



Antimicrobial activity of some essential oils against *S. Aureus* and *Candida Albicans* with mastitic relevance .

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ABSTRACT

A total of 100 milk samples were collected from different animals (39 cows and 61 buffaloes) from El-Kalyobia, El-Monofia and El-Sharkia Governorates. The bacteriological identification revealed that 42 Staphylococcus isolates (29/42 (69%) of Coagulase positive *S.aureus* and 13/42 (30.9%) of Coagulase negative *S.aureus*) from subclinical mastitis and clinical mastitis 22/50 (44%) and 20/50 (40%) respectively. PCR assay showed that *clfA* gene was detected in 10 isolates of *S. aureus* at 638bp. Also four isolates were identified as *Candida albicans* from the same samples. Antimicrobial agents sensitivity test revealed that 20 tested *S.aureus* isolates were resistant to Cefotaxime, Erythromycin, Clindamycin, Penicillin, Amoxicillin/Clavulnic acid, Doxycyclin, Amoxicillin, Gentamycin and Trimethoprim/Sulpha with different rates. The studying of antimicrobial activity of some essential oils showed that MICs for Thyme, Lavander, Garlic, Cinamoon oils found to be effective against *S. aureus* ranged from 80 - 2560, 80 - 2560, 5-640 and 5 - 5120 µg/ml respectively and MBCs ranged from 160 -2560, 20-2560, 20-1280 and 10-5120 µg/ml respectively. MIC₅₀ was detected only for Thyme oil at 2560 µg/ml . Marjoram, Camphor and Tea oils did not have any effect against the tested strain .*Candida albicans* isolates were resistant to antimicrobial agents and all tested essential oils.

Keywords: Mastitis, Essential oils, Antimicrobial agents, Resazurin.

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1. INTRODUCTION

Worldwide, economic losses due to mastitis have been estimated at \$35 billion (Wellenberg *et al.*, 2002). Staphylococcal mastitis was the commonest and economically the greatest concern wherever dairy farming practiced the chief reservoir of bacterium was an infected udder. Seriousness of mycotic infection of mammary glands depends upon the species of the fungus involved mycotic mastitis is a challenge, as many of these fungi do not respond to the antibiotics therapy (Tarfarosh *et al.*, 2008). The most common treatment is based on intramammary infusion of antibacterial agents. The treatment of bovine subclinical mastitis caused by *S. aureus* in the lactation can be economically unviable. However,

cure rates obtained with such drugs are not always effective, because it may determine the emergence of resistant bacteria (Zafalon *et al.*, 2007) as well as increase amounts of antibiotic residues in milk (Fagundes *et al.*, 2010). Alternative treatments to bovine mastitis with bacteriocins and plant derived compounds (Baskaran *et al.*, 2009; Mubarack *et al.*, 2011) have been described. The use of medicinal plants as source for relief and illness is an ancient and well documented of the early civilization in China, India and the near east (Mahesh and Satish, 2008). Essential oils (EO) are classified as GRAS (generally regarded as safe), show antibacterial proprieties and resistance has not been reported after prolonged exposure.

Then, the investigation of their antimicrobial activity against bacterial agents of mastitis is justifiable. So This work was planned to detect the antimicrobial activity of essential oils of plants against *S.aureus* and *Candida albicans* isolated from mastitic animals by isolation and biochemical characterization of the causative agents of bovine mastitis, identification of *S.aureus* by using PCR and determination of antimicrobial activity of some essential oils against the prevalent isolates of *S.aureus* and *Candida albicans* with determination and evaluation of MICs and MBCs of essential oils for the tested strains.

2. MATERIAL AND METHODS:

2.1. Samples collection

The present study was carried out on a total of 100 lactating cows and buffaloes, from different farms in El-Kalyobia, El-Monofia and El-Sharkia Governorates. Milk samples collected as follow 39 cow (30 clinical and 9 subclinical mastitis) 61 buffaloes (20 clinical and 41 subclinical mastitis). Milk samples were collected according to National Mastitis Council NMC (1990).

2.2. Bacteriological examination

A loopful of each sample is cultured onto nutrient agar (oxid) then stained by Gram's stain (Murray et al., 1999); Subculturing onto mannitol salt agar, Baird Parker agar (Baired et al., 1996) and blood agar (Cruickshank et al., 1975). Each colony showed typical character of *S.aureus* and Gram positive reaction was subjected to biochemical identification (catalase and coagulase tests). For isolation of *C. albicans*, each sample cultured onto Sabouraud dextrose agar then microscopical and germ tube tests were applied.

