



## Alternative Approaches for Mitigation of Drug Resistance in Streptococcus Species

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### ABSTRACT

During infection, *S. agalactiae* can form biofilms which is associated with increased antimicrobial resistance. Additionally, bacteria when present in a biofilm, are resistant to various physicochemical stresses. Therefore, this study is aimed to determine the *in vitro* anti biofilm activities of cinnamon oil and/or silver nanoparticles and augmentin against multidrug resistant *S. agalactiae* isolates recovered from clinical mastitic dairy cattle. Antimicrobial susceptibility testing of 146 *Streptococci* isolates revealed that 42.5% (62/146) of *Streptococci* isolates exhibited a feature of multidrug resistance (MDR), being resistant to more than 3 antibiotics of different classes. Cinnamon oil, silver nanoparticles and augmentin exhibited marked inhibitory activities against *S. agalactiae* isolates with inhibition zone diameters up to 40 mm, 35 mm and 38 mm respectively. Besides, broth microdilution test of cinnamon oil revealed maximum activities against the tested isolates with minimum inhibitory concentrations (MICs) up to 0.0025 µg/mL. The combination of cinnamon oil and silver nanoparticles combination gave the best result as the MIC values have been reduced to 4 folds. The cinnamon oil alone was capable of reducing the number of cells just after 4 h according to time killing assay.

The above results highlight the hopeful antibacterial and antibiofilm features of cinnamon oil and silver nanoparticles in the treatment of emergent resistant *S. agalactiae* mastitic infections.

**Key words:** *S. agalactiae*, Biofilm, Antibiofilm, AgNPs, Cinnamon

(<http://www.bvmj.bu.edu.eg>)

(BVMJ-35(2): 393-412, 2018)

### 1. INTRODUCTION

*Streptococcus agalactiae* (group B streptococci, GBS) is a noteworthy infectious mastitis pathogen where it causes subclinical and mellow direct clinical mastitis in dairy

cows, resulting in critical financial misfortunes dairy ranchers (Keefe, 1997). This microorganism inadequately gets by in nature, yet can persevere inconclusively

inside the mammary organ and could be transmitted to solid dairy animals through poor draining cleanliness. Execution of the standard mastitis aversion program has annihilated *S. agalactiae* from a few nations in Western Europe (Merl *et al.*, 2003; Zadoks and Fitzpatrick, 2009). During the last decade, key virulence factors of group B Streptococci concerned with the survival, spread and persistence of the bacterium within the host (Nizet and Rubens, 2000 and Spellerberg, 2000). Colonization and infection of target tissues of GBS required the capacity of these bacteria to adhere and to keep on mucosal epithelial surfaces then, the formation of biofilm like communities could facilitate microbial survival and propagation by enhancing resistance to host defenses and nutrient lack (Melchior *et al.*, 2006; Raza *et al.*, 2013 and Rosini and Margarit, 2015). Microscopic organisms inside biofilms have expanded protection from antimicrobial specialists. In addition, the creation of biofilms was corresponded with pathogenicity and destructiveness of microscopic organisms (Saginur *et al.* (2006). Natural drugs have been critical wellsprings of items for the creating nations in treating basic irresistible ailments and defeat the issues of opposition and reactions of the as of now accessible antimicrobial specialists (Kianbakht and Jahaniani , 2003). Various *in vitro* thinks about have revealed the utilization of plant separates in mix with anti-infection agents, with critical decrease in the MICs of the anti-

toxins against some safe strains (Darwish *et al.*, 2002; Al-hebshi *et al.*, 2006 and Betoni *et al.*, 2006). Silver nano particles (AgNPs) utilized as antimicrobial agents, especially against multidrug safe microscopic organisms (Li *et al.*, 2010; Cardozo *et al.*, 2013 and Theophel *et al.*, 2014). Additive substance or synergistic antibacterial impacts of silver nano particles joined with elective methodologies for treatment of obstruction (eugenol, phenazine-1-carboxamide, and cinnamaldehyde) and regular (ampicillin, kanamycin, erythromycin, chloramphenicol, amoxicillin, ciprofloxacin, and moxifloxacin) antimicrobial specialists (Ghosh *et al.*, 2013; Theophel *et al.*, 2014 and Biasi-Garbin *et al.*, 2015). In the present study, we give evidence that GBS detaches can shaped biofilms on nonliving and living surfaces and sort 2a-pili are associated with biofilm development making more bond capacity and protection from antimicrobial agents utilized. Thus, we propose a potential novel technique to repress bacterial grip, antibacterial resistance and to prevent associated diseases.

## 2. MATERIALS AND METHODS

### *Clinical samples*

Two hundred milk samples were randomly collected from mastitic dairy cattle showing decreased milk yield with pus descended in milk, bloody or clotted milk. Out of them, one hundred and six (n=106) were obtained from different dairy farms from different Governorates in Egypt, while 94 samples were collected from sporadic cases in

Sharqia Governorate. Sampling was done during the period from September 2015 to January 2016 with no history of antibiotic application during sample collection. Samples were aseptically collected in sterile screw capped bottles. The samples were transported in an ice box and bacteriological- examination was performed within 24 h.

