Original Article

Inhibition of *Streptococcus Agalactiae* Biofilm Formation in Response to Purified Phytochemical Antimicrobial Materials

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Abstract

Group B streptococci (GBS) are globally recognized to cause adverse pregnancy outcomes, such as stillbirths and miscarriages, and are one of the main causes of newborn sepsis and meningitis. The high resistance of GBS to antibiotics becomes difficult or impossible to treat, becoming increasingly common, causing a global health crisis. It complicates their eradication, potentially leading to the development of chronic infections. A total of 181 specimens were obtained from pregnant women. Out of these specimens, 22 isolates were bacteriologically identified as S.agalactiae. They were collected from Al-Anbar Province hospitals. Twenty-two isolates were identified as GBS depending on cultural and microscopical properties, automated (VITEK-2 system), and molecular identification based on atr gene, which is an essential gene expressed isolates in all S.agalactiae. The antimicrobial susceptibility test was done by using the disc diffusion method for(12) antimicrobials. The results was appeared the highest resistance to Erythromycin (100%), Cefotaxime (100%), Ceftriaxone (100%), Meropenem(100%), Tetracyclin (95.45%), Cefepime (90.90%), Ampicillin (90.90%), Penicillin (86.36%), Clindamycin(81.81%), Azithromycin (81.81%), Chloramphenicol (40.90%), Levofloxacin (22.72%). Biofilm formation estimation by using a microtiter plate (MTP) was performed. Out of 22 isolates of S.agalactiae, 20(90.90%)isolates produced biofilm as indicated by MtP. Out of 20 biofilm-producing isolates, 3(15%), 10(50%), and 7(35%)were weak, moderate, and strong, respectively. The inhibitory effects of Gallic acid, Cinnamic acid, Salicin, (-)-Epigallocatechingallate, Linoleic acid, Metronidazole, Amoxicillin, Erythromycin, and Levofloxacin were tested against biofilms of streptococcus agalactiae from pregnant women. The salicin was found to have strong bactericidal activity against biofilm. Inhibition of biofilm formation and growth after incubation with different concentrations of phytochemical compounds and antibiotics solution were assessed by the crystal violet and 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide reduction assay. The phytochemical compounds and antibiotics solution significantly inhibited the initial cell attachment of the GBS but were less inhibitory towards 8 h preformed biofilms formed on polystyrene surface except for erythromycin; the inhibition was very low because of the resistance of GBS to erythromycin. However, there was a synergistic effect between erythromycin and gallic acid,or Metronidazole by using Checkerboard technique.

Key words: Antimicrobial, Biofilm, Streptococcus agalactiae, Synergism.

Introduction

Streptococcus agalactiae is Gram-positive cocci, catalase-negative, facultative anaerobes, and oval-shaped (Raabe & Shane, 2019). This group includes 10 different serotypes, the first nine of which have been found throughout history (Ia, Ib, II, III, IV, V, VI, VII, and VIII),), and the tenth of which was discovered currently (IX) (Raabe & Shane, 2019; Slotved, Kong, Lambertsen, Sauer, & Gilbert, 2007).

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Different serotypes are identified by type-specific capsular polysaccharides, which serve as a virulence factor through which GBS evades the host immune system (Toniolo et al., 2015). *S.agalactiae* is an important cause of mortality and morbidity in neonates, maternal women, the elderly, and immunocompromised adults (Saad et al., 2018). It causes infections in women during pregnancy and puerperium and invasive infections in newborns (Palacios-Saucedo et al., 2022).

