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Antibacterial efficacy of some essential oils in combination with locally used disinfectants against clinical and environmental strains of *Staphylococcus* spp.

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Abstract:
Through a period of three months (October to December, 2014), 37 clinical and environmental *Staphylococcus* isolates were collected from three different hospitals in Baghdad (Baghdad Teaching Hospital, Fattima-AL-Zahra Hospital for Pediatric and Obstetricand AL-Kindi Teaching Hospital), 25(67.57%) isolates were from clinical specimens and 12(32.43%) isolates were from hospital environment. out of 37 isolates, 28(75.68%) isolates were Coagulase-Negative Staphylococci (CONS) while 9 (24.32%) isolates were *Staphylococcus aureus* (coagulase-positive staphylococci). Three different disinfectants commonly used in our hospitals were tested to determine the antibacterial activity against *Staphylococcus* isolates and the result in showed a high level resistance of *Staphylococcus* isolates to the disinfectants under test when used alone. The result also showed that the concentrated SURFANIOS and Sekulyse disinfectants were more effective on the *Staphylococcus* isolates under test in comparison to the diluted concentration of those disinfectants.
Antibacterial activity of *Syzygium aromaticum* (cloves oil), *Eucalyptus globules* (calptus oil) and *Rosmarinus officinalis* (rosemary oil) was done in different concentrations (100, 50, 25 and 12,25 mg/ml) for each essential oil. The findings revealed that the cloves oil is the only essential oil which had antibacterial activity against *Staphylococcus* spp.strains in the 100,50and 25 mg/ml concentrations, while the other two essential oils have no antibacterial activity against studied isolates. The antibacterial activity of essential oils in combination with disinfectants against Staphylococci strains were observed using agar well diffusion method and the result showed that the essential oils in combination with disinfectants showed considerably more antibacterial activity than the essential oils and disinfectants alone when compared with them.

Introduction:
The genus *Staphylococcus* is a member of the *Staphylococcaceae* family (1), and it contains at least 40 species (2). Most are harmless and reside normally on the skin and mucous membranes of humans and other organisms (3).*Staphylococci* are also omnipresnt distributed in very different niches in nature, including soil, water, and air, and in a variety of foodstuffs, such as meat, cheese, and raw milk (4). These organisms are resistant to detrimental environmental conditions and can berecovered from non-physiological environments even months after inoculation, a peculiar characteristic of *Staphylococci* is their ability to grow in high salt concentrations, and most of them grow in media with 10% of NaCl (5). The species in the genus are classified to two major group based on the production of enzyme coagulase, coagulase positive, including species *S.aureus*, *S. intermedius*, *S. schleiferi*, and *S.delphini*; and coagulase negative, including more than 30 different species (6).
Disinfectants play a vital role in worldwide infection control as a crucial weapon against transmission of nosocomial pathogens/infections combating global disease outbreak. Reduction of microbial contamination impact depends primarily on improved cleaning and proper disinfection of the hospital environment, mainly high risk areas, as these measures are crucial to stemming down distribution of these microbial contaminations. Nowadays, there is limited awareness among health care workers about choosing an appropriate disinfectant, chiefly in small health care settings. Usually, an agent with broad-spectrum antimicrobial activity is chosen based on the literature provided by manufacturers (7).
The mode of action of disinfectants is thought to be linked to destruction of proteins, lipids or nucleic acids in the cells or its cytoplasmic membrane, although microorganisms vary in their sensitivity to chemical germicide (8). The extensive use of disinfectants has given rise to the emergence of *Staphylococci* resistant to disinfectants (9).
The term “essential oil” was used for the first time in the 16th century by Paracelsus von Hohenheim, who mentioned to the effective component of a drug as “Quinta essential”.The first bactericidal experiment of EOs have been performed by De la Croix in 1881 . However, since those times the use of EOs in medicine gradually decreased (10).
Syzygium aromaticum belong to the family *Myrtaceae*. Clove oil extracted from *S. aromaticum* has biological activities, such as antibacterial, antifungal, insecticidal and antioxidant properties, and is used commercially as a savoring agent and antimicrobial material in food (11).
Eucalyptus globules belong to the family of *Myrtaceae*. Its leaves have been applied as traditional remedies for treatment of various diseases such as pulmonary tuberculosis, influenza, fungal infections and diabetes. Because of their antioxidant activity, leaf extracts of *E. globulus* have been used as food additives (12). Rosemary