#### *Phenotypic characterization of Streptococci isolates*

Primary isolation and serotyping of streptococcal isolates was carried out onto Edward's agar medium (Oxoid, Hampshire, England, UK). Single, well-isolated colony from overnight cultures was subcultured onto blood agar for testing their haemolytic patterns (Edwards, 1933). The isolates were identified by standard bacteriological methods including cultural characteristics, Gram's stain and biochemical tests such as catalase, CAMP, sodium hippurate and esculin hydrolysis tests (Ayers and Rupp, 1922; Christie *et al.*, 1944 and Hardie , 1986). Serotyping of the isolates was done in the Serology Unit, Animal Health Research Institute, Dokki, Giza, Egypt using commercial antisera (Oxoid, UK) according to the manufacturer's instructions.

#### *Genotypic characterization of S. agalactiae*

Genomic DNA was extracted from phenotypic streptococci isolates by using the QIAamp DNA Mini kit (Qiagen, GmbH, Germany) according to the manufacturer's instructions. Oligonucleotide primers for streptococci genus specific gene (*tuf*),

5'GTACAGTTG CTTCA GGACGTATC-3' and 5'-ACGTTTCGATTTTCATCACGTTG -3' (Ke *et al.*, 2000) and *S. agalactiae* species specific gene (*cfb*), 5'-TTTCACCAGCTGTATTAGAAGTA-3' and 5'-GTTCCCTGAA CAT TATCTTTGAT -3') (Picard *et al.*, 2004) were used. PCR amplifications were performed with a PTC-100™ programmable thermal cycler (MJ Research Inc., Waltham, USA) in a total reaction volume of 25 µL consisting of 12.5µL of Emerald Amp GT PCR Master Mix (2X) (Fermentas, Inc. Hanover, USA), 1µL of each primer (Sigma Aldrich, Co., St. Louis, USA), 6 µL template DNA and the volume was completed to 50 µL by nuclease-free water. The amplification condition for *tuf* and *cfb* primers were performed as following thermal cycling condition sets: initial denaturation at 94°C for 5 min, 35 cycles of amplification (secondary denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec for *tuf* while *cfb* primer at 55°C for 30 sec and extension at 72°C for 30sec) and a final extension step at 72°C for 7 min . An aliquot of each amplicon was loaded on 1.5% agarose gel (Sigma-Aldrich, Co., St. Louis, MO, USA) containing 0.5 µg/mL ethidium bromide (Sigma-Aldrich, Co., St. Louis, MO, USA)( Sambrook *et al.*, 1989). A 100 bp DNA ladder (Fermentas, Inc. Hanover, USA) was used as a molecular weight standard. The amplified DNAs were electrophoresed at 100 V for 60 minutes on a mini horizontal electrophoresis unit (Bio-Rad, USA). The gel

was then visualized and photographed under an UV transilluminator (Spectroline, Westbury, USA). For each PCR experiment, appropriate positive and negative controls were included.

#### *Antimicrobial susceptibility testing*

##### *-Disc diffusion method*

Susceptibility of streptococci isolates to various antimicrobials was evaluated by Kirby-Bauer disc diffusion test (Bauer *et al.*, 1966) and the results were evaluated according to the guidelines of Clinical and Laboratory Standards Institute (CLSI, 2014). Antimicrobial discs (Oxoid, Hampshire, England, UK) including amoxicillin (25 µg), amoxicillin/clavulanic acid (20/10 µg), cloxicillin (1 µg), cefoperazone (75 µg), ceftriaxone (30 µg), cephalexin (30 µg), impenem (10 µg), streptomycin (10 µg), ciprofloxacin (5 µg), trimethoprim/sulphamethoxazole (23.75/1.25 µg), tetracycline (30 µg), clindamycin (2 µg) and erythromycin (15 µg) were used.

##### *Agar well diffusion method*

The antimicrobial activities of 7 MDR *S. agalactiae* isolates against cinnamon oil (Sigma Aldrich, Germany), AgNPs (Nakaa company, Egypt) and augmentin (GlaxoSmithKline, USA) were applied using agar well diffusion method (Valgas *et al.* (2007). It was performed using 18h cultures in 10 mL Müller Hinton broth adjusted approximately to  $1-1.5 \times 10^8$  CFU/mL. The bacterial suspension was spread over the plates of Müller-Hinton agar (Oxoid,

Hampshire, England, UK) using a sterile cotton swab in order to get a uniform microbial growth on both control and test plates. The used essential oil was dissolved in 10% aqueous dimethyl-sulfoxide (DMSO) under aseptic conditions. A well was made in MHA and filled with 100 µL of the respective cinnamon oil prepared in DMSO, while silver nanoparticles and augmentin drug were dissolved in sterile distilled water then the agar surface was inoculated with the culture. A well was filled with aqueous DMSO as negative control. The plates were left for 30 minutes at room temperature to allow the diffusion of oil and other agents and then were incubated at 37°C for 24 h. After the incubation period, the zones of inhibition around the wells were measured and recorded.

