

SYNERGISTIC EFFECT OF VOLATILE OILS AND ANTIBIOTICS AGAINST SOME GRAM POSITIVE AND NEGATIVE PATHOGENIC BACTERIA

[8]

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ABSTRACT

Eight most currently used antibiotics were examined for their antibacterial properties against Gram-ve bacteria as *Pseudomonas aeruginosa*, *E. coli*, *Proteus vulgaris* and Gram+ve as *Staphylococcus aureus*, *Streptococcus pneumoniae*. Nitrofurantoin was the most effective against the tested bacteria, the inhibition zones ranged between 16-20mm and the MIC between 65-85ug/ml followed by ampicillin (11-18mm), ciprofloxacin (9-12mm) and gentamicin (6-9mm). The erythromycin was the lowest effective against the tested bacteria. Also, seven volatile oils were applied by contact and fumigation methods to study their effect on the tested bacterial strains. The fumigation method gave the highest inhibitory effect more than contact method and the thyme oil gave maximum inhibitory action (inhibition zone 20-28mm) against all the tested bacteria, and the MIC ranged between 0.1-0.15mg/ml followed by marjoram oil (19-25mm) and the MIC between 0.1-0.2mg/ml, cinnamon oil (12-16mm) and the MIC between 0.2-0.3mg/ml. Anise and chamomile oils did not give any response against all the tested bacteria. The combination between thyme and other tested oils gave a synergistic effect for inhibitory action against all the tested bacteria, if compared with thyme oil alone. The combination between thyme and marjoram oil gave the maximum inhibition zones (20-29mm), followed by thyme with cinnamon oil (20-27mm), thyme with geranium gave (18-27mm), thyme with peppermint (17-27mm), thyme with chamomile (16-27mm) and thyme with anise oil (15-26mm). The combination of thyme oil with different tested antibiotics gave the lowest inhibitory effect than combination between thyme and other volatile oils against all the tested bacteria. The protein and DNA content of treated bacteria with thyme oil were increased by 38.46-47.37% and 34.26-46.94% respectively, if compared by non-treated bacteria.

Keywords: *Pseudomonas aeruginosa*, *E. coli*, *Proteus vulgaris*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, Antibiotics, Plant extract, Volatile oils

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INTRODUCTION

Resistance of bacteria to antimicrobial agents has become a worldwide problem, both in hospitals and in the community (Molstad and Otto, 1999). Essential oil extracts of various plants have been reported to have inhibitory effect against diverse types of microorganisms including Gram-positive and Gram-negative bacteria, fungi and viruses (Sue *et al* 2000). The development of antimicrobial agents has advanced significantly in recent years and a large number of new drugs are available for clinical practice, yet the use of these drugs has caused changes in the bacteria that cause infection resulting in the appearance of drug-resistant strains (Ooishi and Miyao, 1997).

Numerous studies have also shown that many of these oil exert potent antimicrobial effects on a wide variety of human pathogens and food spoilage microorganisms (Abo-Ghalia *et al* 2004). Diab *et al* (2004) reported that, the treatment of urinary tract infection by *Pseudomonas aeruginosae* was completely treated by imipenem and cefotaxime antibiotics.

This study aimed to differentiate between the inhibitory effect of volatile oils, antibiotics either used separately or in combination between each of them against some pathogenic bacterial (Gram +ve and Gram -ve) strains.

MATERIAL AND METHODS

Bacterial strains

All bacterial isolates were provided by Prof. Dr. Hosam Ibrahim El-Sharkawy Prof. of Microbiology, Faculty of Medicine, Zagazig University. Identification of

bacterial isolates were carried out by typical colonial morphology and Gram stain then biochemical tests according to Cheesbrough (1985).

Volatile oils used

Seven volatile oils were used in this study purchased from Sekem company, Egypt. These oils used for testing their antimicrobial activities against bacterial strains. The testing oils were as follows: marjoram (*Margorana hortensis*), thyme (*Thymus vulgaris*), geranium (*Pelargonium graveolens*), peppermint (*Mentha piperata*), anise (*Pimpinella anisum*), cinnamon (*Cinnamomum zylanicum*) and chamomile (*Marricaria recutica*)

Antimicrobial assay

Seeded agar plates were prepared using 25ml of molten Muller-Hinton agar for bacterial growth as described by Bauer, *et al* (1966). A well of 5mm in diameter was made in solidified agar and 10ul of tested oil or combined with antibiotics was added to the well, plates were incubated at 28-30°C for 24 h. Oils were mixed with 1-2drops of tween 80 for emulsified before using. Inhibition zones were measured in mm. MIC is recorded as the lowest concentration of the antibiotics that inhibites the growth of tested organisms.