Colonization of the mother is the main factor in mother-to-child GBS transmission (Seale, Blencowe, et al., 2017). GBS colonization and persistence in various hosts are based on its ability to adhere to host cells and tissues. As a result, bacterial cell aggregation and the production of biofilms are facilitate (Rosini & Margarit, 2015). The ability to produce biofilms varies among GBS strains, and these variations are related to phylogenetic lineage, isolation source, and capsular serotype (Parker et al., 2016)0. Biofilms offer protection against harsh environments that can include antimicrobials, high pH and immune cells (D'Urzo et al., 2014). Antimicrobial resistance (AMR) is a serious threat to public health worldwide because of its global spread. AMR not only substantially raises the cost of providing medical treatment but also increases mortality and morbidity. The antimicrobial drugs become less effective gradually because of the unnecessary use of antibiotics (Zhu, Huang, & Yang, 2022). *S.agalactiae* may be innately resistant to various antibiotics and may potentially develop resistance to them through a variety of ways (Petchiappan & Chatterji, 2017). Phytochemicals were used as antibiofilm because have a high level of antibacterial activities, which are essential for medicinal treatments, the pharmacological activity of medicinal plants may be related to their secondary metabolites, which are comprised of smaller molecules than primary metabolites such as proteins carbohydrates, and lipids. Medicinal and aromatic plants can synthesize antibacterial and antifungal medications, which are comparatively less toxic to humans (Larayetan, Ololade, Ogunmola, & Ladokun, 2019). Phytochemical compounds used in this study as antibiofilm included: (Cinnamic acid, (-)-Epigallocatechingallate, Gallic acid, Linoleic acid, Salicin) these Phytochemical compounds possess the most biological activities, such as antioxidant, anticancer, anti-inflammatory, antimicrobial.

METHODS:

Sample collection

Samples were collected from August 2021 to the end of December 2021, by collecting 181 samples from pregnant women in the third trimester of pregnancy, the specimens include vaginal swabs from females admitted into Alanbar Province hospitals.

Identification of S. agalactiae

A total of 181 isolates from the vaginal swabs were collected. were cultured by strewing them onto a plate of agar containing 5% sheep blood. For 18–24 hours, the plates were incubated at 37 °C in 5% CO_2. Using common microbiological *S. agalactiae* morphological identification procedures, including the Gram stain and the Catalase test, the isolates were recognized as GBS. CAMP test, bacitracin test, automated identification by (VITEK-2 system), and molecular identification based on *atr* gene.

Antimicrobial susceptibility test

The following 12 antimicrobial discs (Bioanalyse, Turkey) were selected: Ampicillin, Penicillin, Cefepime,Ceftriaxone, Cefotaxime, Meropenem, Azithromycin, Erythromycin, Tetracycline,Levofloxacin, Chloramphenicol, Clindamycin. According to the Clinical Laboratory Standard Institute's advice, 5% Mueller-Hinton agar-containing sheep blood was used for the antimicrobial susceptibility testing (AST) of GBS (CLSI 2021).

Determination of minimum inhibitor concentration (MIC)

MIC of Phytochemical compounds and antibiotics solution were evaluated by Resazurin Microtitrer-plate Assay (REMA).

Synergism between Erythromycin and Gallic acid, Metronidazole

The use of the checkerboard approach to combine erythromycin, and gallic acid, or metronidazole: The checkerboard method was used in 96 well microplates to examine any possible synergistic interactions between erythromycin and gallic acid, or metronidazole.

Biofilm formation

Production of biofilm was measured using quantitative assays, defined by Bertelloni by a microplate reader using 96-well sterile flat-bottomed polystyrene microtiters (Bertelloni, Cagnoli, & Ebani, 2021). and studied (Cinnamic acid, (-)-Epigallocatechingallate, Gallic acid, Linoleic acid, Salicin, Metronidazole, Amoxicillin, Erythromycin, and Levofloxacin) against biofilm development.

Biofilm biomass assay

For s. agalactiae isolates, the modified crystal violet (CV) assay proposed by (Djordjevic, Wiedmann, & McLandsborough, 2002) was used to evaluate cell attachment. To measure absorbance at 595 nanometers, a microplate reader was employed. The biomass formation inhibition % for each concentration of the test materials was calculated using the mean absorbance (OD595 nm) and the equation below:

Percentage inhibition = 100 - [(OD595 nm experimental well with test matrial / OD 595 nm control well without test material) *x*100].