2.3. Antimicrobial sensitivity test :

Determination of the susceptibility of isolated Staphylococci to some antimicrobial agents (Table, 2) was applied

by diffusion method (Barry and Thornos Berry, 1985) Metric ruler was used to measure the diameter of the zone of inhibition according to (CLSI, 2011).

2.4. Identification of *S.aureus* using PCR assay:

Extraction of DNA according to QIAamp DNA mini kit instructions. Preparation of PCR Master Mix according to Emerald Amp GT PCR mastermix (Takara) Code No. RR310A kit. Oligonucleotides were designed as clumping factor (*clfA*) gene as follow: the forward primer *clfA-F* (GCAAAATCCAGCACAAACAGGAAACGA) and the reverse primer gene *clfA-R* (CTTGATCTCCAGCCATAATTGGTGG) (Mason et al., 2001).

2.5. Determination of MICs of Essential oils (CLSI, 2009).

Seven essential oils were used including Thyme, Lavender, Garlic, Cinamoon, Marjoram, Camphor and Tea oils obtained from National Research Center. The prepared medium were dispensed (100 µl/well) into sterile U bottom 96 well microtiter plates using multichannel pipette then 100 µl of aforementioned prepared essential oils were dispensed into the wells in column 1. final essential oil concentration were 5-5120 µl/ml. 100 µl of bacterial suspension equivalent to No. 3.0 McFarland, Inocula of approximately 4.5x 10⁵ CFU/ml were prepared with sterile 0.85 % NaCl and dispensed to each well. Then incubate at 35°C/16-18 hours. The MICs were detected before and after adding the Resazurin (Sigma-Aldrich, USA) indicator as the lowest concentration of the antimicrobial agent that inhibited visible growth of bacteria. (Palomino et al., 2002).

2.6. Determination of MBCs of essential oils

Determination of MBCs of essential oils to the susceptible isolates of *S. aureus* according to CLSI, (2009) where MBC defined as the lowest concentration of the antimicrobial agent that prevent visible growth and kill bacteria.

3. RESULTS

3.1. Results of cultural , morphological and biochemical characters of isolated *S.aureus*

S.aureus isolates on mannitol salt agar exhibited a yellow halo zone surrounding their growth because of their ability to ferment mannitol. 29 *S.aureus* isolates from samples produced clear zone of beta hemolysis . 13 isolates showing white non hemolytic colonies on blood agar .Growth of 29 isolates on Baired Parker agar appeared as black, shiny, convex and surrounded by a clear zone of about 2-5 mm in diameter .All Staphylococci are catalase positive. The ability of *S.aureus* isolates to coagulate citrated rabbit plasma was exhibited in 29 isolates indicating positive result, while 13 samples were not able to coagulate citrated rabbit plasma and considered as coagulase negative Staphylococci (Table 1).

3.2. Result of antimicrobial sensitivity test :

Antibiotic sensitivity test for 17 isolates of Coagulase positive *S.aureus* and 3 isolates of Coagulase negative *S.aureus* revealed that isolates were resistant to Cefotaxime 95.2% , Erythromycin 95.2%, Clindamycin 90.4% , Penicillin 85.7 % , Amoxicillin/Clavulnic acid 85.7% , Doxycyclin 85.7%, Amoxicillin 57.7%, Gentamycin 28.5% , Trimethoprim/Sulpha 14.2% , Norfloxacin, Ciprofloxacin and Ofloxacin 0% . These results showed a high

degree of *S.aureus* resistance to most antimicrobial agents. The highest resistance of seven staphylococci isolates was to 7 antimicrobial agents representing 33.7% (Table 2).

3.3. Result of PCR

The *clfA* (clumping factor) gene was amplified for 10 isolates of *S.aureus* at 638 bp. (Figure1).

3.4. Detection of MICs and MBCs of essential oils

Antimicrobial activity of some essential oils were demonstrated as follow: Thyme oil found to be effective against 20 *S.aureus* isolates by MIC from 80 - 2560 µg/ml and MBC from 160 -2560 µg/ml (Figure 2). Thyme oil had MIC₅₀ which was 2560 µg/ml. Lavender oil found to be effective on 4 *S.aureus* isolates by MIC from 80 - 2560 µg/ml and MBC from 320-2560 µg/ml and did not have MIC₅₀ (Figure, 3). Garlic oil found to be effective on 10 *S.aureus* isolates by MIC ranged from 5 - 640 µg/ml and MBC from 20 - 1280 µg/ml (Figure 4) and did not have MIC₅₀ . Cinamoon oil found to be effective on 5 strain of *S.aureus* isolates by MIC from 5 - 5120 µg/ml (Figure 5) and MBC from 10 - 5120 µg/ml and did not have MIC₅₀ .On the other hand, Marjoram , Camphor and Tea oils were not effective against any of the tested isolates (Table 4). In addition, *Candida albicans* isolates were resistant to all tested essential oils.

