Comparative study on the susceptibility of *Staphylococcus aureus* to antibiotics and some volatile oils

Ammar A M*, Alhumiany A A**, Moussa E H* and Ebrahim A A A*
*Dept. of Bacteriology, Mycology and Immunology, Fac. Vet. Medicine, Zagazig University
**Dept. of Medical Microbiology Fac. Applied Medical Science Trubah, Taif University, KSA

ABSTRACT

The using of antibiotics for the chemotherapy of bacterial infections represents one of the most remarkable achievements of last century. Plant essential oils and extracts have been used many thousands of years ago in alternative medicine and natural therapies. The current research aims to study the susceptibility of the *Staphylococcus aureus* isolates to 13 different antibiotics and testing the effect of five volatile oils extracts on isolated bacteria. Twelve samples including wound, osteomeylitis and brain abscess were collected from patients, these samples were cultured on suitable enriched and selective media, identified by microscopical examination and biochemical tests. Susceptibility of the bacterial isolates to13 different antibiotics and five volatile oils extracts using agar diffusion method were done. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were also studied. The effect of combination between five antibiotics and five volatile oils on 12 isolates of *S.aureus* was also studied using agar diffusion method.

INTRODUCTION

S.aureus is one of the most important pathogens causing severe morbidity and fatal infections. The rapid revolution of antibiotic resistance to *S.aureus* is of considerable concern. The proportion of multidrug resistant *S.aureus* (MRSA) has raised world wide during the last two decades *(1).*

S.aureus can express several different types of protein toxins which are probably responsible for symptoms during infections. Some toxins damage the cellular membranes while other lyse erythrocytes, but it is unlikely that hemolysis is a relevant determinant of virulence *in vivo*. Leukocidin causes membrane damage to leukocytes but is not hemolytic *(2, 3).*

S. aureus cells express on their surface proteins that promote attachment to host proteins such as laminin and fibronectin that form the extracellular matrix of epithelial and endothelial surfaces. In addition, most strains express a fibrin/fibrinogen binding protein (clumping factor) which promotes attachment to blood clots and traumatized tissue. Most strains of *S.aureus* express both fibronectin and fibrinogen-binding proteins. Interaction with collagen may also be important in promoting bacterial attachment to damaged tissue where the underlying layers have been exposed *(4).*

The invasion of host tissues by *Staphylococci* apparently involves the production of a huge array of extracellular proteins *(5).*

The best characterized and most potent membrane damaging toxin of S. aureus is α-toxin, it is expressed as a monomer that binds to the membrane of susceptible cells. Subunits of toxins then oligomerize to form heptameric rings with a central pore through which cellular contents leak In humans platelets and monocytes are particularly sensitive to α-toxin. Susceptible cells have a specific receptor for α-toxin which allows the toxin to bind causing small pores through which monovalent cations can pass, the mode of action of α-hemolysin is likely by osmotic lysis *(6).*

Coagulase is an extracellular protein which binds to prothrombin in the host to form a complex called Staphylothrombin, the protease activity characteristic of thrombin is activated in the complex, resulting in the conversion of fibrinogen to fibrin. Coagulase is a traditional marker for identifying S. aureus in the clinical microbiology laboratory *(7, 8).*

There has been an increased interest in looking at antimicrobial properties of biologically active compounds. There has been an increased interest in investigating

antimicrobial properties of extracts from aromatic plants particularly essential oils. Therefore, it is reasonable to expect a variety of plant compounds in these oils with specific as well as general antimicrobial activity and antibiotic potential *(9).*

Essential oil from cinnamon bark also contains cinnamyl acetate (8.7%) which increases the activity of the parent compound. granulation cytoplasmic membrane rupture and inactivation and/or inhibition of intracellular and extracellular enzymes, these biological events could take place separately or concomitantly culminating with mycelium germination inhibition *(9-11).*