(*Rosmarinus officinalis*) which has long been known asa spice and medicinal herb belongs to the *Lamiaceae* family andreceiving increasing concern due to its antimicrobial, anti- inflammatory and anti-oxidative constituents. Rosemary oils are largely used in traditional medicine, in perfumery, phytocosmetic and in liquor manufacturing (13).

This study was aimed to:

- Determine the antimicrobial activity of some disinfectants usually used in our hospitals separately and in combination with each other.
- Determine the antimicrobial activity of some essential oils on *Staphylococcus* isolates.
- Study the antimicrobial activity of essential oils and disinfectants in combination against isolates.

Methods:

Collection of isolates

Thirty seven non-duplicate clinical and environmental *Staphylococcus* spp. isolates were collected from three hospitals in Baghdad including: Baghdad Teaching Hospital, Fattima-AL-Zahra Hospital for Pediatric and Obstetricand AL-Kindi Teaching Hospital. Clinical bacterial strains were isolated from different inpatient specimens including: blood, urine and wound while the environmental strains were isolated from different hospital environment sitesincluding:Medical equipment, Patients' beds, tables, sinks and floors.

Identification of isolates

The clinical and environmental isolates were identified in the laboratories of the three hospitals mentioned above by using Vitek-2 system and API-STAPH System (Bio-Merieux, France) according to the manufacturer's instructions.

Screening for antibacterial activity of disinfectants

The antibacterial assay of disinfectants was carried out using agar well diffusion method. Three different disinfectants (table -1), which commonly used in our hospitals were tested separately using agar well diffusion method.

Table -1: Specifications of disinfectants used in this study

Disinfectants	Composition	Indication
SURFANIOS	**N-(3-aminopropyl)-N-dodécylpropane-1,3-diamine (N°CAS 2372-82-9:51mg/g), chlorure dedidécyl diméthyl ammonium (N°CAS 7173-51-5 : 25 mg/g),excipients**	**used for cleaning and disinfecting of surfaces**
Sekulyse	**benzalkonium chloride**	**used for cleaning and disinfecting medical devices**
ANIOSPRY 29	**Ethanol,chloride, Didécyldiméthyl ammonium, chlorhydrate, polyhexaméthyl ènebiguanide, excipients**	**used for cleaning and disinfecting of materials and surfaces**

These three disinfectants were used in the concentrations which usually used in the hospitals (table-2). One of these disinfectants was ready to use without dilution (number 3) and the other two disinfectants need dilution with water during the use (number 1 and 2) which diluted to 0.25% and 0.5% respectively, according to the manufacturer's instructions.

Bacterial suspension of turbidity equals to $1.5x10^8$ CFU/ml was prepared. Using a sterile cotton swab lawn cultures of the test organisms were made on the Muller Hinton agar plates. A well of 6mm diameter was made using a sterile cork borer. Separately, 100µl of each disinfectant was placed in 6mm diameter well. Also in one 6mm diameter well, 100µl of D.W. was placed as a negative control for antibacterial activity of disinfectants then the plates were incubated at 37ºC for 18-24 hours. The zone diameter of inhibition (ZDI) of different disinfectants has been determined against Staphylococcal isolates (14).

Table -2: Disinfectants used in this work.