Table (1): Incidence of staphylococci spp. according to coagulase test

	Number of Staphylococci	% in relation to number of Staphylococci	% in relation to number of sample
Coagulase positive	29	69 %	29 %
Coagulase negative	13	30.9 %	13 %
Total	42	42 %	42 %

Table (2): Antimicrobial sensitivity test

Antimicrobial agents	S %	I%	R%
Amoxicillin	23%	19.4%	57.1%
Penicillin	14.2%	0%	85.7%
Amoxicillin/ clavulnic acid	4.7%	4.7%	85.7%
Norfloraxillin	85.7%	14.2%	0%
Ciprofloxacin	52.3%	47.6%	0%
Oflaxacin	85.7%	14.2%	0%
Cefotaxime	0%	4.7%	95.2%
Gentamycin	38.9%	33.3%	28.5%
Erythromycin	4.7%	0%	95.2%
Clindamycin	4.7%	4.7%	90.4%
Trimethoprim/sulpha	47.6%	38.9%	14.2%
Doxicyclin	14.2%	0%	85.7%

S: sensitive I: intermediate R: resistant

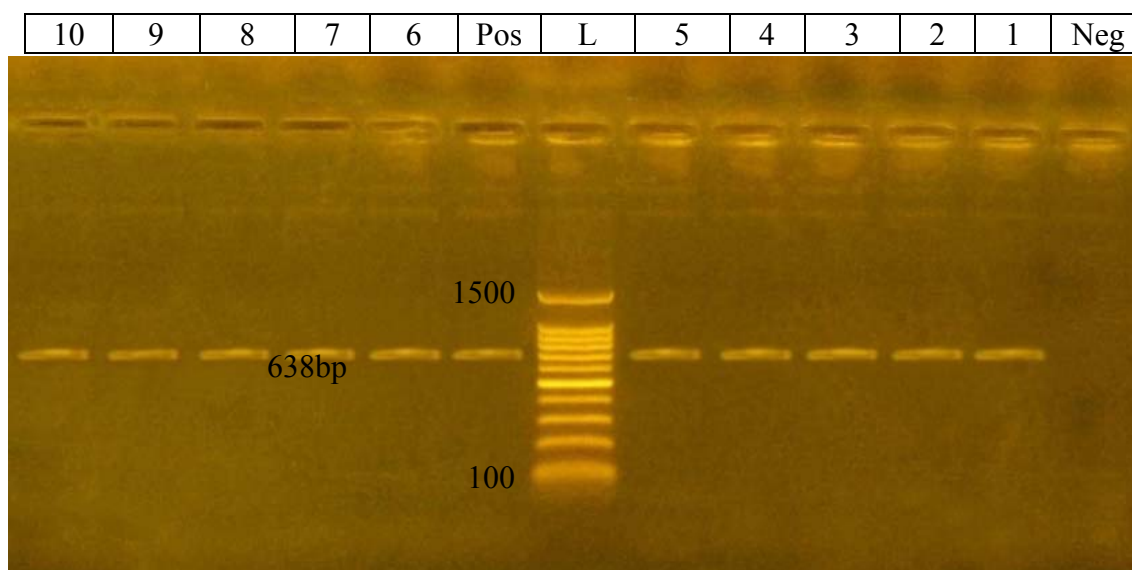


Figure (1): Amplification of *clfA* gene in 10 *S. aureus* strains. Lane L: 100-1500 bp DNA ladder, Neg: Negative control, Pos: Positive control, Lane 1- 10: positive *S. aureus* at 638bp

