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ORIGINAL ARTICLE

Antimicrobial Activities of Some Essential Oils against Bacteria isolated from patient with sore throat infection

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ABSTRACT

Background: *Essential oils possess antibacterial activity, so they are used to control the antibiotic resistant bacteria isolated from sore throat patients.* ***Objective:*** *This study represents a trial to solve the problem of resistance of some harmful pathogenic bacteria to traditional antibiotics through testing the antibacterial effect of some safe essential oils extracted against the resistant strains.* ***Methodology:*** *The study was conducted on 100 patients with different age and sex who complained with sore throat infection from Outpatients and Inpatients Departments of Otorhinolaryngology and Pediatrics, Benha Teaching Hospital during the period from May 2017 to August 2019. 100 Throat swabs were subjected to culture, isolation and identification of isolated bacteria which were tested for their susceptibilities to antibiotics, essential oils and combinations of both antibiotics and essential oils. The highest effect of essential oils on bacterial cell was detected by scanning and transmission electron microscope.* ***Results:*** *The results showed that essential oils have antibacterial activity against some multidrug resistant bacteria(MDR) and a combination between antibiotics and essential oils is a second chance to face the resistance as they were more effective against different bacterial isolates than using each one of them individually.* ***Conclusion:*** *The essential oils might be exploited as natural antibiotic for the treatment of several infectious diseases overcoming the problem of bacterial resistance to traditional antibiotics.*

INTRODUCTION

Sore throat is pain or irritation of the throat. It is usually caused by pharyngitis or tonsillitis[1]. Sore throat is one of most common infectious problem that facing Otorhinolaryngology, Pediatrics and General Medicine doctors[2] .

Bacteria cause 15-30% sore throat cases. They play an important role in tonsillar inflammation. Most cases of bacterial sore throat are caused by group A beta-hemolytic streptococci (GABHS). *Strep.pyogenes* adheres to adhesion receptors that are located on the tonsillar epithelium[3].

Careless and overdose of antibiotics often result in a condition where the body becomes more susceptible to diseases and then treatment becomes complicated as the organisms build up resistance against the antibiotic drugs that are administered more often than required [4,5].

Many reports were published for discussing the probable risks of antibiotic resistance, but now the situation is hazardous, as the crisis differed from the past. It is also essential to make a note of; new discoveries of antibiotics to fight the antibiotic resistant pathogens are restricted[6,7].

From that point of view, searching for an alternative treatment for MDR infections became a mandatory demand[8]. Essential oils are odorous and volatile compounds which are only found in 10% of plant kingdom[9]. Essential oils have shown to possess antibacterial, antifungal, antiviral, insecticidal and antioxidant properties[10]. The combination of antibiotics with essential oils may perform a new way to treat infectious diseases. Many researchers have studied the effect of the combination between antibiotics and different essential oils extracted from medicinal plants. The results showed that those combinations were potent for reducing bacterial resistance to drugs[11].

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) are good techniques used to examine the ultra-structural changes in bacteria induced by antimicrobial peptides[12].

This study aimed at testing some essential oils as antibacterials against bacteria resistant to antibiotics and infecting patients with sore throat.

METHODOLOGY

This work was carried out in Microbiology and Immunology Department, Faculty of Medicine, Benha University and the Department of botany and Microbiology, Faculty of Science, Menoufia University, during the period from May 2017 to August 2019.

Approval for the research design was obtained by the ethical committee, Faculty of Medicine, Benha University.

Samples:

Sterile swabs were used to obtain samples from the surface of tonsils, tonsillar fossae and the posterior pharyngeal wall. Specimens were immediately placed in nutrient broth (NB) transport media and then transferred to laboratory of Microbiology Department, Faculty of Medicine, Benha University.

Culture and isolation:

Each sample was inoculated on Blood, MacConkey, Nutrient and Cled agar plates and incubated at 37°C for 24 hours. The suspected growth was sub cultured on nutrient agar plate and incubated at 37°C for 24 hours for identifying the growth characters[13].