No.	Ddisinfectants	How used in hospitals	Manufacturing Company/ Origin
1	SURFANIOS	dilute to0.25%	Laboratoires ANIOS/ France
2	Sekulyse	dilute to0.5%	Ecolab/ France
3	ANIOSPRY 29	ready to use	Laboratoires ANIOS/ France

Determination of antibacterial activity of essential oils (Eos) against *Staphylococcus* spp.

Agar well diffusion method which previously mentioned above was employed to determine the antibacterial

activity of *S. aromaticum* (cloves oil), *E. globules* (calptus oil) and *R. officinalis* (rosemary oil) as disinfectants (figure-1), these essential oils were obtained from Emad factory/ Iraq in 100% concentration. Essential oils were solubilized in Dimethyl sulfoxide (DMSO) were prepared and diluted with distal water to obtain a final concentrations (100, 50, 25 and 12,25 mg/ml). With a sterile cork borer, bores were punched on the Mueller-Hinton agar plates which were previously swabbed with the bacterial suspension, 100 µl of essential oil was poured into each bore, also in one bore,100µl of DMSO was placed as a negative control for antibacterial activity then the plates were incubated at 37° C for 18-24 hours. The zone diameter of inhibition has been measured by millimeter (mm) using a metric ruler.

Figure -1: Essential oils used in this study; A= (cloves oil), B= (calptus oil) and C= (rosemary oil).

Antibacterial effect of EOs in combination with disinfectants against *Staphylococcus* spp.

The antibacterial activity of EOs in combination with disinfectants was carried out using agar well diffusion method (14). Essential oils (100% concentration) were solubilized in DMSO (dimethyle sulfoxide) and then each essential oil was added to each disinfectant volume to volume as shows in table-3. With a sterile cork borer, wells were punched on the Mueller- Hinton agar plates which were previously swabbed with the bacterial suspension, 100 µl of essential oil in combination with disinfectants was poured into each well and the plates were incubated at 37° C for 18-24 hours. The zone diameter of inhibition has been measured by millimeter (mm) using a metric ruler.

Table -3: Essential oils in combination with disinfectants.

No.	Essential oils	Disinfectants
1	cloves oil	SURFANIOS
2	calptus oil	SURFANIOS
3	rosemary oils	SURFANIOS
4	cloves oil	Sekulyse
5	calptus oil	Sekulyse
6	rosemary oils	Sekulyse
7	cloves oil	ANIOSPRY 29
8	calptus oil	ANIOSPRY 29
9	rosemary oils	ANIOSPRY 29

Results:

Isolation and Identification of *Staphylococcus* isolates

During a period of three months (from October to December, 2014), 37 clinical and environmental *Staphylococcus* isolates were collected from three different hospitals in Baghdad. Out of these 37 isolates, 12(32.43%) isolates were from hospital environment and 25(67.57%) isolates were from clinical specimens. As in figure -2, out of 25 clinical *Staphylococcus* isolates, 15(60%) isolates were from blood, 5(20%) isolates were from urine , 3(12%) isolates were from wounds and 2(8%) isolates were from ears.

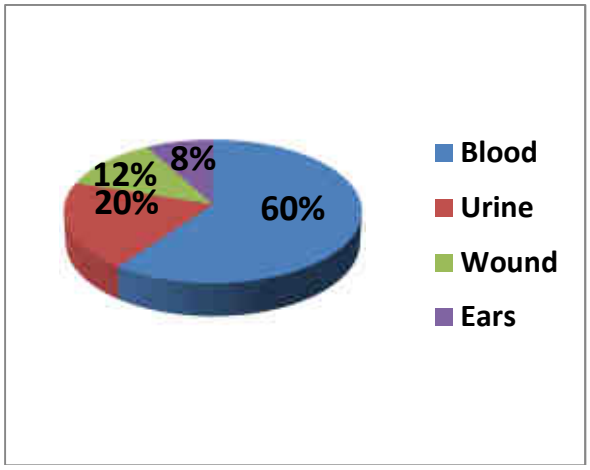

Figure -2: Percentage of *Staphylococcus* isolates in according to clinical specimens type.