##### *Minimum inhibitory concentration*

The minimum inhibitory concentrations (MIC) of silver nanoparticles, augmentin drug and cinnamon oil were determined using the broth micro-dilution method (Kwiecinski *et al.*, 2009). The inoculum size was approximately prepared as  $5 \times 10^5$  CFU/mL. A double fold serial dilution was made for each reagent in custom-designed 96-well panels (Corning, New York, USA) starting from a concentration of 1024 µg/mL. Controls of broth medium with the microorganism or reagent alone were included as positive and negative controls. The microtitre plates were incubated at 37°C for 24h. The first dilution with no microbial growth was recorded as MIC of the reagent.

Subsequently, aliquots from each well were plated onto Müller Hinton agar and incubated at 37° C for 24 h to determine the MBC (minimum bactericidal concentration) of the reagents. The plates were checked for growth of bacterial colonies and MBCs were evaluated as the lowest concentration at which no growth was observed on the plates.

#### *Time kill curve assay*

Planktonic cells of *S. agalactiae* isolates ( $1-5 \times 10^5$  CFU/mL) were incubated in MHB containing MIC levels of cinnamon, augmentin and silver nanoparticles. At determined time points (0, 1, 2, 4, 6, 8, 10, and 24 h), aliquots were aseptically transferred to MHA plus 5% sheep blood plates and the colony forming unit counts were determined after incubation at 37°C for 24h (Biasi-Garbin *et al.*, 2015).

#### *Phenotypic detection of biofilm formation by S. agalactiae*

-Congo red agar (Reid, 1999)

Congo red agar plates were inoculated with test organisms and incubated at 37°C for 24 h aerobically. Black colonies with a dry crystalline consistency indicated biofilm production.

#### *-Quantitative detection of biofilm by microtiter plate method*

The biofilm assay was performed using flat-bottom micro titer plates as described previously (Tendolkar *et al.*, 2004). *S. agalactiae* isolates were grown at 37°C in tryptic soy broth (oxoid, Uk). The bacterial cells were then pelleted at 6,000xg for 10

min, and the cell pellet were resuspended in 5 mL of fresh medium. The optical densities (ODs) of the bacterial suspensions were measured using spectrophotometer (Jenway Ltd., Essex, UK) and normalized to an absorbance of 1.00 at 600nm. The cultures were diluted 1:40 in fresh TSB then 200µL of cells were dispensed into 12 wells in a single row of a sterile 96-well flat-bottom polystyrene micro titer plate. After incubation at 37°C for 24 h, the planktonic cells were aspirated and the wells were washed three times with sterile phosphate-buffered saline (PBS). The plates were inverted and allowed to dry for 1h at room temperature. For biofilm quantification, 200 µL of 0.1% aqueous crystal violet solution was added to each well, and the plates were allowed to stand for 15 min. The wells were subsequently washed three times with sterile PBS to wash off the excess crystal violet. Crystal violet bound to the biofilms was extracted with 200 µL of an 80:20 (v/v) mixture of ethyl alcohol and acetone, and the absorbance of the extracted crystal violet was measured at 595 nm on ELISA reader (stat fax 2010, Germany). As a control crystal violet binding was measured for wells exposed only to the medium with no bacteria. All biofilm assays were performed in triplicate. Interpretation of biofilm production was according to the criteria described previously (Stepanovi *et al.*, 2007). Based on these criteria, optical density cut-off value (ODc) is defined as: average OD of negative control +  $3 \times$  SD (standard deviation) of

negative control, and the biofilms producers are categorized as:  $OD \leq OD_c$ =not a biofilm producer,  $OD_c < OD \leq 2x OD_c$ =weak biofilm producer,  $2x OD_c < OD \leq 4x OD_c$ =moderate biofilm producer,  $4x OD_c < OD =$  strong biofilm producer.

#### *Antibiofilm activity of cinnamon, augmentin and silver nanoparticles*

For analysis of cinnamon, augmentin and silver nanoparticles effect on mature biofilm, 20  $\mu$ L of each cell suspension was added to each well containing 180 $\mu$ L of trypticase soya broth (TSB). After 24 h of biofilm formation, the medium was aspirated off and each well was rinsed with sterile PBS. A 200 $\mu$ L aliquots of TSB containing different concentrations (double fold serial dilution) of each type (cinnamon, augmentin and silver nanoparticles) were added and the plates were incubated for another 24h with alternative substances free wells and biofilm-free wells included as positive and negative controls, respectively. The optical density was measured at 595 nm with an ELISA reader. Experiments were carried out in triplicate manner (Shafreen *et al.*, 2011). The reduction of biofilm formation of each clinical strain was expressed as antibiofilm activity (%), calculated as follows:  $(OD_{control} - OD_{sample} / OD_{control}) \times 100$ .

#### *Checkerboard method*

Two fold serial dilutions of the compounds were prepared in TSB and placed on 96-well micro titer plates. Bacteria ( $1.0 \times 10^5$  cells) were grown with cinnamon or

silver nanoparticles or augmentin individually and in combinations at 37°C for 24 h. The interactions of the two compounds were analyzed by the fractional inhibitory concentration index (FICI), which is defined as the sum of FIC cinnamon and FIC silver nanoparticles or augmentin. FIC of the material is the concentration that kills when being used in combination with another divided by the concentration that has same effect when used individually (Yadav *et al.*, 2013).