Antibiotics susceptibility test

Antibiotics susceptibility test was done by the disc-diffusion technique using commercially available antibiotic disc as recommended by Bauer, *et al* (1966).

Effect of combined between thyme and marjoram oils on protein and DNA content.

Each bacterial strains was treated with lowest MIC concentration of thyme with marjoram oils and incubated at 28-30°C for 24h., centrifuged and washed several times with sterile distilled water. The pellet was dried at 55-60°C then collected for extraction and measuring the protein and DNA content colorimetrically.

Extraction and measurement of protein

The dried pellets of the tested bacteria were extracted with 1N (NaOH) solution at 70°C for 30 min as recommended by **Peiterson, (1977)**. The extracted protein was determined as described by **Lowry, et al (1951)** using bovine albumin as a standard protein in concentrations ranging from 10µg to 100µg/ml.

Extraction and measurement of DNA

DNA was extracted according to **Boom, et al (1990)**. Dried pellets were transferred to epindorff tubes containing 1ml of TE buffer (10mM Tris-HCl and 1mM EDTA pH 8). The samples were boiled at 100°C for 10 min and centrifuged at 14000 rpm for 5 min. the supernatant containing DNA was transferred to a new tube and applied to measurement according to **Burton, (1968)** where it depends on measuring the color developed after treating the extracted DNA with diphenylamine reagent. The absorbance was measured at 600 nm.

RESULTS AND DISCUSSION

Eight antibiotics namely ampicillin (30 µg, Am), nitrofurantoin (300 µg, Nt) ciprofloxacin (5 µg, Cp), amikacin (3 µg, Am), cephradine (30 µg, Ce), gentamycin (30 µg, Gm), impenem (10 µg, Im)

and erythromycin (30 µg, Er) were used as illustrated in Table (1) with standard disc diffusion method. The presented data revealed that the highest effective antibiotic was obtained by nitrofurantoin against either Gram-ve or Gram+ve bacteria, resulted to inhibition zone ranged between 16-20 mm. Ampicillin represent the second inhibitory effect antibiotic against either Gram-ve or Gram+ve of the tested strain except *Staphylococcus aureus*, since amikacin gave the same inhibition zone as nitrofurantoin being 16 mm. It is also interesting to mention that, all the tested strain were resistance to erythromycin except *Streptococcus pneumonia* and *Proteus vulgaris*.

Gentamycin gave the lowest inhibition zones against Gram-ve bacteria which ranged between 6-8 mm. These results were agreement with **Gupta et al (1999)** who showed that the susceptibility of Gram-negative bacteria *E.coli* to nitrofurantoin reach to 70%. **David et al (2000)** found that 63% of *E. coli* isolates were resistant to gentamicin, also **Fawzy (2004)** reported that 34% of *E.coli* isolates from diarrhea were sensitive to gentamycin. **Diab et al (2004)** revealed that 90% of *Pseudomonas aeruginosa* isolates from out patient was sensitive to ciprofloxacin antibiotic. The results in Table (2) illustrated that the MIC values of ampicillin and nitrofurantoin differed according to bacterial isolates, in general the MIC of nitrofurantoin higher than ampicillin on all the tested bacterial strains.

The efficacy of seven volatile oils on bacterial strains were illustrated by contact method in Table (3) and by fumigation method in Table (4). These results indicated that, the thyme oil with contact

Table 1. Antibiotics susceptibility test against the tested bacterial strains

Bacterial strains	Inhibition zones (mm)							
	Am	NT	Cp	Ak	Ce	Gm	Im	Er
Gram -ve								
<i>Pseudomonas aeuroginosa</i>	18	20	10	17	12	8	15	-ve
<i>E.coli</i>	16	18	11	15	10	6	12	-ve
<i>Proteus vulgaris</i>	15	16	9	15	11	8	11	6
Gram+ve								
<i>Staphylococcus aureus</i>	11	16	12	16	14	13	13	-ve
<i>Streptococcus pneumonia</i>	18	18	14	15	12	14	14	7

Am = ampicillin Nt = nitrofurantoin Cp = ciprofloxacin Ak = amikacin Ce = cephradine
 Gm = gentamicin Im = imipenem Er = erythromycin .