Peppermint leaf and peppermint oil have a long history of use for digestive disorders. Recent evidence suggests that enteric coated peppermint oil may be effective in relieving some of the symptoms of irritable bowel syndrome. A combination product including peppermint oil and caraway oil seems to be moderately effective in the treatment of non-ulcer dyspepsia. Topical application of peppermint oil may be effective in the treatment of tension headache, because of its relaxing effects on smooth muscle peppermint oil given via enema has been modestly effective for relief of colonic spasm in patients undergoing barium enemas. Peppermint oil is well tolerated at the commonly recommended dosage but it may cause significant adverse effects at higher dosages *(12).*

MATERIAL AND METHODS

Bacterial strains

Specimens (include wound, osteomyelitis and brain abscess) were collected from 12 subject, 8 were collected from wound infection cases, 3 samples were collected from cases of osteomyelitis and one sample obtained from brain abscess). The cases were taken from Ultra Lab. In Zagazig city.

Media and antibiotic discs: Nutrient agar, Nutrient broth, Baired parker agar medium *(13),* Mannitol salt agar, Muller–Hinton agar (MHA) (Oxoid Ltd., England) and DNase agar medium (Becton Dickinson USA).

The antibiotics tested at microgram and they include: Amoxicillin-clavulanic acid (30μg), Ampicillin-sulbactam (20μg), Cefepime (30μg), Cefoperazone (75 μg), Cefotaxime (30μg), Cephradin (30μg), Ceftriaxone (30μg), Cefuroxime sodium (30 μg), Cephalexin (30μg), Aztreonam (10μg), Imipenem (10μg), Meropenem (10μg) and Oxacilline (10 μg).

Chemicals and reagents: Half McFarland standard solution and Gram stain *(14).*

Volatile oils: Five volatile oils were kindly obtained from Sekem Company, Salam city, Egypt, these volatile oils were: Garlic, Thyme, Cinnamon, Peppermint and Origanum.

Methods

Morphological identification: the twelve samples were cultured on nutrient agar with 7.5% gram NaCl and incubated at 37°C for 24-48hs. Microscopical identification was also including for observation of the grape like colonies by using Gram stain. Subculture of positive colonies on manitol salt agar at 37°C for 24-48h was done, the colonies were surrounded by yellow halo zone, thus indicate mannitol fermentation. Also culturing on Baired parker media was included at the same conditions and black color with halo zone appeared.

Biochemical identification: the twelve samples were tested firstly by catalase test as follow, Two to three ml of 3% hydrogen peroxide solution was poured into a test tube, using a sterile wooden stick or a glass rod several colonies of the suspected colonies were picked and immersed in the hydrogen peroxide solution. Active bubbling means catalase positive organism *(15).*

Coagulase test: This test was done using 0.2 ml of plasma, 0.8 ml of over night broth culture of the test organism, gently mixed then the tubes were incubated at 37°C for up to 4 hs. After 1 h. if no clot was formed examination was done after 3 hs. If no coagulum was formed after four hours incubation at 37°C, the tubes were left over night in the room temperature and reexamined again as some strains of *S. aureus* produced a delayed clot *(16).*

Deoxyribonuclease (DNase) test: The DNA agar plates were prepared and divided into

sections to denote the strain to be applied to them. A colony of the *Staphylococcus* isolate was spot inoculated onto a small area of the medium. The plate was incubated aerobically at 37°C for 18-24 hours, then flooded with few milliliters of 1 mol/L (1 N) hydrochloric acid to precipitate un hydrolyzed DNA. After standing a few minutes, the plate was examined. The DNase test was positive when the spot cultures were surrounded by clear, unclouded zones*(16)*.

Thermostable nuclease production: Prepare microslides by spreading 3 ml toluidine blue-deoxyribonucleic acid agar on the surface of each microscope slide. After agar solidified, cut 2 mm diameter wells (10-12 per slide) in agar and remove agar plug by aspiration. Then add about 0.01 ml of heated sample (15 min in boiling water bath) of broth cultures used for coagulase test to well on prepared slide. Incubate the slides in moist chamber 4hs at 35°C, the bright pink halo extending at least one mm from periphery of well indicates a positive reaction *(17)*.