Biofilm metabolic activity assay

According to (Schillaci, Arizza, Dayton, Camarda, & Stefano, 2008) metabolic activity of the biofilms developed

by S. agalactiae was measured using the MTT assay. The microplate reader was then used to measure the absorbance at 570 nm (Patel, Gheewala, Suthar, & Shah, 2009).

Determination of Biofilm Inhibitory Activity of phytochemical compounds and antibiotics solution

A- Inhibition of Initial Cell Attachment

Sandasi assessed phytochemical compounds and antibiotics solution impact on the initial cell attachment during biofilm development. Solutions of test materials (equivalent to 0.25 MIC, 0.5 MIC, 1 MIC, and 2 MIC) were made using two different microtiter plates. The MTT assay was used to quantify metabolic activity, and the modified crystal violet assay (CV) was used to measure biofilm development (Sandasi, Leonard, & Viljoen, 2010).

B- Inhibition of preformed biofilm

phytochemical compounds and antibiotics solution impact on biofilm development and maturity was calculated by (Sandasi et al., 2010). Before adding test materials, biofilms were allowed 24 hours to form. The plates were incubated for 8, 12, 16, 20, and 24 hours after test substance was applied to developed biofilms. Then, using a modified CV test, biofilms were evaluated for biomass attachment, and MTT experiments were run on the biofilm cells that had already developed (Sandasi et al., 2010).

DNA isolation and quantification

Genomic DNA was extracted from bacterial culture using DNA isolation kits (Geneaid, Korea) according to the manufacturer's instructions. DNA concentration and purity were determined using a Nano-drop device and stored at 20 °C to prevent degradation. According to manufacturer's instructions, 1 X (TAE) buffer, 1% agarose gel, and molecular weight markers (100 bp) were all prepared.

PCR reactions mixtures and conditions

Amplification of *atr* gene was done using standard PCR and *atr* primers 5'-CAA CGA TTC TCT CAG CTT TGT TAA-3' and 5'-TAA GAA ATC TCT TGT GCG GAT TTC-3', with end product 780bp(Mudzana et al., 2021). 20 µl reaction mixture was all done according to the manufacturer's instructions(BIONEER, Korea). Conditions for PCR thermal cycling included a first denaturation phase lasting 4 minutes at 94 °C and 35 cycles (denaturation 94 °C for 1 min, annealing at 58 °C for 45 sec, extension 72 °C for 1 min) and a final elongation step at 72 °C for 7 min.

Results and Discussion

This study took four months to complete, commencing in August and end in December 2021. From 181 clinical specimens, there are twenty-two isolates were identified as GBS. The isolation rate of GBS from pregnant women was (12.15%), and most of the participants were between the age range of 25-37 years. With regard to the clinical history of the participants, the participants had multigravida(54.54%) or abortion (22.73%) or stillbirth (9.09%) or neonatal death (13.64%). The rate of S.agalactiae isolated from pregnant women depends on many factors such as virulence of isolates, health status of patients, impact of environmental factors and hormonal changes that occur during pregnancy, and the resulting microbiota imbalance that raises the risk of GBS infections, which can lead to complications for both mothers and their children. Many local studies were shown the rates of GBS in Iraq, such as Hassan explained the rate of GBS in Baghdad (18%) (Hassan et al., 2019).

Also, there are international studies that show GBS rates with explaining the clinical history of the patients. Such as in Southeast Ethiopia, the prevalence of S.agalactiae based on the clinical history of the patients which was (75.8%) were multigravida, (25.3%) had a history of abortion, (12.1%) had a history of stillbirth, and (15.4%) had a history of neonatal death(Tesfaye et al., 2022b). The prevalence rate was in Egypt (17.89%) and Kuwait (16.4%) (Abdallah et al., 2021).

Identification of S. agalactiae

The results of tests for identifying *S.agalactiae* using microscopic diagnostics was positive Coccus (chain or pair) and negative to catalase. *S. agalactiae* is β-hemolytic on blood agar. Major virulence factor employed by GBS during pathogenesis, positive to bacitracin , and positive to CAMP test is also used to differentiate (*S.agalactiae*) from other streptococcal species. In this instance, we have a positive result, indicating that the colony tested is *S.agalactiae*. CAMP factor encodes the *cfb* gene since the *cfb* gene is so prevalent in GBS strains, the CAMP test or PCR check for the *cfb* gene was commonly employed to distinguish GBS from other Streptococcus species. All bacterial isolates (100%) with *S. agalatiae* were identified molecularly using the *atr* gene. Figure (1).

