day**	**10 th day**
Control	99.9%	98.6%	98.6%	99.5%	99.9
***Aeromonas hydrophila* + Thyme oil**	20%	26.67%	42%	55.33%	60%
***Aeromonas hydrophila* + Black seed oil**	-27%	-29.73%	-135%	13.5%	32.43%

Table 4: Reduction rate of *E. coli* with the addition of 0.17% EOs through inoculation.

	2nd day	**4th day**	**6th day**	**8th day**	**10 th day**
Control	53.13%	-62.5%	62.5%	-93.8%	28.13%
***E.coli* + Thyme oil**	100%	100%	100%	100%	100%
***E.coli* + Black seed oil**	46.67%	55%	60%	73.33%	80%

Table 5: Reduction rate of *E. coli* with the addition of 0.17% EOs through dipping.

	2nd day	**4th day**	**6thday**	**8th day**	**10 th**
Control	4.7%	41.9%	-32.6%	90%	77.4%
***E.coli* + Thyme oil**	-12.2%	92.45%	86.33%	75.5%	75.5%
***E.coli* + Black seed oil**	Zero	-142.86%	Zero	57.14%	57.14%

Table 6: Reduction rate of *E. coli* with the addition of 0.35% EOs through dipping.

	2nd day	**4th day**	**6th day**	**8th day**	**10 th day**
Control	4.5%	63.63%	28.8%	-112%	-400%
***E.coli* + Thyme oil**	100%	100%	100%	100%	100%
***E.coli* + Black seed oil**	89.55%	94.55%	94.55%	94.55%	96.82%

In case of 0.1% concentration during inoculation with thyme oil, reduction rate was 20% at 2nd day, 26.67% at 4th day, 42% at 6th day, and gradually increased till reach 55.33% at 8th day, 60% at 10th day. While in case of black seed oil with the same concentration, there was reduction in *A. hydrophila* count that noticed after 8th day with reduction rate 13.5%, till reach 32.43% at 10th day, after that cheese became deteriorated as in table (3).

Kirrella et al. (52) found that thyme oil had the most effective EO at concentration of 1% that extend shelf life of samples till 12 day and reduce *Aeromonas* count while 0.5% concentration keeps samples till 9th day only in healthy status.

E. coli:

Several *E. coli* isolates from raw milk and dairy products can cause severe foodborne illnesses in humans including hemolytic uremic syndrome, thrombotic thrombocytopenic purpura, hemorrhagic colitis, and bloody diarrhea (53), neonatal meningitis *E. coli* (NMEC), which is among the primary causes of meningitis in neonates worldwide (54) that may reach to critical illness and death (55).

In this study, we found that in case of 0.17% concentration during inoculation with thyme oil, reduction rate was 100% from 2nd day and still until 10th day in the same rate. On the other hand, in case of black seed oil the reduction rate was 46.67% at 2nd day, 55% at 4th day, 60% at 6th day, 73.33% at 8th day and reach 80% at 10th day. While the same concentration in dipping method, the count of *E. coli* starts to decrease at 4th day with reduction rate 92.45%, then reduction rate decreases slightly and became 86.33% at 6th day, 75.5% at 8th day and 75.5% at 10th day in case of thyme oil; but in case of dipping with black seed oil, the reduction of *E. coli* count started at 8th day with reduction rate 57.14% and still at the same rate at 10th day as in tables (4,5).

In case of 0.35 % concentration during dipping method, both thyme and black seed oil had high reduction rate and thyme oil had the highest

reduction rate that reach 100% from 2nd day and still at the same rate till 10th day, while reduction rate in case of black seed oil was 89.55% at 2nd day, 94.55% at 4th day, 94.55% at 6th day, 94.55% at 8th day and 96.82% at 10th day (table 6).

Control of pathogenic *E. coli* strains using thyme oil as a food additive with 1% thyme oil the reduction % reached 99.8% at 1st week and disappear at 2nd week of refrigerated storage (56). Salman et al. (23) reported that antibacterial effect of thyme oil against *E. coli* ranging from 0.02 to 1 % and Salem et al. (57) reported that thyme oil with concentration (1% and 1.5%) was acceptable till 8th day of storage, and (0.5%, 1% and 1.5%) decreased count of *E. coli* with reduction rate of 18.28% and 28.92% on 6th and 8th of storage, respectively.

Thyme oil acts as bacteriostatic and bactericidal against *E. coli* O157: H7 (58). Thymol, carvacrol and linalool in thyme are mainly the reason of the highest antimicrobial effects against bacteria (59). Hoel et al. (50) mentioned in their study that the inhibitory effect of thyme oil ranged from (0.063 to 1%) and it had the maximum antimicrobial activity against *E. coli*. Also Chandan (33) had similar report.

Black seed oil has antibacterial properties (60), but it had no inhibitory effect against *E. coli* O157:H7 while thyme oil considered the strongest EO (61). Reduction the inhibitory activity of EOs may be due to low temperatures that resulted in decreasing bacterial cell membrane permeability due to higher quantities of saturated fatty acids are contained in the membrane (62). Also, presence of fat, water, carbohydrates, proteins, salt, antioxidants, preservatives and some additives in food may decrease the inhibitory activity of EOs (63).

Silva et al. (44) reported that plant-based materials usage like thyme in food industry was necessary to inactivate *E. coli* O157:H7.

Oussalah et al. (64) and Al-Nabulsi et al. (61) reported that *E. coli* O157:H7 inhibited with 0.05% thyme oil. While Hossain et al. (65) found the effective concentration against *E. coli* O157:H7 was 0.01% while Selim (66) reported that the most effective thyme oil concentration was 0.25-0.5% on to reduce *E. coli* O157:H7 count. Alsawaf and Alnaemi (67) reported that black seed oil had an excellent effect on *E. coli* at concentration ranged from 0.3 to 1%, but Gill and Holley (26) and Al-Salman (68) had another aspect which said that black seed oil affect Gram +ve bacteria than Gram –ve bacteria and it was agree with our study.

Conclusion

Our study indicated that thyme oil is the most effective essential oil on Gram-negative bacterial count reduction while black seed oil had a moderate effect on reduction rate. In addition, some natural oils with strong aroma may be unacceptable from some consumers.

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