Antimicrobial assay of twelve isolates: the inoculum was allowed to develop certain turbidity, and then adjust its turbidity by comparing with 1/2 McFarland turbidity standard for each 12 samples, then after using sterile swab which was dipped into ready prepared 1/2 McFarland and streaking the plates in one direction, then streaking at right angles to the first streaking, and finally streaking diagonally, end by using the swab to streak the outside diameter of the agar. The streaked plates were allowed to dry, then 6 antibiotic discs were applied to each plate finally these plates were incubate for 18 h. at 37°C. the inhibition zone was measured using a metric ruler *(18)*.

Antimicrobial assay of volatile oils: Diffusion method was used in this survey, culture plates seeded with the desired tested bacteria, then 30µl of volatile oils were pipette into sterile filter paper discs, the plates were put in the refrigerator for 2 hours for diffusion of the antibacterial substances, then were incubated at 37°C for 24 hr, the plates were viewed for any inhibition zones *(19)*.

Minimum inhibitory concentration (MIC) for volatile oils: the test was done using broth microdilution technique as described previously *(20,21)*.

RESULTS

Antibiotic susceptibility of 12 isolates of S. aureus was shown in Table 1. The obtained results revealed that the most effective antibiotic was Imipenem (IPM) 66.7% (8 out of 12), Meropenem (MEM) 50% (6 out of 12), Cephalexin (CL) and cefotaxime(CTX) 41.7%(5 out of 12) , Amoxicillin-clavulanic acid (AMC), Cefuroxime (CXM) and oxacillin (OX) 33.3% (4 out of12). High resistance percentage was Ampicillin-sulbactam (SAM) (91.7), while low resistance percentage was seen with imipenem (IPM) 33.3%.

Table 1. Antibiotic susceptibility of 12 isolates of S. aureus

Antibiotics	Isolate No.											
	1	2	3	4	5	6	7	8	9	10	11	12
AMC	S	S	S	R	S	R	R	R	R	R	R	R
SAM	R	R	R	R	S	R	R	R	R	R	R	R
FEP	S	S	S	R	I	R	R	R	R	R	R	R
CFP	I	I	I	S	I	R	R	R	R	I	R	R
CTX	S	I	S	R	I	S	R	S	R	S	R	R
CE	S	S	R	S	R	I	R	R	R	R	R	R
CRO	I	S	I	I	I	R	S	R	R	R	R	R
CXM	I	S	S	S	S	R	R	R	R	R	R	R
CL	S	S	S	R	S	R	R	R	R	S	R	R
ATM	S	I	R	R	I	R	R	S	R	R	R	R
IPM	S	S	R	S	R	S	S	R	S	S	R	S
MEM	S	S	I	S	S	R	S	S	R	R	R	R
OX	S	R	R	S	R	R	S	R	R	R	R	S

S= Sensitive I=Intermediate R=Resistant

AMC = Amoxicillin-clavulanic acid, **SAM** Ampicillin-sulbactam, **FEP** = Cefepime,**CFP** = Cefoperazone, **CTX** = Cefotaxime, **CE** = Cephradin, **CRO** = Ceftriaxone, **CXM** = Cefuroxime sodium, **CL** = Cephalexin, **ATM** = Aztreonam , **IMP** = Imipenem, **MEM** = Meropenem and **OX** = Oxacillin

Sensitivity test of S. aureus (strain No. 10) against 5 different volatile oils shown in fig. (1) Which revealed that cinnamon oil *(Cinnamomum zeylanicum)* was the only sensitive one (16mm).