### **3. RESULTS**

#### Isolation and identification of streptococcal mastitis

Bacteriological analysis of 200 milk samples randomly collected from mastitic milking cattle either from dairy farms from different Governorates in Egypt or sporadic cases from Sharqia Governorate revealed that *Streptococcus* spp. were recorded in 146 samples (73%) Table(1). On Edward's agar media, *Streptococcus* spp. showed dew drop colorless colonies, among them, 38 (26%) were  $\alpha$  haemolytic, 75 (51.3%) were  $\beta$  haemolytic and 33 (22.6%) were  $\gamma$  haemolytic when the media was supplied by 5% sheep blood. Among the  $\beta$  haemolytic isolates, 27 (18.5%) produced CAMP reaction that may be identified as either *S. agalactiae* or *S. uberis*. Biochemical characteristics of bacterial isolates identified them simply. Sodium hippurate test identified *S. agalactiae* and *S. uberis* from other streptococci isolates

which gave positive reaction (violet ring) (30/146; 20.5%) while other streptococci were sodium hippurate negative (yellow ring) (116/146; 79.5%).

In addition, esculin hydrolysis biochemical test was used for identification of *S. uberis* and *E. faecalis* (63/146; 43.2%), which produced black colonies when cultivated on bile esculin agar due to esculin hydrolysis. For differentiation between them, the isolates were cultured on MacConkey's agar media, on which *S. uberis* was negative (23/63; 36.5%), while 40 isolates (63.5%) grew which were then identified as *E. faecalis*.

All bile esculin negative Streptococci isolates (83/146) were tested for their susceptibility to bacitracin antibiotic disc. The results recorded that 45/83 (54.22%) were sensitive for bacitracin ensuring them as *S. pyogenes*. In contrary, 38/83 (45.78%) of the isolates were bacitracin resistant and seven isolates of them were recognized by CAMP positive reaction,  $\beta$ -haemolysis on blood agar and sodium hippurate hydrolysis test positive as *S. agalactiae* isolates with a percentage of 4.79 % of the total recovered isolates. Serological identification was applied for the unidentified isolates (n= 31) and the results revealed 25(80.6%) Group C streptococci and 6 (19.4%) Group D non enterococcus *Streptococci*.

Conventional PCR was done for molecular identification of streptococci isolates (n=30) which were  $\beta$  haemolytic and positive sodium

hippurate test and the results revealed 7 *S. agalactiae* isolates with amplicons of 153 pb Fig (1 &2).

#### *Antimicrobial susceptibility testing*

All streptococci isolates were tested for their susceptibility to different antimicrobial agents of several classes. Absolute susceptibility was observed for impenem followed by ciprofloxacin (73%) which could be used as a drug of choice for treatment; meanwhile, 100% of streptococci isolates were resistant to streptomycin, clindamycin, ceftriaxone, cephalixin and cefepime and cloxacillin. Additionally, more than half of isolates were resistant to amoxicillin clavulanic acid (91.7%), 86% were resistant to tetracycline, 80% were resistant to amoxicillin, 79% were resistant to erythromycin and 54.7% were resistant to sulfamethoxazole-trimethoprim. All streptococci isolates were MDR. The higher percentage of resistance to antimicrobials classes was documented for 5 classes (62/146; 42.5%).

#### *Antimicrobial activities of cinnamon oil, silver nanoparticles and augmentin.*

The effectiveness of the essential oil was confirmed by agar well diffusion assay and its inhibition zone diameter was measured. It was observed that DMSO control wells revealed no inhibition for all examined isolates. Cinnamon oil exhibited marked inhibitory activities against *S. agalactiae* isolates with inhibition zones` diameters ranged between 36-40 mm. Silver

nanoparticles exhibited inhibitory activities against *S. agalactiae* isolates but less than cinnamon oil with inhibition zones` diameter ranged between 28-38 mm. Also, Amoxicillin clavulanic exhibited inhibitory activities with inhibition zones` diameters ranged between 36-40mm.

Minimum inhibitory concentrations and minimum bactericidal concentrations of cinnamon oil, silver nano particles and augmentin using broth microdilution assay were variable. Interestingly, cinnamon oil with 10.24 µg/mL as stock concentration recorded the lowest MIC values (up to 0.0025µg/mL)while MICs of 0.25 µg/mL and 8µg/mL were recorded for augmentin and silver nanoparticles, respectively when starting with a stock solution of 512 µg /mL. The more efficient agent against *S. agalactiae* was cinnamon as its values were ranged from 0.04-0.0025µg/mL, then augmentin (2-0.25µg/mL) and at the end silver nanoparticles (16-8µg/mL).

#### *Combination between cinnamon, augmentin and silver nanoparticles alternatively*

Two types of treatments for *S. agalactiae* isolates on microtiterplate were used which showed either additive, synergistic, antagonistic or no interaction between them (Table 2). When the two compounds were combined, the MIC values of cinnamon and silver nanoparticles reduced 4folds to be 0.000625-0.0025µg/mL,0.3-0.125 µg/mL, respectively. In addition, the MIC values of augmentin and silver

nanoparticles reduced 4folds to be 0.125-0.3µg/mL and the MIC values of both cinnamon and augmentin decreased 4 folds (0.00015/0.0083µg/mL).