Identification of isolated bacteria:

It was based on colony characteristics of the organisms as hemolysis on blood agar, changes in physical appearance in differential media and enzyme activities of the organisms. The colonies on the different culture media were isolated and identified according to the conventional methods described by Bergey's manual of systematic bacteriology[14].

Preparation of the bacterial suspension:

It was done by picking up 5-10 colonies of each isolate with a sterile wire loop and suspended into 2.5 ml of sterile distilled water. The density of suspension was determined by comparing with density standard on McFarland 0.5 barium sulphate solution[15].

Antibiotic susceptibility testing:

Antibiotic discs (Oxoid) including Penicillin G, Linezolid, Vancomycin, ceftriaxone, Erythromycin, Levofloxacin, Clindamycin, Tetracycline were used for Gram positive bacteria and Amikacin, Gentamycin, Amoxicillin/Clavulinic, Ampicillin/Sulbactam, Cefotaxime, Levofloxcin, Ciprofloxacin(5 µg) and Cefepime were used for Gram negative bacteria .

Using Muller Hinton agar (Oxoid), bacterial suspension was streaked on the surface of the plates. The antimicrobial discs were evenly distributed. After 24h incubation at 37˚C, inhibition zone diameters were measured. The results were interpreted by comparing the measured zone diameter with the interpretive criteria recommended by CLSI[16].

Effect of some essential oils on certain human pathogenic bacteria:

Preparation of essential oils:

Sixteen natural essential oils were used Anise oil, Pepprmint oil, Rosmary oil, Eucalyptus oil, Clove oil, Cinnamon oil, Origanum oil, Chamomile oil, Ginger oil, Lavender oil, Thymus oil , Lemon grass, Tea tree oil, Basil oil, Carawy oil, Camphor oil were purchased from Captain Company in the local market from Benha and stored in full dark vials at 4 ºC[17,18].

Screening for the antimicrobial potential of the different essential oils:

Seven tested resistant bacterial isolates were screened for their susceptibility to different essential oils using the agar well diffusion method[19]. The MIC of the tested different essential oil were determined using well diffusion method of Muller Hinton agar. Different dilutions of the oils were prepared (100%, 88%, 66%, 44%) using viscous liquid tween 80 (0.2 %). The MIC was recorded visually as the lowest concentration which inhibited bacterial Growth[20].

Antibacterial activity of combined antibiotics and essential oils against multidrug resistant bacteria:

Multidrug resistant (MDR) bacterial isolates were sub cultured on nutrient agar plates. A suspension was prepared using few separate colonies for each isolate and 1-2 ml of phosphate buffer. Each suspension was diluted using phosphate buffer to obtain cell count of about 10^6 CFU/ml. An inoculum of 100 µl of the MDR bacterial isolate at 10^6 CFU/ml was spread on nutrient agar plates and left to dry at 37ºC for 15 min. The antibiotic disks were loaded in 10 µl of 50 mg/ml essential oils The disks impregnated with essential oils were placed on bacterial inoculated plates at 37ºC for 24 h. Inhibition zones were measured in mm and classified as sensitive, intermediate and resistant according to NCCLS[21].

The effect of essential oils on bacterial cells by scanning and transmission electron microscope:

Picking up 5-10 colonies of bacterial isolate of bacteria were transferred to a 10mL test tube contain nutrient broth media. Essential oil was incubated with bacteria at 37°C for 18 hours. Changes in the morphology of the bacteria was photographed by scanning electron microscope (model JEOL, JSM-5200 LV, Japan); also changes in the ultrastructure of the bacteria was photographed by transmission electron microscope (model JEOL-JEM-100 SX electron microscope, Japan) at the Electron Microscope Unit of the Faculty of Medicine, Tanta University[12].

RESULTS

Out of 100 patients, 50% of females had positive growth, while 36% of males had positive growth and 14% of cases were negative.