Table 2. MIC of ampicillin and nitrofurantoin antibiotics against the tested strains

Bacterial strains	Ampicillin (ug/ml) MIC	Nitrofurantoin (ug/ml) MI C
Gram-ve		
<i>Pseudomonas aeuroginosa</i>	14	80
<i>E.coli</i>	20	72
<i>Proteus vulgaris</i>	18	65
Gram+ve		
<i>Staphylococcus aureus</i>	16	85
<i>Streptococcus pneumonia</i>	18	82

MIC = Minimum Inhibitory Concentration.

Table 3. Effect of different volatile oils against the tested strains by contact method

Volatile oils	Inhibition zones (mm)				
	<i>Pseudomonas aeruginosa</i>	<i>E. coli</i>	<i>Proteus vulgaris</i>	<i>Staphylococcus aureus</i>	<i>Streptococcus pneumonia</i>
Marjoram	20	19	18	20	18
Thyme	22	22	20	18	17
Geranium	0	12	0	10	9
Peppermint	0	10	0	9	0
Anise	0	0	0	0	0
Cinnamon	16	15	14	15	13
Chamomile	0	0	0	0	0
Control	0	0	0	0	0

Table 4. Effect of different volatile oils against the tested bacterial strains by fumigation method

Volatile oils	Inhibition zones (mm)				
	<i>Pseudomonas aeruginosa</i>	<i>E.coli</i>	<i>Proteus vulgaris</i>	<i>Staphylococcus aureus</i>	<i>Streptococcus pneumonia</i>
Marjoram	25	22	20	21	19
Thyme	27	28	23	20	20
Geranium	0	13	0	12	9
Peppermint	0	12	0	11	8
Anise	0	0	0	0	0
Cinnamon	16	14	14	13	12
Chamomile	0	0	0	0	0
Control	0	0	0	0	0

and fumigation methods was the highest effective against all Gram-negative bacteria followed by marjoram and cinnamon oils, the remaining oils have low or not effect on Gram negative and positive bacteria. On the other hand, marjoram oil was the highest effective against Gram positive followed by thyme and cinnamon oil. There has been increasing interest in the use of natural substances with antimicrobial properties in preference to synthetic substances for controlling diseases (**Dac-Vinh et al 2000; Hussain et al 2003; Dorman and Deans, 2004; and Abo-Ghalia et al 2004**). These results were agreement with **Chao et al (2000)** who reported that Gram-ve bacteria have a cell wall covered by an outer membrane composed of lipopolysaccharide (LPS) and some proteins, this structure may prevent either the uptake of the oils or protect the peptidoglycan layer from the oils. The outer LPS membrane of Gram – ve bacteria present a permeability barrier to hydrophobic substances that can enter and inhibit the growth of Gram +ve bacteria (**White, 1995**). Gram +ve bacteria had not the outer membrane and the peptidoglycan layer is on the outside and more available to contact with the oils. On the other hand, **Zambonelli et al (2004)** suggested that the alterations caused by thymol are due to its ability to damage the cellular membranes and to interfere with the membrane enzymatic reactions which are fundamental for cellular membrane. Bacteriostatic (MIC) effect of thyme, marjoram and cinnamon which selective the most effective oils (by contact method) were tested against selected strains. The results were reported in Table (5) which illustrated that the lowest MIC of the thyme followed by marjoram and cinnamon oils against

Gram positive and negative bacteria. MIC of the thyme oil ranged between 0.1-0.15mg/ml, whereas marjoram from 0.1-0.2 and cinnamon oil from 0.2-0.3 mg/ml. **Abo-Ghalia et al (2004)**, reported that the MIC of thyme oil 0.32 mg/ml against *Staphylococcus aureus*, *Streptococcus pyogenes*, *E. coli* and *Proteus vulgaris*, however *Klebsiella sp.* and *Streptococcus faecalis* were inhibited by 0.64 mg/ml. Thyme oil had a bacteriostatic concentrations against *E. coli* and *S. enteridis* at 0.05% and 0.04% respectively, as reported by **Smith et al (1998)**.