#### *Time kill curve assay*

Synergistic activities of cinnamon oil, silver nanoparticles and augmentin alternatively (previously shown by broth dilution assays) were examined in a multi-time point assay and the time-response curves were plotted. Bacteria were inhibited to various degrees in the presence of cinnamon, silver nanoparticles and augmentin. When treated with cinnamon at 0.0025µg/mL, AgNPs at 16µg/mL and augmentin at 0.25µg/mL, complete inhibition of *S. agalactiae* growth after 24 h exposure was found. However when the bacteria were exposed to cinnamon + AgNPs, 100% inhibition was achieved within 10 h of exposure, upon exposure to cinnamon+augmentin, 100% inhibition was achieved within 12 h., while upon exposure to AgNPs+augmentin, 100% inhibition was achieved. This data suggested the cinnamon oil enhanced anti-bacterial effect of AgNPs. The synergistic interaction was observed after 4 hour incubation where there were decreasing in *S. agalactiae* growth than seen in AgNPs alone.

#### *Phenotypic detection of biofilm by Congo red agar*

*S. agalactiae* isolates (n=7) were cultured on Congo red agar for production of biofilm. Biofilm producing isolates (5/7,



71.4%) could convert red color of media into black color due to consuming the sucrose in the media.

*Quantitative detection of biofilm by S. agalactiae isolates*

On Trypticase soya broth, 7 *S. agalactiae* isolates were cultured for biofilm production quantitatively. Five (71.4%) *S. agalactiae* produced weak biofilm, while moderate and strong biofilm producing isolates were also recorded one for each (14.3%).

*Antibiofilm activities of cinnamon oil, augmentin and silver nanoparticles*

Different concentrations of cinnamon oil, augmentin or silver nanoparticles against *S. agalactiae* were applied as antibiofilm and measured by the ELISA reader. In comparison with biofilm of each *S. agalactiae* isolate, biofilm was inhibited by different concentrations of cinnamon with variations (21-98%), most of isolates were inhibited at different concentrations (51.2-0.025 $\mu$ g/mL) so, cinnamon oil was effective against biofilm formation by *S. agalactiae*.

*Combination between the antibacterial agents as antibiofilm*

Combinations of cinnamon oil, augmentin and or silver nanoparticles were applied to increase their efficacy to be used as antibiofilm. With the combination of cinnamon oil and augmentin, most of isolates were inhibited at concentration of 32-64  $\mu$ g/mL, so this combination was less effective than cinnamon oil or augmentin alone in

treatment of biofilm formation by *S. agalactiae*. In combination of silver nanoparticles and cinnamon oil, most of isolates were inhibited at concentrations of 0.25-0.125 $\mu$ g/mL, so this combination was more effective than each of cinnamon or silver nanoparticles alone in treatment of biofilm formation by *S. agalactiae*. In combination of silver nanoparticles and augmentin, most of isolates were inhibited at concentration of 1/1  $\mu$ g/mL, so this combination was more effective than each of them alone in treatment of biofilm formation by *S. agalactiae*.

Table (1): Incidence of *Streptococcus* species in clinically mastitic dairy cattle in different localities in Egypt

Source of samples(No)		Positive growth on Edward`s media <sup>a</sup>	<i>S. agalactiae</i> <sup>b</sup>	<i>S. uberis</i> <sup>b</sup>	<i>S.pyogene</i> <i>s</i> <sup>b</sup>	<i>Enterococcus</i> <sup>b</sup>	Group C streptococci ( <i>S. dysagalactiae</i> ) <sup>b</sup>	Group D non- enterococcus, streptococci <sup>b</sup>
Sporadic	Belbies (19)	14 (73.68)	1 (7.14)	2 (14.29)	4 (28.57)	4 (28.57)	3 (21.43)	0 (0.00)
	Awladsief (38)	24 (63.16)	0 (0.00)	1 (4.17)	8 (33.33)	10 (41.67)	3 (12.50)	2 (8.33)
Sharkia	Bordien (27)	1 (3.70)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	1 (100.00)
	Abohamad (3)	1 (33.33)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	1 (100.00)
	Diarnegm (7)	3 (42.86)	0 (0.00)	0 (0.00)	1 (33.33)	0 (0.00)	2 (66.67)	0 (0.00)
Farms	Mandoor (19)	18 (94.74)	0 (0.00)	7 (38.89)	4 (22.22)	5 (27.78)	2 (11.11)	0 (0.00)
Sharkia	Alharaky (16)	16 (100.00)	2 (12.50)	2 (12.50)	4 (25.00)	8 (50.00)	0 (0.00)	0 (0.00)
Giza	Alalamia (10)	10 (100.00)	1 (10.00)	3 (30.00)	3 (30.00)	2 (20.00)	1 (10.00)	0 (0.00)
Suez	Suez (15)	15 (100.00)	0 (0.00)	0 (0.00)	1 (6.67)	5 (33.33)	8 (53.33)	1 (6.67)
	Atefsalem (11)	9 (81.82)	0 (0.00)	0 (0.00)	3 (33.33)	0 (0.00)	5 (88.89)	1 (11.11)
	Dina (17)	17 (100.00)	1 (5.88)	5(29.41)	8 (47.06)	3 (41.18)	0 (55.56)	0 (0.00)
Alexandria	Talaat (8)	8 (100.00)	0 (0.00)	3 (37.50)	2 (25.00)	2 (25.00)	1 (12.50)	0 (0.00)
	Said elalfy (10)	10 (100.00)	2 (20.00)	0 (0.00)	7 (70.00)	1.(10.00)	0 (0.00)	0 (0.00)
Total	200	146 (73.00)	7 (4.79)	23 (15.75)	45 (30.82)	40 (27.40)	25 (17.12)	6 (4.11)