Table 1: Correlation between patient's age and type of infection

Variable	Organisms		P
	G (-ve) (n= 20)	G (+ve) (n= 66)	
Age (mean± SD)	36.8±21.2	25±22.3	0.03^
Age groups (n, %)			
≤ 16 Y.O	3 (15%)	32 (48.48%)	0.01#
16-50	9 (45%)	19 (28.79%)	
≥ 50	8 (40%)	15 (22.73%)	

Gram-positive infection was significantly correlated with younger age, P = 0.03,

Table 2: Correlation between the clinical complain infection type

Variable	Organisms		P
	G (-ve) = 20	G (+ve) = 66	
Fever	11 (55%)	35 (53.03%)	0.8
Cough	0 (0%)	7 (10.61%)	0.04#
Sore throat	8 (40%)	22 (33.33%)	0.5
Vomiting	1 (5%)	1 (1.52%)	0.4
Abdominal pain	0 (0%)	1 (1.52%)	1

Cough symptoms were significantly associated with gram-positive infection, P = 0.04. Also the highest rate of positive infection was detected in patients complaining of fever.

Table 3: Frequency of Gram positive organisms;

Organisms	(Total n = 66)	
Staph.aureus	29	43.94%
Strep.pyogenes	37	56.06%

The commonest Gram positive organism was *Strep.pyogenes*.

Table 4: Frequency of Gram negative organisms;

Organisms	(Total n = 20)	
E. coli	5	25%
K.pneumoniae	12	60%
P.aeruginosa	3	15%

The commonest Gram negative organism was *K.pneumoniae*

Antibiotic sensitivity testing:

For Gram positive bacteria, the most effective antibiotic to *Strep.pyogens is* Vancomycin then Penicillin and mostly resistant to Ceftriaxone, the most effective antibiotic to *Staph.aureus* was Vancomycin and resistant to Penicillin.

For Gram negative bacteria, the most effective antibiotic to *P.aeruginosa is* Amikacin and mostly resistant to Ampicillin/ Sulbactam, the most effective antibiotics to *E. coli* is Amikacin and Clindamycin and mostly resistant to Cefepime, the most effective antibiotics to *K. pneumoniae* is Ciprofloxacin and mostly resistant to Clindamycin.

There were 7 isolates resistant to all antibiotics. These were *Staph. aureus* (4 isolates), *K.pneumoniae* (2 isolates) and *E.coli* (one isolate).

Table 5: Antimicrobial response against some essential oils

Oil		***Staph. aureus*** **(n=4)**	***K. pneumoniae*** **(n=2)**	***E. coli*** **(n=1)**
Anise oil	R	4 (100%)	2 (100%)	1 (100%)
Peppermint oil	R	3 (75%)	1(50%)	1 (100%)
	S	1 (25%)	1(50%)	0 (0%)
Rosemary oil	R	1 (25%)	2 (100%)	1 (100%)
	S	3 (75%)	0 (0%)	0 (0%)
Eucalyptus oil	R	3 (75%)	2 (100%)	1 (100%)
	S	1 (25%)	0 (0%)	0 (0%)
Chamomile	R	3 (75%)	1(50%)	1 (100%)
	S	1 (25%)	1(50%)	0 (0%)
Cinnamon oil	R	4 (100%)	2 (100%)	1 (100%)
Origanum oil	R	4 (100%)	2 (100%)	1 (100%)
Clove oil	R	4 (100%)	2 (100%)	1 (100%)
Ginger oil	R	4 (100%)	2 (100%)	1 (100%)
Lavender oil	R	4 (100%)	1(50%)	1 (100%)
	S	0 (0%)	1(50%)	0 (0%)
Thymus	R	4 (100%)	2 (100%)	1 (100%)
Lemon grass	R	3 (75%)	2 (100%)	1 (100%)
	I	1 (25%)	0 (0%)	0 (0%)
Tea tree oil	R	2 (50%)	1(50%)	1 (100%)
	S	2 (50%)	1(50%)	0 (0%)
Basil oil	R	4 (100%)	2 (100%)	1 (100%)
Caraway	R	3 (75%)	2 (100%)	1 (100%)
	I	1 (25%)	0 (0%)	0 (0%)
Camphor oil	R	4 (100%)	2 (100%)	1 (100%)