According to Figure -3, out of 37 isolates, 28(75.68%) isolates were Coagulase-Negative Staphylococci (CONS) while 9 (24.32%) isolates were belong to *Staphylococcus aureus* (coagulase- positive staphylococci). During this study we noticed that the majority of coagulase-negative staphylococci were within the blood specimens and environmental specimens.

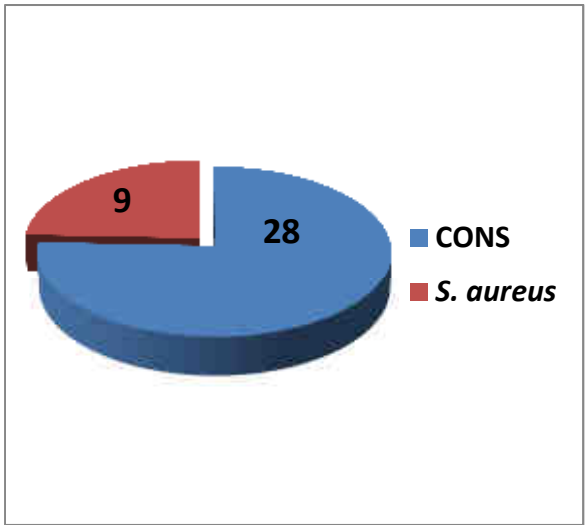

Figure -3: Numbers of Coagulase-Negative Staphylococci (CONS) and *Staphylococcus aureus* isolates.

Antibacterial activity of disinfectants against *Staphylococcus* isolates

Susceptibility of 37 *Staphylococcus* isolates towards three different disinfectants (SURFANIOS, Sekulyse and ANIOSPRY 29) commonly used in our hospitals were tested separately and the zone diameter of inhibition in millimeter (mm) of each disinfectant has been determined and summarized in table -4. Result in this table shows a high level resistance of *Staphylococcus* isolates to the disinfectants under test.

Table -4: Zone diameter of inhibition in millimeter (mm) of disinfectants.

Isolate NO.	SURFANIOS	Sekulyse	Sekulyse
1	14	16	16
2	18	R	R
3	20	R	R
4	17	R	R
5	13	R	R
6	15	R	R
7	20	R	R
8	16	20	20
9	20	20	20
10	25	R	R
11	20	12	12
12	21	R	R
13	17	R	R
14	20	19	19
15	20	R	R
16	R	16	16
17	R	R	R
18	R	R	R
19	16	R	R
20	R	R	R
21	R	R	R
22	R	R	R
23	R	R	R
24	20	R	R
25	18	20	20
26	22	R	R
27	20	R	R
28	R	R	R
29	R	R	R
30	17	R	R
31	18	R	R
32	23	19	19
33	15	20	20
34	17	R	R
35	14	R	R
36	20	11	11
37	21	R	R

On the other hand, SURFANIOS and Sekulyse disinfectants were also tested for antibacterial activity when concentrated to duplicate, 0.5% and 1% respectively. The result in table-5, showed that the concentrated SURFANIOS and Sekulyse disinfectants were more effective on the *Staphylococcus* isolates under test in comparison to the diluted concentration which is used in the hospitals according to the manufacturer's instructions, this may be attributed to increasing in MICs values of tolerant or resistant isolates.

Table -5: Zone diameter of inhibition in millimeter (mm) of SURFANIOS and Sekulyse disinfectants when concentrated to duplicate.