No: Number. Data are represented by No (%).

a:Percentages were calculated according to number of mastitis milk samples.

b:Percentages were calculated according to number of positive Streptococci isolates on Edward’s media

Table 2: Results of checkerboard test for cinnamon and silver nanoparticles, cinnamon/augmentin and augmentin/ silver nanoparticles against *S. agalactiae*

GB S stra ins	cinnamon	MIC		Cinnamon/ silver nano particles	FICI	Effect	Cinnamon/Aug mentin	FICI	Effect	Augmentin / silver nano particles	FICI	Effect
		Silver nano particles	Augme -ntin									
1	0.04	8	1	0.00015/0.125	0.02	Synergisti c	0.0025/0.125	0.2	Synergistic	0.3/0.3	3.0	Synergisti c
2	0.0025	8	1	0.000625/0.3	0.3	Synergisti c	0.000625/0.03	0.3	Synergistic	0.3/0.3	0.3	Synergisti c
3	0.01	8	1	0.000625/0.3	0.1	Synergisti c	0.0025/0.125	0.4	Synergistic	0.3/0.3	0.3	Synergisti c
4	0.01	16	0.5	0.0025/0.125	0.3	Synergisti c	0.0000375/0.0 02	0.00 8	Synergistic	0.125/0.12 5	0.3	Synergisti c
5	0.005	16	0.25	0.0025/0.125	0.06	Synergisti c	0.01/0.5	4	Antagonisti c	0.125/0.12 5	0.51	Additive
6	0.02	16	1	0.000625/0.3	0.05	Synergisti c	0.00015/0.008	0.02	Synergistic	0.125/0.12 5	0.13	Synergisti c
7	0.04	8	2	0.000625/0.3	0.05	Synergisti c	0.0000375/0.0 02	0.00 2	Synergistic	0.3/0.3	0.2	Synergisti c

GBS: Group B streptococci MIC: minimum inhibitory concentration.

FICI: fractional inhibitory concentrations index

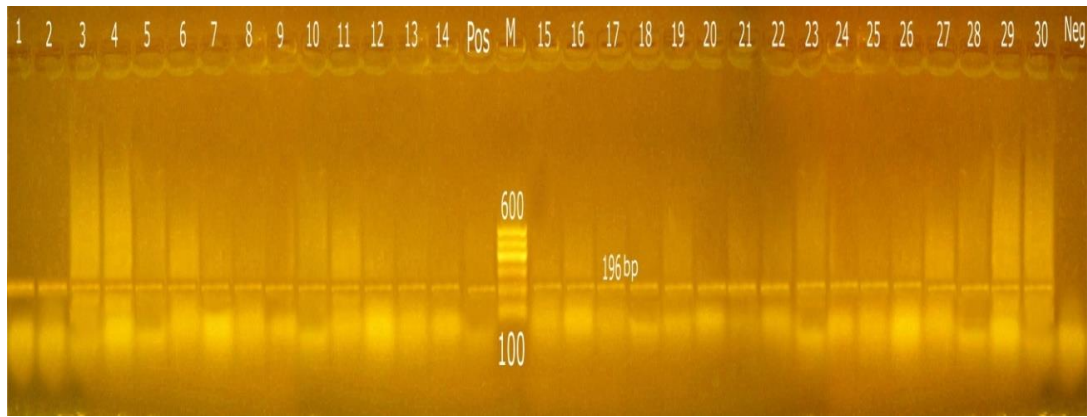


Fig. (1): PCR amplification of 196 bp *tuf* gene of Streptococci isolated from bovine mastitis cases. Lane M: 100 bp DNA ladder"Marker", Lanes 1-30 were positive Streptococci isolate. Lane Pos: positive control, Lane Neg: negative control.