Table 5 shows the most effective essential oil to *Staph. aureus* is Rosemary oil and the most effective essential oil to *K. pneumoniae* is peppermint, chamomile and lavender oil, while *E.coli* isolate were resistant to all essential oil. From 7 resistant isolates, 4 isolates (3 *Staph. aureus* and 1 *K. pneumoniae*) showed sensitivity to some oils.

Table 6: MIC of different oil extract against different *Staph. aureus*:

	Oil type	**IZ (mm) at different concentration**				**P$^{\$\$}$**
		100%	**88%**	**66%**	**44%**	
***Staph.aureus* 1**	**Rosemary oil**	24.6	22.5	22.1	21.2	0.03
	Chamomile oil	21.2	21	0	0	0.03
	Tea tree oil	21.4	0	0	0	*
	Eucalyptus oil	21	0	0	0	*
***Staph. aureus* 2**	**Rosemary oil**	21	21	0	0	0.15
	Peppermint oil	21.3	0	0	0	*
***Staph. aureus* 3**	**Rosemary oil**	22.3	21.8	0	0	0.14
	Tea tree oil	22.4	21.3	21.1	0	0.14

Table 6 shows that; the Rosemary oil had the best effect on Staph 1 isolates whatever the concentration of oil extract used, P = 0.03 for concentration and 0.02 for oil type.

Table 7: MIC of different oil extract against *K.pneumoniae* isolate

Oil type	IZ (mm) at different concentration				P$^{\$\$}$
	100%	88%	66%	44%	
Pepprmint oil	21.7	21.1	0	0	0.04
Chamomile oil	22	0	0	0	*
Lavender oil	21.8	21.3	0	0	0.04
Tea tree oil	22	21.7	21	0	0.04
P$^{\$\$}$	0.2	0.2	*	*	

Table 7 shows that MIC of different oil extract against all isolates of with *K. pneumoniae* isolate all had equal effect at concentrations of 100% and 88%, P = 0.2.

The effect of essential oils on bacterial cell by scanning and transmission electron microscope:

The most effective essential oil (Rosemary oil) on the most effected bacteria (*Staph. aureus) was* examined under electron microscope

Scanning electron microscope:

It shows alternation in the structure of the cell envelope, cell membrane and generally alternation in the external structure of the cell (Photo 1).

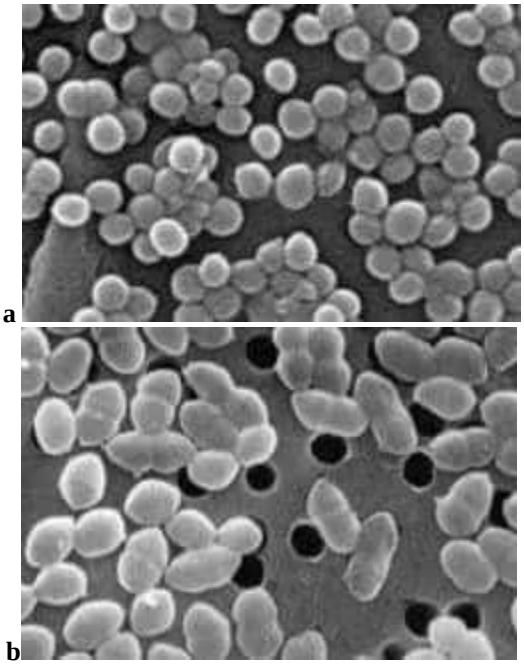

Photo 1: Photographs of scanning electron microscope reveal the effect of antibacterial substances on *Staphylococcus aureus* (a) represents control; (b) represent effect of rosemary oil.