Isolate NO.	SURFANIOS 0.25%	SURFANIOS 0.5%	Sekulyse 0.5%	Sekulyse 1%
1	14	24	16	28
2	18	29	R	28
3	20	36	R	28
4	17	30	R	28
5	20	15	R	22
6	15	22	R	28
7	20	27	R	30
8	16	26	20	31
9	20	31	20	32
10	25	29	R	30
11	20	26	21	32
12	21	24	R	36
13	17	22	R	26
14	20	36	19	31
15	20	28	R	30
16	R	22	16	32
17	R	22	R	28
18	R	25	R	20
19	26	29	R	26
20	R	22	R	25
21	R	25	R	28
22	R	22	R	28
23	R	24	R	28
24	20	28	R	28
25	18	22	20	33
26	22	31	R	29
27	20	29	R	27
28	R	26	R	28
29	R	24	R	35
30	17	31	R	30
31	18	25	R	35
32	23	28	19	30
33	15	25	20	25
34	17	24	R	25
35	14	26	R	28
36	20	28	11	28
37	21	32	R	35

Determination of antibacterial activity of *Syzygium aromaticum*, *Eucalyptus globulus* and *Rosmarinus officinalis* essential oils (Eos) as disinfectants against *Staphylococcus* strains

Antibacterial activity of *S. aromaticum* (cloves oil), *E. globules* (calptus oil) and *R. officinalis* (rosemary oil) as disinfectants was done in different concentrations (100, 50, 25 and 12,25 mg/ml) for each essential oil alone using agar well diffusion method and the result showed that the cloves oil is the only essential oil which gave antibacterial activity against *Staphylococcus* strains in the 100, 50 and 25 mg/ml concentrations. While no antibacterial activity was found in the other two essential oil (calptus oil and rosemary oil) against *Staphylococcus* strains (figure-4).

Figure -4: Antibacterial activity of essential oils: 1=inhibition zone of cloves oil in 50 mg/ml concentration , 2, 3, 4, 5and 6= calptus oil and rosemary oil in different concentrations and C= negative control (DMSO).

Antibacterial effect of essential oils in combination with disinfectants against *Staphylococcus* strains

The antibacterial activity of essential oils in combination with disinfectants against *Staphylococcus* strains were observed using agar well diffusion method by measuring the diameter of the growth inhibition zone.

The essential oils in combination with disinfectants showed a positive synergistic effect and good antibacterial activity against *Staphylococcus* isolates as shown in figure- 5 and table -6. Data presented in table -6 showed that the essential oils in combination with disinfectants showed considerably more antibacterial activity than the essential oils alone and disinfectants alone when compared with them (table -7).

All the essential oils in combination with disinfectants gave good antibacterial activity but cloves oil showed maximum antibacterial activity compare with other two essential oils (table -6).

Figure -5: Antibacterial activity of essential oils in combination with disinfectants: 1, 2, 3, 4 and 5 essential oils + disinfectants, C= negative control (DMSO).

Table -6: Zone diameter of inhibition in millimeter (mm) of essential oils in combination with disinfectants against *Staphylococcus* isolates**.**

Isolate NO.	Essential oils + disinfectants								
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**
1	20	28	25	20	23	R	20	40	36
2	20	15	20	10	25	R	10	20	15
3	20	30	20	R	20	R	15	10	15
4	20	36	20	20	16	R	20	25	20
5	30	25	20	R	R	R	20	20	R
6	20	20	25	R	20	R	15	20	18
7	20	25	20	20	15	15	20	30	20
8	15	20	25	22	18	21	19	21	16
9	18	20	25	R	R	R	20	28	32
10	15	30	20	R	R	20	15	20	15
11	20	20	20	R	20	R	R	30	15
12	30	25	20	20	20	15	R	15	25
13	20	20	28	20	25	15	20	20	R
14	36	36	25	25	20	20	35	35	20
15	15	20	20	15	R	20	R	R	18
16	25	20	20	R	R	R	R	38	R
17	25	20	30	R	R	R	18	20	20
18	20	15	20	R	20	R	R	R	R
19	20	15	25	20	25	R	18	20	R
20	25	22	20	R	20	18	20	20	10
21	25	35	20	R	20	R	20	20	R
22	40	40	20	20	25	R	20	20	R
23	35	18	20	25	20	18	15	20	25
24	20	18	20	R	25	15	20	20	15
25	20	R	20	R	R	R	R	20	R
26	36	36	38	36	25	20	R	R	26
27	36	25	20	35	35	26	20	35	R
28	36	36	20	35	35	26	36	36	20
29	36	36	36	36	36	R	R	20	36
30	15	20	20	R	38	R	20	36	15
31	36	20	25	R	38	20	R	20	R
32	15	20	20	R	R	R	R	15	R
33	18	15	15	20	R	R	20	25	20
34	18	20	20	R	R	R	15	20	R
35	25	20	20	R	R	R	15	R	20
36	20	22	25	R	R	R	20	R	22
37	30	36	30	R	15	R	36	36	30