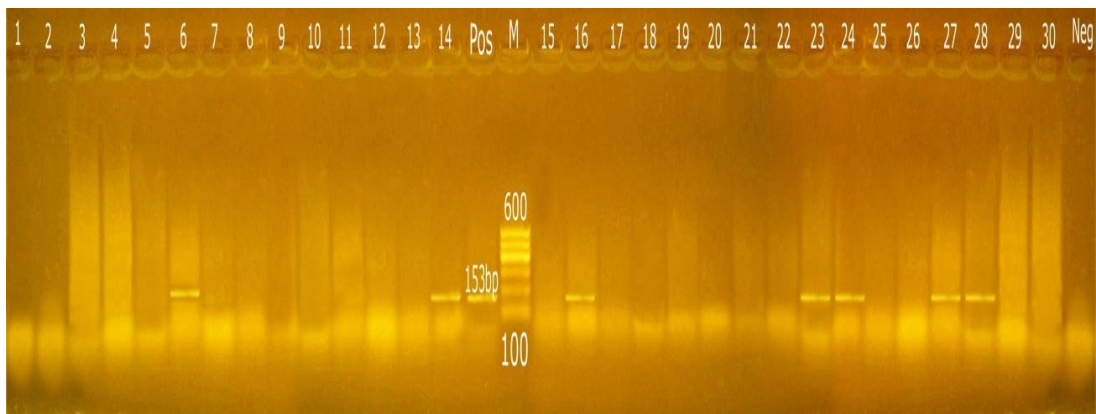


Fig. (2): PCR amplification of *cfb* gene of *S. agalactiae* isolates. M: 100 bp ladder"Marker", lanes No. 6, 14, 16, 23, 24, 27 and 28 were positive, lane Neg: negative control, lane pos: positive control.

#### 4. DISCUSSION

*Streptococcus agalactiae* is a noteworthy infectious pathogen causing clinical and subclinical mastitis. In spite of the fact that this bacterium has a short life expectancy in the earth, it can survive inconclusively inside the mammary organ as a commit pathogen of the udder, Both prompt decreasing milk production, crowds flare-ups, so this is of real significance for the dairy business. Projects to decrease the effect of *S.*

*agalactiae* infection have been authorized in numerous nations over years as annihilation from a crowd may not be accomplished if *S. agalactiae* ends up impervious to the anti-toxin utilized, either by hereditary change or by creation of a covering to shield cells from the anti-microbial (Rosini and Margarit , 2015). Herein, Streptococci was recorded in 146 out of 200 examined milk samples (73%).This is inconsonance with the study of

Amosun *et al.*, 2010) who detected *Streptococci* in milk samples with a percentage of 65%. Indeed, in this study, the incidence of *Streptococci* was recorded as follow, *E. faecalis* (27.4%), group D streptococci (21.2%), group C streptococci (17.1%), *S. uberis* (15.7%), *S. pyogenes* (13.7%) and *S. agalactiae* (4.7%) which was agreed with Edyta Kaczorek *et al.*, 2017) who reported that *S. uberis* (39.3%), *S. dysagalactiae* (30.4%), *S. agalactiae* (20%) and other streptococci (10.4%) in Poland.

In this study, antimicrobial susceptibility testing of streptococci isolates revealed absolute susceptibility to imipenem followed by ciprofloxacin (73%) which could be used as a drug of choice for treatment; meanwhile, 100% of streptococci isolates were resistant to streptomycin, clindamycin, ceftriaxone, cephalexine, cefepime and cloxacillin. Additionally, more than half of isolates were resistant to amoxicillin clavulanic acid (91.7%), while 86% of isolates were resistant to tetracycline, 80% were resistant to amoxicillin, 79% were resistant to erythromycin and finally 54.7% were resistant to sulfamethoxazole-trimethoprim. Less frequent resistance was reported by *S. agalactiae* (46.3%), *E. faecium* (23.1%) and *S. equines* (20.0%) (Klimiene *et al.*, 2011) was not agreed with the present study. Nonetheless, information from the investigation of Nam *et al.*, 2009) was very high contrasted and a report from Sweden,

which demonstrated 0% protection from oxytetracycline in *S. uberis* and *S. agalactiae* revealed by Giannechini *et al.*, 2002). The abnormal state of antibiotic medication opposition could be related with antibiotic medication use in Korea, in which the extent of antibiotic medications represented over half of the offers of antimicrobial operators for creatures (KFDA, 2003&2004).  $\beta$ -Lactams are known as the principal line antimicrobial operators while treating streptococcal udder contaminations (Denamiel *et al.*, 2005). As a few examinations have illustrated (Erskine *et al.*, 2002 and Giannechini *et al.*, 2002), cephalothin and penicillin were observed to be consistently successful against streptococcus separates, over 96% and 92% of secludes indicated susceptibilities to these 2  $\beta$ -lactams, individually shown by Nam *et al.*, 2009). Oxacillin indicated moderately low action contrasted and other  $\beta$ -lactams tried and the predominance of protection from oxacillin was lower than those of different examinations by Erskine *et al.*, 2002 and Rossitto *et al.*, 2002). Other than  $\beta$ -lactams, erythromycin appeared to be the most dynamic antimicrobial specialist, and 28.6% of streptococcal separates demonstrated protection from this medication. This finding is like those of past reports from France (21%) (Gue'rin-Fauble' *et al.* 2002) and Argentina (27.6%), (Denamiel *et al.*, 2005) however considerably higher than that of an investigation on dairy crowds in Uruguay (0–

3%) (Giannouchi *et al.*, 2002 ). Level of erythromycin-sensitive *S. uberis* (34.3%) seen by Nam *et al.*, 2009 was like the finding in a report from the United States (31.9%) (Erskine, 2002) however was significantly higher than that of a report from Finland, (Pitkälä *et al.*, 2004) in which no *S. uberis* disengages were impervious to this medication. The prevalence of resistance to gentamycin and erythromycin was the most variable among the species that showed lower portions of resistance to gentamycin ranged between 20 to 42.4%. Although *S. agalactiae* was susceptible to erythromycin, others showed various rates of resistance to this drug, ranging from 12.5% to 42.8% (Nam *et al.*, 2009). while in this study a higher prevalence of resistance to erythromycin (79%), streptomycin and clindamycin (100%) in all species.