Detection *atr* gene is the high specificity test for GBS screening in pregnant women. It was found only in *S. agalactiae* and encodes for the amino acid glutamines transporter, which has a high degree of specificity for *S. agalactiae*. Because it is a housekeeping gene, the probability of mutation is low (Schörner et al., 2014).

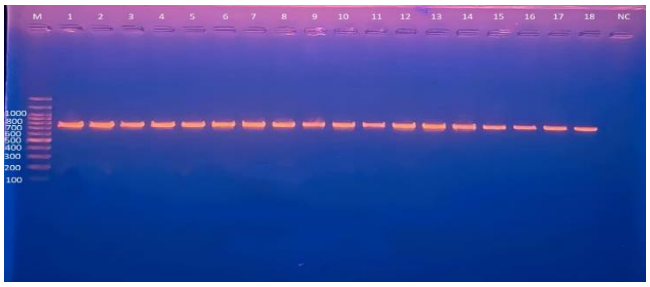

Figure 1. Molecular identification by atr gene.

Antibiotic susceptibility profiles

The results of this study showed the highest resistance to Erythromycin (100%), Cefotaxime (100%), Ceftriaxone (100%), Meropenem (100%), Tetracyclin (95.45%), Cefepime (90.90%), Ampicillin (90.90%), Penicillin (86.36%), Clindamycin (81.81%), Azithromycin (81.81%), Chloramphenicol (40.90%), Levofloxacin (22.72%). The

emergence of resistant organisms in al-Anbar has recently become a significant therapeutic challenge. The sensitivity and resistance to antimicrobial agents of *S. agalactiae* in northern Iraq were evaluated using VITEK 2 Compact System. The results showed that the isolates with the highest percentages of resistance were related to clindamycin (100%), erythromycin (72.4%), tetracycline (68.9%), Cefotaxime (51.7%), Ampicillin (43.1%), and levofloxacin (6.8%) (Rasul, Mustafa, & Abdulrahman, 2020). Also, in Iran, All isolates were susceptible to penicillin. Resistance to tetracycline, erythromycin, and clindamycin was detected as 96.6%, 28.1%, and 16.4% of strains, respectively (Ghamari, Jabalameli, Emaneini, & Beigverdi, 2022). Evaluation of *S. agalactiae* penicillin-resistant bacteria collected over a five-year period in Italy (from 2015 to 2019) showed that the resistance to penicillin increases with time (Genovese, D'Angeli, Di Salvatore, Tempera, & Nicolosi, 2020). This increase in bacterial resistance has been associated with increased antimicrobial use and improper antimicrobial prescribing. This produces selection pressure that results in antibiotic resistance in exposed bacteria, and as a result, horizontal gene transfer results in the persistence of antibiotic resistance genes in populations in the same ecological niches (Alves-Barroco, Rivas-García, Fernandes, & Baptista, 2020). Low access to PBPs, a decline in PBP binding affinity, or the degradation of the antibiotic by beta-lactamases are the three main mechanisms that contribute to gram-positive bacteria developing resistance to beta-lactam antibiotics (Hayes, O'Halloran, & Cotter, 2020). There have been reports of reduced penicillin susceptibility in GBS, and these are brought on by amino acid changes in PBPs that influence how well penicillin medicines bind to certain bacteria (Metcalf et al., 2017). Antibiotics like macrolides-lincosamide can develop resistance through a number of different mechanisms, such as efflux pumps, ribosomal modifications, and drug inactivation (Hayes et al., 2020).

Determination of minimum inhibitor concentration by using (REMA) method

The MIC results of antibiotics solution and Phytochemical Compounds are shown in Table (1).