The data presented in Table (6) clearly show that the highest figures of synergistic inhibitory effect against all the tested strains, which express as inhibition zones (mm) were obtained in the treatment contained equal mixture of thyme and margoram (10ul) than other combination of rest oils. **Cappelletty and Rybak (1996)** reported that the combinations of antimicrobial agents are considered to be synergistic if the effect of the combination is greater than the effect either agent alone or greater than the sum of the effect of the individual agents. Antagonism results occurred if the combination provides an effect more than the effect of either agent alone or more than the sum of the effects of the individual agents. The combination of thyme oil with different antibiotics were tested and the results in Table (7) revealed that the combination of thyme oil with nitrofuratoin gave the highest inhibitory effect against all tested bacterial strains and the action of thyme oil with all tested antibiotics increase the synergistic inhibitory effect against resistant bacterial isolates with treated antibiotics only. The combination between amoxycillin and 10 μ l anise oil gave a

Table 5. MIC of thyme, marjoram and cinnamon oils against the tested strains

Bacterial strains	Thyme oil (mg/ml)	Marjoram oil (mg/ml)	Cinnamon oil (mg/ml)
Gram-ve			
<i>Pseudomonas aeuroginosa</i>	0.10	0.15	0.25
<i>E.coli</i>	0.15	0.20	0.30
<i>Proteus vulgaris</i>	0.10	0.15	0.20
Gram+ve			
<i>Staphylococcus aureus</i>	0.10	0.10	0.20
<i>Streptococcus pneumonia</i>	0.15	0.10	0.25

Table 6. Efficacy of combination between thyme oil and different volatile oils against the tested strains

Treatment	Inhibition zones (mm)				
	<i>Pseudomonas aeuroginosa</i>	<i>E.coli</i>	<i>Proteus vulgaris</i>	<i>Staphylococcus aureus</i>	<i>Streptococcus pneumonia</i>
Thyme + marjoram	29	28	24	20	21
Thyme + geranium	26	27	22	19	18
Thyme + peppermint	27	26	23	20	17
Thyme + cinnamon	27	27	22	20	20
Thyme + anise	26	26	23	18	15
Thyme + chamomile	27	27	22	20	16

Table 7. Effect of combination between thyme oil and different antibiotics against the tested strains

Treatment	Inhibition zones (mm)				
	<i>Pseudomonas aeruginosa</i>	<i>E.coli</i>	<i>Proteus vulgaris</i>	<i>Staphylococcus aureus</i>	<i>Streptococcus pneumonia</i>
Thyme + ampicillin	22	20	20	18	17
Thyme + nitrofurantoin	23	22	21	19	21
Thyme + cefprofloxacin	20	20	18	17	17
Thyme + amikin	21	20	19	18	18
Thyme + cephradine	20	20	20	17	19
Thyme + gentamicin	21	21	18	16	18
Thyme + imipenem	22	20	19	16	17
Thyme + erythromycin	20	21	20	15	17

Table 8. Effect of thyme oil (10ul) on protein and DNA content of the tested Gram positive and negative bacteria

Bacterial strains	Protein content (mg/gm)			DNA content (ug/gm)		
	Control	Treated	% of increasing	Control	Treated	% of increasing
Gram-ve						
<i>Pseudomonas aeruginosa</i>	40	58	45.00	110	148	34.55
<i>E.coli</i>	38	56	47.37	108	145	34.26
<i>Proteus vulgaris</i>	41	58	41.46	105	142	35.24
Gram+ve						
<i>Staphylococcus aureus</i>	38	54	42.10	98	144	46.94
<i>Streptococcus pneumonia</i>	38	54	38.46	105	150	42.86

$$\% \text{ of increasing} = \frac{\text{Treated} - \text{Control}}{\text{Control}} \times 100$$

synergistic effect against the multiresistant isolates of *Ps. aeruginosa* where this combination increases the inhibition zone of the amoxicillin disc against all chosen multiresistant isolates of *Ps.aeruginosa*, **Zaid (2001)**. **Fawzy (2004)** revealed that the combination between antibiotic and 10ul thyme oil gave a synergistic effect against *E. coli*, *Salmonella typhi* and *Shigella* sp.

The effect of thyme oil on protein and DNA content of the tested bacterial strains were reported in Table (8), these results indicated that the protein and DNA content of all the tested bacterial strains increased if compared with non treated chosen isolates, but the percentage of increasing different according to the treated bacterial isolates. The protein and DNA content of *Pseudomonas aeruginosa* increased by ratio 45.0 and 34.55% respectively. The maximum increasing rate of protein and DNA content were attained at *E. coli* and *Staphylococcus aureus* by ratio 47.37 and 46.94%, respectively. **Wesam, (1994)** reported that the cephalosporins treatments were associated with inharmonious effect on the nitrogen metabolism of *Bacillus megaterium* and *E. coli*. It increased the protein-N of *B. megaterium*, on the contrary, the protein-N was decreased significantly in treated *E. coli* with cephalosporins. Such inharmonious behavior of cephalosporins can be explained at the basis of the low concentration that increased the protein synthesis through the acceleration of protein building, while the higher concentration decreased the protein synthesis (**Egorov, 1985**). As regards the effect of thyme oil treatment on DNA content, **Fawzy, (2004)** revealed that DNA content of treated isolates increased compared with controls. The data report-