In this study, cinnamon oil exhibited marked inhibitory activities against *S. agalactiae* isolates with inhibition zone diameters up to 40mm by agar well diffusion assay when using a concentration of 1024µg/mL. There were no reports of similar studies connected with antibacterial activity of cinnamon oil against GBS in mastitis in Egypt. The cinnamon oil was active against *Streptococcus mutans* clinical strains at different concentrations: 100; 50; 25; 12.5 and 6.25 % for which corresponding inhibit zones: 62; 51; 35; 21 and 16 mm were obtained. The authors applied disc-diffusion method using 50 µL of the oil per 6 mm disc

Fani and Kohanteb , 2011). In this study, cinnamon oil showed antibacterial effect against *S. agalactiae* with MIC values ranged from 0.04 to 0.0025µg/mL. In a study documented previously that both cinnamaldehyde and eugenol standards showed profound activity against both *Streptococci* spp. and Gram negative anaerobes, active concentration for both compounds ranged between (0.15 – 0.8 µg/mL) (Abidin *et al.*, 2013). Additionally, the findings were reported on the activity of cinnamon bark oil against both Gram positive and Gram negative bacteria shown by Unlu *et al.*, 2010) who agreed with the present study as streptococci was a Gram positive organism. In a previous research, the MIC values for cinnamon oil were 12.8µg/mL for multidrug resistant and non-multidrug resistant streptococci clinical strains with the use of agar dilution method recorded by Fani and Kohanteb, 2011). In addition, the MIC values of the extracted oil from *Cinnamomum zeylanicum* against clinical strains of *S. agalactiae* isolated from the vagina and anus of women were between 0.125 and 1.0µg/mL (Monika *et al.*, 2014).

Interestingly, the synergistic effect of cinnamon oil with silver nano particles and cinnamon oil with augmentin in this study was very effective as recorded in a previous study of Mohammad *et al.*, 2016) which declared the profound synergistic effect of honey and cinnamon against *S. mutans*. The combination of eugenol with AgNPbio

significantly reduced the MIC value of both compounds, and the calculated FICI indicated a synergistic effect between them against all GBS strains. At 125 µg/mL, AgNPbio alone inhibited the growth of all GBS strains. When the two compounds were combined, the MIC values of eugenol and AgNPbio decreased 4- to 8-fold and 4- to 256 fold, respectively (Biasi-Garbi *et al.*, 2015), and this combination similar to combination of cinnamon oil and silver nanoparticles in present study.

Silver nanoparticles synthesized by an eco friendly method using the filamentous fungus *F. oxysporum* showed inhibitory activity against planktonic cells of all GBS strains in study reported previously by Biasi-Garbi *et al.*, 2015), this agreed with the present study that reported silver nanoparticles exhibited inhibitory activities but less than cinnamon oil against *S. agalactiae* strains with inhibition zone diameter of 35mm. Also, augmentin exhibited inhibitory activities with inhibition zone diameters up to 38 mm. Silver nano particles (AgNPs) were a good bactericidal agents against multidrug resistance pathogens reported by Abdullah and Alwan, 2017), this idea was in consistent with other studies documented by Namasivayam *et al.*, 2011 ; Amany *et al.*, 2012) which reported that AgNPs were a better bactericidal for most multi drug bacterial resistance such as *E. coli* and *S. aureus* and all mentioned studies agreed with our study as *S. aureus* was Gram

positive organism and studies on *S. agalactiae*. The combination of these nanoparticles with several compounds has shown potent antimicrobial activity in different microbial species, including those displaying resistance to conventional antibiotics reported by Allahverdiyev *et al.*, 2011; Cardozo *et al.*, 2013; Ghosh *et al.*, 2013; Herman and Herman, 2014). Additive or synergistic effect of essential oil component cinnamaldehyde with chemically synthesized silver nanoparticles against Gram-positive and Gram-negative bacteria has been reported elsewhere by Ghosh *et al.*, 2013). In previous study Biasi-Garbin *et al.*, 2015), eugenol also exhibited an antibacterial activity against biofilms of *S. agalactiae*, showing the ability to inhibit its formation as well as the viability of mature biofilm, under *in vitro* conditions, this agreed with effect of cinnamon oil on biofilm.

## 5. CONCLUSION

The results obtained in this study demonstrated the bactericidal activity of cinnamon oil, silver nanoparticles and augmentin, in addition to the synergistic effect of cinnamon oil with AgNPs against planktonic cells of *S. agalactiae*. Furthermore, this compound inhibited biofilm formed on polystyrene.

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