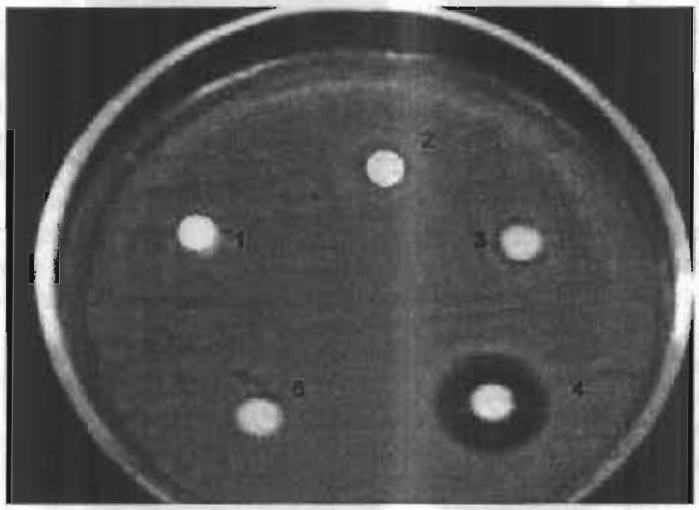

Fig. 1. Sensitivity test of S. aureus (strain No. 10) against 5 different volatile oils
1- Garlic , 2-Thym ,3- Peppermint ,4- Cinnamon and 5- Origanum

MIC and MBC by using Cinnamon oil on 12 isolates of *S.aureus* which was sensitive to cinnamon oil was shown in Table 2 which revealed that cinnamon oil was the most effective volatile oil in this study.

Table 2. MIC and MBC by using Cinnamon oil on 12 isolates of S.aureus

No.	MIC	MBC
1	0.35	0.7
2	0.2	0.35
3	0.2	0.35
4	0.2	0.35
5	0.2	0.35
6	0.2	0.35
7	0.2	0.35
8	0.2	0.35
9	0.2	0.35
10	0.35	0.7
11	0.2	0.35
12	0.2	0.35

(MIC) : Minimum inhibitory concentration
(MBC): Minimum bactericidal concentration

The results of effect of combination between antibiotics and volatile oils are seen in Table 3.

Table 3. Combination between antibiotics and volatile oils effect onto the bacterial growth

IsolateNo.	OX	OX + Cinnamon	CE	CE + Cinnamon	IPM	IPM + Cinnamon	CL	CL + Cinnamon	SAM	SAM + Cinnamon
1	20	20	25	34	30	30	35	38	11	0
2	11	14	18	12	19	20	20	12	10	8
3	10	13	10	20	25	0	18	0	10	0
4	19	23	22	30	30	36	12	10	11	0
5	22	7	0	0	18	0	18	0	20	8
6	0	14	14	30	13	30	10	24	6	0
7	6	20	12	32	12	30	12	20	10	0
8	0	8	10	0	19	0	0	0	6	0
9	8	20	8	32	11	40	8	30	0	0
10	6	10	0	0	10	30	19	0	8	0
11	8	22	12	32	40	42	10	30	0	0
12	20	0	0	0	30	0	0	0	0	0

OX = Oxacillin, **CE** = Cephradin, **IMP** = Imipenem, **CL** = Cephalexin and **SAM** Ampicillin-sulbactam,

DISCUSSION

Antibiotic resistace is highly dangerous problem which threats both animal and human health. *S.aureus* is one of the most important pathogens causing severe morbidity and fatal infections. Concerning the antimicrobial susceptibility patterns, Imipenem (IPM) was the most effective drug among the tested antichemotherapeutics where it inhibited 66.7% of all tested isolates followed by, Meropenem (MEM) which only inhibit 50% of all tested isolates follwed by Cephalexin (CL) and cefotaxime(CTX) which inhibited 41.7% and finally Amoxicillin-clavulanic acid (AMC), Cefuroxime (CXM) and oxacillin(OX) which inhibited 33.3%. The high resistance percentage was Ampicillin-sulbactam (SAM) 91.7% similar results were reported by *(3)* which showed that *S.aureus* isolates were sensitive to Imipenem (66%) and lower percent (40%) was recorded by *(8).*

Concerning the antimicrobial activity of volatile oils. the Cinnamon oil was the highest antibacterial activity (80%) followed by peppermint (20%), on the other hand, garlic, thym and origanum oil showed (2%) similar results were reported *(9)* which showed that S.aureus isolates were sensitive to Cinnamon oil by 79%.