Table 1. MIC of antibiotics solution, and Phytochemical Compounds

inhibitors	MIC
(-)-Epigallocatechingallate	1.25 mg/ml
Amoxicillin	2.5 mg/ml
Cinnamic acid	0.312 mg/ml
Erythromycin	2.5 mg/ml
Gallic acid	2.5 mg/ml
Levofloxacin	0.156 mg/ml
Linoleic acid	5 mg/ml
Metronidazole	1.25 mg/ml
Salicin	0.625 mg/ml

Evaluation of the effect of the combination of phytochemical compounds and antibiotic solution using checkerboard technique

The advent of resistant bacteria has limited the efficacy of conventional antibiotics, necessitating the development of alternate ways for dealing with infections caused by drug-resistant bacteria (Chi & Holo, 2018). One possibility for increasing or restoring antimicrobial efficacy against multidrug-resistant bacteria is the discovery or development of adjuvants, which includes the development of substitute antibiotics (Montero et al., 2018). Because microorganisms are rapidly finding techniques to resist antibiotics, it is highly challenging to identify new antibiotics. One strategy that was employed to combat the bacteria potential to develop resistance to antibiotics currently on the market was the checkerboard technique (Ayaz et al., 2019). Combinations of natural substances may promote or facilitate synergytesting techniques by enhancing or enabling an antibacterial agent's interaction with its target within the pathogen, which use susceptibility approaches to determine the cumulative activity of two or more compounds. Such inhibitors are useful for usage with antibiotics linked to high resistance rates since lower concentrations of both drugs can be utilized in this method (Sanhueza et al., 2017). Antibiotics and natural products together reduce the MIC of antibiotics while improving the susceptibility of multidrug-resistant bacteria to these medications. This occurrence of synergism aims to reduce microbial resistance and toxicity (Newman & Cragg, 2016).

Checkerboard assays of GBS gave synergistic profiles when erythromycin was combined with gallic acid, and Metronidazole. The MIC values for the erythromycin, gallic acid, and Metronidazole. FICI values when erythromycin was combined with gallic acid was (0.0117), and FICI values when erythromycin was combined with Metronidazole was (0.0468). FICI values less than 0.5 indicate a synergistic effect between the tested materials.

The synergistic combination of natural substances with already accessible antibiotics is an effective strategy to combat the resistance problem. The term "synergism" is used when two substances' combined therapeutic impact is greater than the sum of their individual effects. Previous findings demonstrated from this study are that the combination between erythromycin and other materials exerts synergistic effects evaluated as metabolic activity reduction and restores sensitivity to erythromycin in erythromycin-resistant strains of GBS. Therefore, combining herbal medicines and phytochemicals with antibiotics and other therapeutically significant medications is a relatively new and efficient method for managing resistant microorganisms.

Several chemicals have been studied for their ability to change microbial resistance, some of which are effective against numerous targets, such as inhibiting PBP, improving bacterial outer membrane permeability, and inhibiting bacterial efflux pumps (Ayaz et al., 2019).

Biofilm formation

In MtP assay the characterization of *S.agalactiae* isolates varied between strong 7/22 (31.81%), moderate10 (45.45%), weak 3 (13.63%), and no biofilm producers 2/22 (9.09%), as shown figure (4). Contrary to our study, in another study in 2016, the production of biofilm in china was only (13.8%) from isolates (Jiang et al., 2016). Bacterial biofilms are an essential virulence factor with a vital role in the pathogenesis of bacteria; it is essential due to increased resistance to host defenses, which promotes microbial survival and growth

(Rosini & Margarit, 2015). There is a study that found that *s. agalactiae* colonizing pregnant females have a more ability to form biofilm than GBS isolated from different symptomatic infections (Atawia, Abdallah, Zaki, & Eltaieb, 2018).