Transmission electron microscope:

It shows alternation in the morphology of the cell, coagulation of the cytoplasmic content, vaculation in the cell, leavage of intracellular constitutant, and generally alternation in the internal structure of the cell.

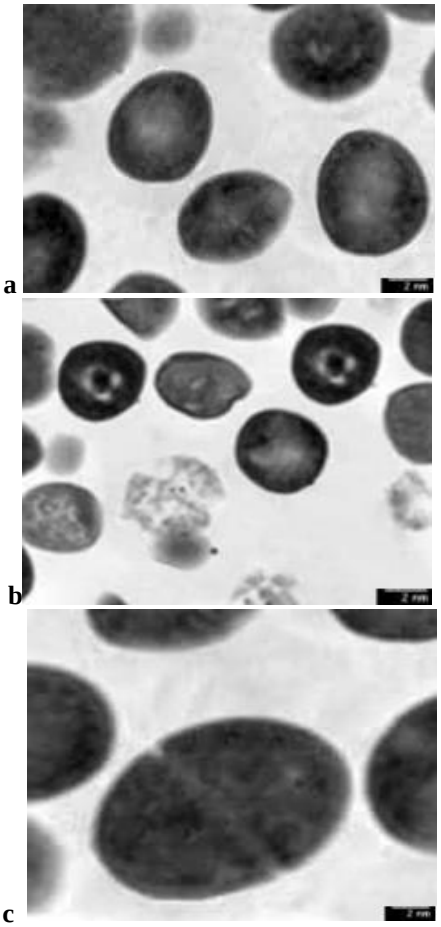

Photo 2: Photographs of transmission electron microscope reveal the effect of antibacterial substances on *Staph. aureus*; (a) represents control; (b-c) represents effect of rosemary oil.

Antibacterial activity of combination between the essential oils tested with the tested antibiotics against the resistant strains:

Table 8: Effect of combination between essential oils and the antibiotics against gram positive resistant *Staph.aureus* 4 isolate:

Essential oils	Antibiotics							
	P	CRO	TE	LEV	LZD	DA	VA	E
Anise oil	0	0	4	0	0	0	0	0
Peppermint oil	0	0	0	21	0	0	0	21.3
Rosemary oil	3	0	0	0	7	0	0	0
Eucalyptus oil	0	0	0	0	0	0	0	0
Chamomile	10	0	0	5	0	0	0	0
Cinnamon oil	0	0	0	0	0	0	0	0
Origanum oil	9	5	8	0	0	0	23	0
Clove oil	0	0	0	0	0	0	8	0
Ginger oil	0	0	0	0	0	0	0	0
Lavender oil	8	0	11	0	0	10	0	0
Thymus	0	0	0	0	6	0	0	0
Lemon grass	0	0	0	0	0	0	0	0
Tea tree oil	0	0	0	0	0	0	0	0
Basil oil	0	0	0	0	0	0	0	0
Caraway oil	0	0	0	10	0	0	0	0
Camphor oil	0	0	0	0	0	0	0	0

Table 8 shows that *Staph.aureus* isolate (4) is sensitive to combination of Origanum oil and Vancomycin (with diameter 23 mm) , to combination of peppermint oil and Erythromycin (with diameter 21.3 mm) , and to combination of peppermint oil and Levofloxacin (with diameter 21 mm).
E.coli isolates resist all combinations between antibiotics and essential oils. *K.pneumoniae* isolates resist all combinations between antibiotics and essential oils.

DISCUSSION

Sore throat is a disease caused by pharyngitis or tonsillitis[22].The main cause for expansion of pathogen resistance is self-medication, uncontrolled ridiculous antibiotic use and antibiotic overdose issues are the potential reasons for microbial resistance[23].

With the increase in bacterial resistance to antibiotics, the use of natural antimicrobial compounds is important not only in food preservation, but also in the control of human diseases and plant microbial origin[24, 25].