The obtained results revealed that, Cinnamon oil was the highest antibacterial activity (80%) followed by peppermint (20%), on the other hand, garlic, thym and origanum oil showed (2%). Similar results were reported *(12).*

The obtained results revealed that, mean of MIC of cinnamon oil was 0.2-0.7 which means cinnamon oil was the most effective volatile oil in this study against *S. aureus* isolates. Similar results were obtained *(13).*

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الملخص العربى

دراسة مقارنة على قابلية تعرض الميكروب العنقودى الذهبى للمضادات الحيوية ولبعض الزيوت الطيارة

احمد محمد عمار*، عبدالرحمن عبدالله الحمياني ، عصام حسن محمد* ، احمد عبد الله عباس***
*قسم البكتريولوجيا والفطريات والمناعه – كليه الطب البيطري- جامعه الزقازيق
**قسم الميكروبيولوجي الطبية – كليه العلوم الطبيه التطبيقيه بتربه- جامعه الطائف- المملكة العربية السعودية

خلال السنوات القليلة الماضية توجه الاهتمام إلى استخدام الزيوت الطيارة (زيوت أساسية ذات رائحة طيارة ناتجة عن الأيض الثانوى لبعض النباتات) الي استخدامها في الطب البديل والعلاج الطبيعي ويهدف البحث إلى دراسة مدى حساسية العزلات البكتيرية (المكورات العنقودية الذهبية) إلي المضادات الحيوية المختلفة، وإختبار تأثير خمسة مقتطفات من الزيوت الطيارة على تلك البكتيريا المعزولة. وقد تم جمع العينات (حالات جروح وحالات التهاب الغشاء العظمى وحالات خراريج دماغية) من اثني عشر مريضا وتم استزراع هذه العزلات علي الوسط البكتيري المغذي والاختياري وتم تشخيص هذه العزلات بإستخدام الميكروسكوب و الاختبارات الكيميائيه المختلفه وبعد ذلك تم دراسه حساسيه هذه المكورات العنقويه الي ثلاثه عشره مضادا حيويا وبعد ذلك تم دراسه تأثير مستلخص خمسه زيوت طياره علي هذه العزلات وتم عمل التركيز الادني المثبط والقاتل لهذه الزيوت وفي النهايه تم الخلط بين هذه الزيوت والمضادات الحيويه ومعرفه تأثيرها.

ولقد أوضحت النتائج أن أكثرها فاعلية ضد كل العزلات هو الامبنيم بنسبة ٦٦,٧%؛ الميرونيم ٥٠% ، السيفالوكسين، السيفوتاكسيم ٤١,٦% ، أموكسيسيلين / حمض الكلافيلونيك ، السيفيوركزين ، الاوكساسيلين ، ٣٣,٣%، السيفيبيم ، السيفرادين ٢٥% ، السيفترايكزون ، ازترونام - ١٦,٦% ، السلبكتام ، السيفوبيرازون ٨,٣% .

وأظهرت نتائج دراسة تأثير مستخلص خمسة زيوت عطرية ضد١٢عزله من الميكروب الذهبى العنقودى أن أكثرها فاعلية هو زيت القرفة ٨٠% متبوعاً بزيت النعناع ٢٠% وكانت اقل نسبة ٢% مع زيوت كلا من الثوم والبردقوش والزعتر.

أظهرت دراسة تأثير تركيزات اقل من أقل تركيز مثبط و أقل تركيز قاتل ضد العزلات البكتيرية باستخدام زيت القرفة و ذلك بإضافة تركيزات أقل من أقل تركيز مثبط للقرفة باستخدام طريقة التخفيف بالأنابيب وجد أنه يساوى (٠,٢ –٠,٧) فى كل العزلات .

وأظهرت نتائج المزج بين خمسة مضادات حيوية وزيت القرفة ، وجد أن المزج بينهم له تأثير مثبط ضد جميع العزلات البكتيرية واقوي من كلا منهما منفردا فى جميع المضادات الحيوية ماعدا السلبكتام لم يحدث تحسن بعد المزج بل حدث تضاد فى تسعة عينات. ووجد أن أفضل نسبة تحسن بعد المزج كانت مع الاوكساسيلين حيث حدث تحسن فى تسعة عينات.