Antimicrobial activity against sessile cells

Determination of the antibiofilm effect against biomass in S. agalactiae biofilm

In order to anti-biofilm activity of some antibiotics, Phytochemical compounds and effects were tested on both the initial cell attachment and performed (24h) biofilms. Modified CV assay indicated that the effect of the Phytochemical compounds and antibiotic solutions on biomass attachment exceeds 70% (percentage inhibition) 2 MIC for all test materials, except erythromycin, which was 55% due to high resistance of *s.agalactiae* isolates to erythromycin, also at the MIC and 0.5 MIC the inhibition was above 50% except for erythromycin was under 50%. Even at 0.25 MIC, initial cell attachment was reduced but not like inhibition of 2 MIC or MIC, as shown in figure (2).

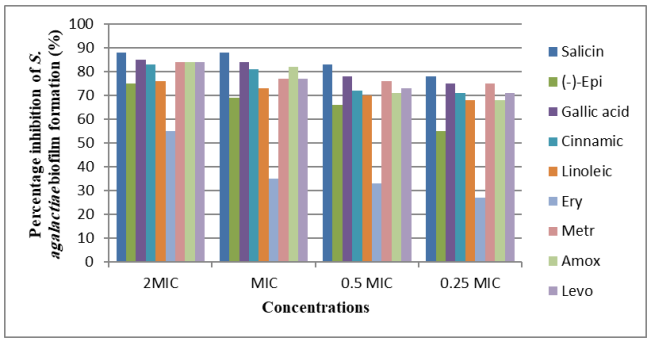

Figure 2. Result of various concentrations of antibiotics, Phytochemical Compounds on initial cell attachment of S.agalactia, shown as Percentage inhibition of S.agalactiae biofilm formation (%).

However, it does not achieved complete inhibition of cell attachment despite using 2 MIC of antibiofilms. Overall, the use of Phytochemical Compounds to modify biofilm formation sites makes them unsuitable for attachment and appears to be a useful method of dealing with microbial adherence (Jadhav, Shah, Bhave, & Palombo, 2013). The antibiotic solution and phytochemical compounds were tested against a preformed biofilm. Biofilm formation involves an initial reversible (weak) attachment phase followed by an irreversible (strong) attachment phase (Oliveira, Brugnera, Cardoso, Alves, & Piccoli, 2010). The findings demonstrate that the MIC of inhibitors was applied to *S. agalactiae* preformed biofilm (24 h) and tested for 8 hours, 12 hours, sixteen hours, twenty hours, and twenty-four hours incubation. We noticed that the percentage inhibition of *S. agalactiae* preformed biofilm increased with increasing incubation time, except for efficacy of Amoxicillin and Erythromycin decreased dramatically with time, figure (3).

The extracellular polysaccharide layer in a constructed biofilm, which may deter the entry of antimicrobials, or the mature biofilm's tight three-dimensional layout, which may obstruct the entry of these compounds into the biofilm, may be responsible for the observed resistance. The fact that most antimicrobial substances work better against cells that are actively proliferating is another factor that could be responsible for this rise in resistance. Lack of nutrition and oxygen causes the cells in biofilms to develop slowly, which may lessen the antibacterial effects of substances used to treat them (Sandasi et al., 2010).

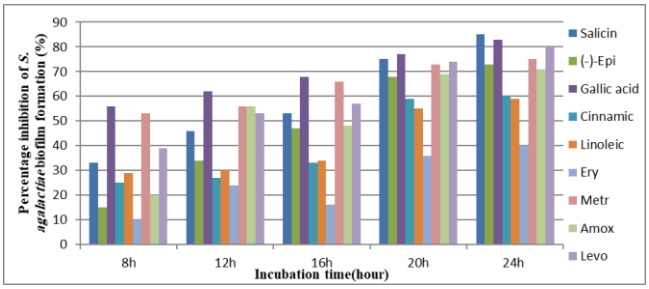

Figure 3. The result is shown as Percentage inhibition of S.agalactiae biofilm formation (%) on 24h preformed biofilm of S. agalactiae.

Determination of the antibiofilm effect against the metabolic activity of S.agalactiae biofilm

MTT assay was used to identify attached viable cells, whereas CV stains both attached viable and non-viable cells. MTT (thiazolyl blue tetrazolium bromide) can only be reduced by living cells into a colorful chemical that can be calorimetrically quantified. Based on the metabolic activity of the cells, the MTT assay only detects live cells (Kouidhi, Zmantar, & Bakhrouf, 2010).