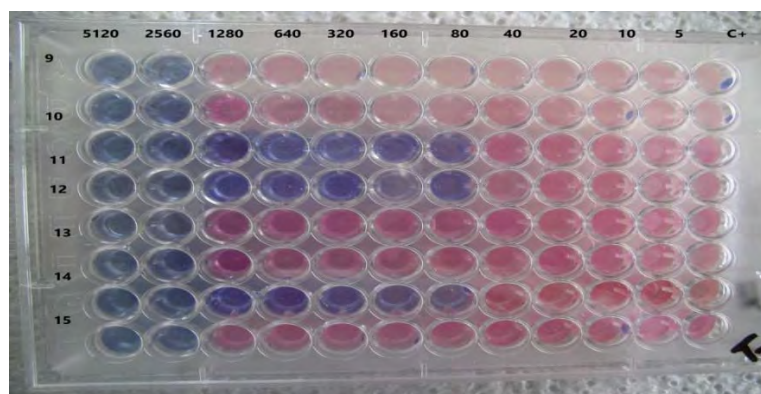


Figure (2): Activity of Thyme oil against different strain of *S.aureus* from 9 to 16: Row 1: result of MIC 2560 µg/ml (the color of first two wells were blue and other wells changed from blue to pink color). Row 3: MIC 80 µg/ml.

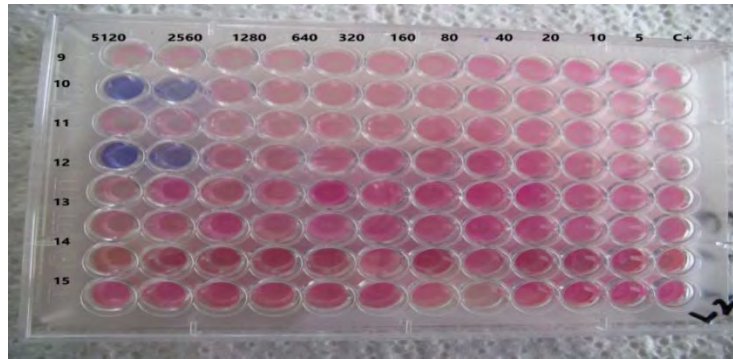


Figure (3): Activity of Lavender oil against different strain of *S.aureus* from 9 to 16: Row 2 & 4 showed MIC 2560 µg/ml (the color of first two wells were blue and other wells changed from blue to pink color).

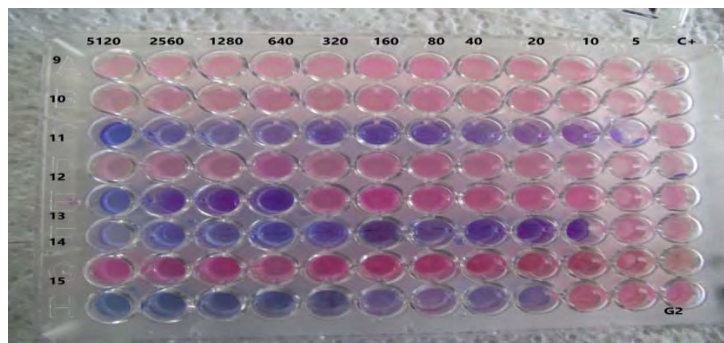


Figure (4): Activity of Garlic oil against different strains of *S.aureus* from 9 to 16: Row 3 showed MIC 5 µg/ml .Row 5 showed MIC was 640 µg/ml.

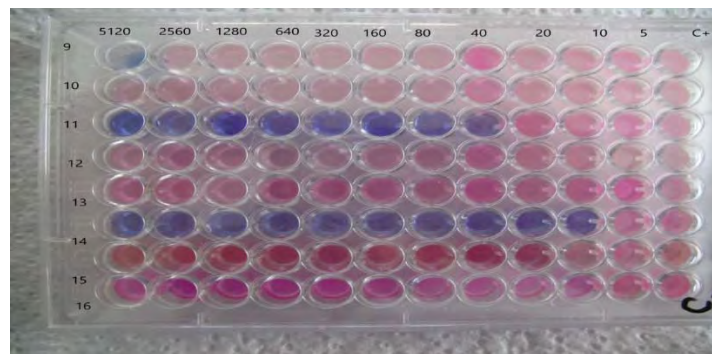


Figure (5): Activity of Cinnamon oil against different strain of *S.aureus* from 9 to 16: Row 3 showed MIC 40 µg/ml and Row 6: MIC was 10 µg/ml.

4. DISCUSSION:

In this study, the incidence of staphylococci in both clinical and subclinical mastitis found to be 40 % from clinical mastitis and 44 % was isolated from subclinical mastitis, this result was nearly similar to Shaimaa, (2012) and Hashemi *et al.*, (2011) Staphylococci spp. was the most prevalent microorganism isolated from mastitis with percentage of 42 % which was in

accordance with Deogo and Jerake, (2003) who revealed that Staphylococci spp. were 39.2% from mastitis. Staph. aureus was identified by coagulase positive and represented (29%) and staphylococci which are coagulase negative represented (13%) this result was in line with Shitandi and Kihumbu, (2004) who found that coagulase negative staphylococci (13.5%). Concerning to *Candida albicans* which isolated in this study depending on culture

and morphological characters was 4 % of all collecting sample, this percentage nearly similar to Tarfarosh *and* Purohit, (2008) who screened Fifty six cases of bovine clinical mastitis for presence of *Candida* spp. Of this *Candida* was isolated from milk of four cases (7.14%).