This study was conducted on 100 patients with sore throat from different age and sex, most of them were females from different age groups. Positive swab cultures were detected in 86% of patients. The prevalence of Gram positive and Gram negative infections was 66% and 20% respectively. The commonest Gram-positive bacteria were *Strep. pyogenes* then *Staph. aureus*. The commonest Gram-negative organism was *K. pneumoniae* followed by *E. coli* then *P. aeruginosa*.

Similarly Bukhari et al.[26], reported 74% of patients with positive culture. The prevalence of gram-positive organisms was higher than the gram-negative (84.7% and 15.3%) respectively. The most frequent isolates were *Strep. pyogenes* and *Staph. aureus* (51.4% and 12.5%) respectively, while *K. pneumoniae* and *P. aeruginosa* were present in 6 and 2% respectively. On the other hand, the results of Mave et al.[27] reported 15 isolates (25%) positive cultures, the prevalence of gram-negative bacteria was 21.6%, while gram-positive organisms were 3.3%. Around 30% of the healthy population carries *Staph. aureus* in their anterior nares and that appears to play a key role in the epidemiology and pathogenesis of infection[28,29].

Considering the correlation between the type infection and age group, our study revealed that; Gram-positive infection was significantly associated with younger age group, P = 0.03, similarly a study by AL-Ibady et al. [30] reported that infection with *Strep. pyogenes* in children is greater than in adults. On contrary Mave et al,[27] reported that the most predominant respiratory organisms found were Gram-negative bacteria in older patients.

In our work the Gram positive bacteria *Strep. pyogenes* was highly sensitive to Penicillin, Vancomycin, Also *Staph. aureus* show high sensitivity to Vancomycin. Also, *Strep. pyogenes* was highly resistant to Ceftriaxone, Tetracycline, and Linezolid, Clindamycin, Erythromycin, and Levofloxacin, and for

Staph. aureus show resistance with Penicillin, Tetracycline, Erythromycin, linezolid, Levofloxacin, Clindamycin

In a study conducted with GABHS sensitivity to different antibiotics among guidance Medical school children from 200 students which were studied, showed that GABHS were sensitive to Vancomycin, erythromycin. Surprisingly all GABHS were resistant to penicillin[31].

This agrees also with a study reporting that from 21 *Strep. pyogenes* all of them were sensitive to penicillin, 13 isolates were sensitive to erythromycin and Tetracycline, and 14 isolates were resistant [32].

On the other hand, Sabetha et al.,[33] reported that (81%) of *Staph. aureus* were sensitive to clindamycin.

The current work evaluated that most effective essential oil against *Staph. aureus* is rosemary and tea tree oils. The most effective essential oil on *K.pneumoniae* is peppermint, chamomile and lavender oil while *E. coli* isolate were resistant to all essential oil.

Our study is in agreement with a study which reported tea tree oil has high antimicrobial activity against *Staph. aureus* with lowest MIC[34]. On the other hand**,** thyme oil is the most effective followed by cinnamon oil against *Staph. aureus* isolates[35]. Also, the antibacterial activity of oregano was reported. The lowest values of minimum inhibitory concentration had a significant yield by oregano essential oil against *E.coli*[36]. Our study shows that essential oils are more effectual towards gram-positive than gram-negative bacteria. This agrees with Su et al.[37]**.**

It may be due to the presence of lipopolysaccharide that encircling the bacterial peptidoglycan layer and limits the diffusion of hydrophobic compounds into the cytoplasm[38]**.**

The current study evaluated the combination strategy. It showed that *E. coli* was resistant to all combination trials, while *Staph. aureus* isolate was sensitive to a combination between origanum oil and vancomycin, also, to a combination between peppermint oil and Erythromycin, and between peppermint oil and Levofloxacin.

This agrees with a study reporting that origanum oil exhibits activity with Vancomycin against *Staph.aureus*[39].

CONCLUSION

The effect of essential oils against MDR *Staph. aureus* was promising either alone or in combination with antibiotics as an option for treating MDR organisms. Moreover, it opens the door for a wide research for using natural products as essential oils.

Conflict of interest:

- The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.
- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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