A= cloves oil+ SURFANIOS, B= calptus oil+ SURFANIOS , C= rosemary oil + SURFANIOS, D= cloves oil+Sekulyse, E= calptus oil +Sekulyse, F= rosemary oil + Sekulyse, G= cloves oil+ ANIOSPRY 29 , H= calptus oil + ANIOSPRY 29, I=rosemary oil+ ANIOSPRY 29.

Figure -7: A Comparison of SURFANIOS in combination with Cloves oil with Cloves oil alone and SURFANIOS alone.

Isolate NO.	SURFANIOS	Cloves oil + SURFANIOS	Cloves oil only
1	14	20	16
2	18	20	21
3	20	20	15
4	17	20	18
5	13	30	12
6	15	20	16
7	20	20	15
8	16	15	15
9	10	18	17
10	15	15	17
11	20	20	13
12	21	30	21
13	17	20	20
14	20	36	17
15	20	15	14
16	R	25	16
17	R	25	12
18	R	20	15
19	16	20	15
20	R	25	11
21	R	25	14
22	R	40	15
23	R	35	15
24	20	20	17
25	18	20	16
26	22	36	17
27	20	36	13
28	R	36	18
29	R	36	12
30	17	18	12
31	18	36	15
32	13	15	19
33	15	18	14
34	17	18	20
35	14	25	20
36	20	20	15
37	21	30	18

Disscusion:

Disinfectants are chemical agents used on inanimate objects to inactivate virtually all identified pathogenic microorganisms. Unlike antibiotics, which are chemotherapeutic drugs mostly used internally to control infections and which interact with specific structures or metabolic processes in microbial cells, disinfectants act non-specifically against multiple targets (15).

Our result showed a high level resistance of *Staphylococcus* isolates toward the disinfectants under test when used alone. The result also showed that the concentrated SURFANIOS and Sekulyse disinfectants were more effective on the *Staphylococcus* isolates under test in comparison to the diluted concentration of those disinfectants.

Clove essential oil, used as an antiseptic in oral infections, inhibits Gram-negative and Gram-positive bacteria as well as yeast. Nuñez and Aquino reported that Clove essential oil can be considered as a potential antimicrobial agent for external use because it is not clearly deactivated by dilution or affected by organic matter. The design of clinical experiments will determine its effectiveness in medical and dental practice (12).

From the above results it can be concluded that the essential oils in combination with disinfectants have great

potential as antibacterial compounds against *Staphylococcus* strains and that they can be used in our hospitals as disinfectants combination with disinfectants usually used in our hospitals.

According to many reports, there is a relationship between the antimicrobial activity and the chemical structures of the most abundant in the tested essential oil. In one study, Ghellai *et al.* mentioned that the antimicrobial activity of the essential oils combined with Povidone-Iodine solution, against strains of *S. aureus* in biofilm cultures was superior to each antimicrobial agent alone. The application of these essential oils may supply a scientific ground in increasing the antimicrobial effect of PVI against bacterial biofilms and accordingly prevention and treatment of nosocomial infections caused by, among others, *Staphylococcus aureus* (16).

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