ed in this concern were linked with the effect of antibiotics on nucleic acid contents. **Gottfredsson et al (1995)** have shown that the post antibiotic effect (PAE) phase after cefprofloxacin exposure is characterized by a progressive increase in DNA synthesis, which could be due to an increase in DNA repair as a result of persistent antimicrobial action during the post antibiotic effect. Alternatively, the increase in DNA could be due to continued attempts at DNA replication, since DNA polymerase activity is not hampered, but this replication is abortive because the circular DNA cannot be separated as a result of gyrase inhibition.

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مجلة اتحاد الجامعات العربية للدراسات والبحوث الزراعية ، جامعة عين شمس ، القاهرة ، ١٤ (١) ، ١٢١-١٣٢ ، ٢٠٠٦

تأثير تآزر الزيوت الطيارة والمضادات الحيوية ضد بعض البكتريا المرضية الموجبة والسالبة الجرام

[٨]

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الايبريسروميسين اقل المضادات الحيوية تأثيرا على السلالات البكتيرية المختبرة.

تم دراسة تأثير سبعة زيوت طيارة بطريقة الملامسة والتبخير على البكتريا الملوثة لغرف العمليات، ووجد أن طريقة التبخير تعطى أعلى تأثير مثبط مقارنة بطريقة الملامسة ضد البكتريا المختبرة، وكان زيت الزعتر أكثر الزيوت له نشاط ضد بكتيري، حيث أظهر تثبيطا من ٢٠-٢٨م، والتركيز المثبط لنمو البكتريا بين ٠,١٥-٠,١ ملليجرام/مل، يليه زيت البردقوش (١٩-٢٥م)، والتركيز المثبط للنمو ٠,٢-٠,١ ملليجرام/مل، وزيت القرفة من ١٢-١٦م والتركيز المثبط للنمو بين

تم استخدام ثمانى مضادات حيوية شائعة الاستخدام لدراسة نشاطها ضد البكتيريا السالبة لجرام مثل سيدوموناس اريجينوزا والاشيرشيا كولاي و بروتياس فولجارس والموجبة لجرام مثل ستافيلوكوكس اوربوس وستربتوكوكس نيومونيا المعزولة من غرف العمليات.وقد وجد أن النيتروفورانتون اكثر تثبيطا للبكتريا المختبرة ، حيث وصل النشاط الضد البكتيري إلى ١٦-٢٠مم والتركيز المثبط للنمو من ٦٥-٨٥ ميكروجرام/مل ، يليه الامبسيلين (١١-١٨م)، والاميكين (١٥-١٧م) ، والامبينيم (١١-١٥م)، والسبروفلاكسيسين (٩-١٢م) ، والجيتاميسين (٦-٩م)، وأظهر

زيت الينسون (١٥-٢٦م). وأظهرت النتائج أن خلط زيت الزعتر مع المضادات الحيوية يعطى تأثير مثبط اقل من الخلط مع الزيوت الطيارة لكل السلالات البكتيرية، وأوضحت النتائج أن المحتوى البروتيني والحامض النووي للبكتريا المعاملة بزيت الزعتر يزيد بنسب بين ٤٦,٣٧-٣٨,٤٦% للمحتوى البروتيني و ٤٦,٢٦-٣٤,٩٤% للحامض النووي مقارنة بالغير معاملة.

٢,٠٣-٠,٢ ملليجرام/مل، وزيت الينسون والبابونج ليس له تأثير مثبط على البكتريا المختبرة. ووجد أن خلط زيت الزعتر مع الزيوت الأخرى ينشط التأثير الضد بكتيري مقارنة بتأثير زيت الزعتر منفردا، وبخلط زيت الزعتر مع البردقوش زادت فعالية النشاط الضد بكتيري الى ٢٠-٢٩م، ثم يليه خلطة مع زيت القرفة (٢٠-٢٧م)، ومع العطر (١٨-٢٧م)، ومع النعناع (١٧-٢٧م)، ومع البابونج (١٦-٢٧م)، ومع

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