Antimicrobial sensitivity test for *S.aureus* isolates recorded that 33.3% of tested isolates were resistant to seven antibiotics, this is conducted with Bjorland *et al.*, (2001) who found that, *S.aureus* exhibited resistance to a wide range of antimicrobial agents including disinfectants. Concerning to resistance to Pencillin, which showed 85.7% of isolates were resistant to Penicillin this was supported by Ebrahimi and Akhavan-Taheri, (2009) who found 87% of the isolates resistant to Pencillin. 85.7% of *S. aureus* was resistant to Doxycycline which conducted with Edward *et al.*, (2009) who reported that ,the examined *Staphylococcus* spp. was highly resistant to tetracycline (more than 70%). In the current study MIC of 7 plant essential oils were detected including Thyme, Lavender, Cinamoon, Garlic, Margoram, Camphor and Tea oils . Thyme found to be effective on all tested *S. aureus* isolates with MIC range 80-2560 µg/ml which supported by Monika *et al.*, (2011) who tested The antibacterial activity of oils against clinical bacterial strains of *Staphylococcus*. The results of experiments showed that the oil from *T. vulgaris* exhibited extremely strong activity against all of the clinical strains. Thyme oil demonstrated a good efficacy against antibiotics resistant strains of the tested bacteria. Lavender oil found to be effective on *S. aureus* isolates with MIC range 80-2560 µg/ml on 5 isolates only from all tested isolates this is nearly similar to study of Monika *et al.*,(2011) who studied The antibacterial activity of oils was against clinical bacterial strains of *Staphylococcus*. The results of experiments showed that. Lavender oil had been less activity against clinical strains of *Staphylococcus*. Concerning to Cinamoon oil which found to be effective on 8 isolates

S. aureus with MIC range 5-5120 µg/ml this is nearly similar to Seenivasan *et al.*,(2006) who found that Cinnamon oil showed significant inhibitory effect against *S.aureus*. This study revealed that Cinnamon oil showed maximum activity with MIC values ranging from 0.8 to 3.2 µg/ml. Garlic oil found to be effective on 11 isolates of *S. aureus* with MIC range from 5-640 µg/ml, this was conducted with Jagadeesh *et al.*, (2011) who estimated antibacterial activity and MIC of essential oil of garlic by using various bacterial pathogens. Among the bacterial pathogens tested against essential oil of garlic, *S.aureus* was found to be highly sensitive. Camphor oil found to be not effective on any of the tested isolate *S. aureus* as the study by Seenivasan *et al.*,(2006) who found that Camphor oil failed to inhibit any of the tested strain . *Staphylococci* found to be not affected by Marjoram oil and this is nearly similar to study of Deans and Katerina, (1990) who evaluated Steam distilled volatile oil from Marjoram (*Origanum majorana* L.) for its antibacterial and antifungal activities and found that *S.aureus* being least affected to Marjoram oil. In addition, Tea oil did not have any effect against *S. aureus* isolates. In the current study, antibiotics and all essential oils did not have any effect on *Candida albicans*. This result disagreed with Iwalokun *et al.*, (2004) who studied the antimicrobial effects of aqueous garlic extract (AGE) against 10 *Candida* spp. and found that The anticandidal effect of AGE resulted in a growth inhibition zone of 27.4-6 3.7 mm with no significant difference ($P < 0.05$) in MIC values at 24 and 48 hours, respectively. Also disagreed with Elena *et al.*, (2011) who investigate the antibacterial and antifungal activity of eight essential oils and found that Lavender oil had antibacterial and particularly high antifungal activity against tested strains.

5. CONCLUSION

It was concluded from the results that further studies are necessary for assessing the importance of intramammary infection caused by other bacterial pathogens and the misused of antibiotics leads to antibiotic resistance in addition to milk residues so the practitioners and specialists should lay a strategic control of epidemics by using plant essential oils to save animal health and increase production. In addition to further evaluation of physiological effects of essential oils in vivo to determine potential suitability for mastitis as a therapy.

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