The results of the MTT assay confirmed that the antibiotic solution and phytochemical compounds significantly inhibited metabolic activity of the biofilms formed by *S.agalactiae.* MTT test results show the highest anti-adhesion activity at 2 MIC, inhibition begins to decrease as the concentration of each antibiofilm decreases. Least inhibition was at 0.25 MIC, due to the low concentration of the inhibitor, so to inhibit biofilm formation we need a high concentration of the inhibitor as shown figure (4).

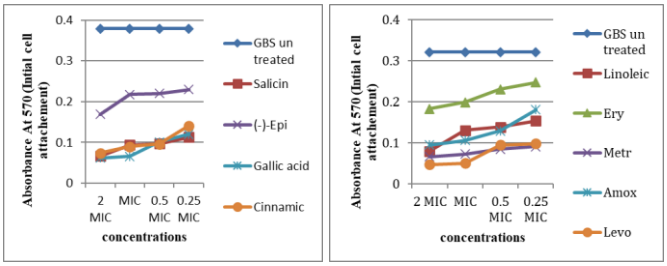

Figure 4. Effect of antibiotics, Phytochemical Compounds on the metabolic activity of S.agalactiae initial cell attachment at different concentration of test materials.

However, in the case of preformed biofilms the antibiotics, and Phytochemical Compounds inhibited the metabolic activity of *S.agalactiae* at MIC. The metabolic activity suppression was found to increase with increased time of exposure, thus the activity being highest at 24h exposure as shown figure (5).

Despite extensive research into natural compounds, mostly phytochemicals, as anti-biofilm agents in in vitro and in vivo

settings, there are no medicines that the FDA has approved. Most of them failed in phase II and phase III clinical investigations (Lu et al., 2019). This failure could be due to the chemical's availability in people after injection, which lessens the compounds' efficacy.Combining techniques like antibiotics with organic anti-biofilm compounds could be one way to address this issue and get better results (Mishra et al., 2020).

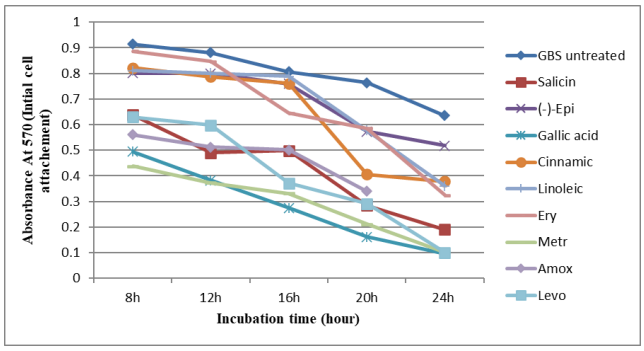

Figure 5. Effect of antibiotics, Phytochemical Compounds on the metabolic activity of preformed biofilm cells of S.agalactiae incubated for 8h, 12h, 16h 20h, and 24h, as determined by the MTT assay. Metabolic activity of the antibiotics, Phytochemical Compounds treated cells was inhibited considerably as compared to the untreated control cells.

Conclusion

In current work, GBS colonization rate (about 12.15%) and the prevalence of this bacteria among pregnant women in Anbar of Iraq. The data showed that most GBS strains were resistant to antimicrobials. Levofloxacin and Chloramphenicol antibiotics can still be considered the best choice drugs for prophylaxis and treatment of early-onset GBS infections. The phytochemical compounds have antibacterial activity against the sessile phase of biofilm in antibiotic-resistant *S.agalactiae*. Streptococcus agalactiae is treated with phytochemical compounds combined with antibiotics using a checkerboard approach, which increases the bacteria's susceptibility to antibiotics.

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Author Contribution

Safa Nadem Alani, performed the study, examined and reviewed results, and manuscript writing with the help and supervision of Safaa Abed Latef AlMeani.

Conflict of Interest

The authors declare no conflict of interest.

Ethical Clearance

The study was approved by the Ethical Approval Committee.

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