Validation of Antibacterial Activity of Gossypol against *Enterococcus faecalis*

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November 2022

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Abbreviations:

E. faecalis-Enterococcus faecalis

SDS-Sodium Dodecyl Sulphate

H_2O_2-Hydrogen Peroxide

DMSO-Dimethyl Sulfoxide

HOCl-Hypochlorous Acid

MOPS-n Morpholino propane Sulphonic acid

OD-Optical Density

Hrs.-Hours

Abstract:

Antibiotic resistance is a growing concern today that affects the entire world, necessitating the development of new antibacterial medications. Nosocomial infections pose a serious challenge for patients and hinder effective treatment. The second most common cause of nosocomial infections is *Enterococcus faecalis*, and it depends on the lactate dehydrogenase enzyme's capacity to maintain redox balance for growth, resistance, and virulence. As demonstrated before by computational technique, our study attempted to assess the antibacterial effect of Gossypol on *Enterococcus faecalis* by inhibiting Lactate Dehydrogenase Enzyme. We used six different stressors, including 0.01% SDS, 2.5mM H2O2, 8% Ethanol, 10% DMSO, 10% Glucose, and 0.25% HOCl, in addition to our inhibitor Gossypol. Gossypol was employed in the following concentrations: 5, 10, 25, 50, 75, 100, 150, 200 micrograms per milliliter using a large test tube method, absorbance on a UV-visible spectrophotometer, and on a microscale using a 96-well flat-bottom plate with a Microplate reader. Between control (Culture Media) and vehicle control, which is DMSO, there is no discernible difference. At higher dosages of Gossypol, such as 100 and 200 micrograms per milliliter, there is significant growth inhibition; nevertheless, 5, 10, 25, 50, 75, and 100 micrograms per milliliter show no significant inhibition. Minimum Inhibitory Concentration is 100 microgram per milliliter. We used our six different stress factors with MIC value of Gossypol. There is no discernible growth inhibition when Gossypol is employed in conjunction with stress factors such 0.01% SDS, 2.5mM H2O2, and 0.25% HOCl. However, at 100 micrograms per milliliter at the fourth and fifth hours, glucose 10% exhibits a strong inhibitory impact, though not by a great deal. Along with MIC value of Gossypol, 100 micrograms per milliliter, 8% ethanol and 10% DMSO significantly slowed the development of the bacteria. The lactate dehydrogenase enzyme in Enterococcus faecalis was effectively inhibited by gossypol. Future in vivo studies are required to demonstrate the antibacterial activity of Gossypol in greater detail, as well as to compare it to antibiotics as an adjunctive treatment.

Chapter 1

1. INTRODUCTION:

1.1 *Enterococcus faecalis* and Diseases Associated with it:

E. faecalis is a Gram-positive bacterium that is non-motile, facultative anaerobe, and has a spherical shape.(Paganelli, Willems et al. 2012)*Enterococcus faecalis* is a bacterium that lives in the intestines of people and many animals. It is a leading source of severe, nosocomial infections that are difficult to treat owing to inherent and acquired antibiotic resistance.(Rana, Sauvageot et al. 2013)

Although Enterococci make up less than 1% of an adult's intestinal microflora, they have become a major source of nosocomial infections in the United States in the previous decade(Tannock and Cook 2002). *E. faecalis* accounts for 65 to 80 percent of all enterococcal nosocomial infections in this group(Mundy, Sahm et al. 2000)

E. faecalis, a gram-positive bacteria with a sphere-shaped structure organized as diplococci, is a common pathogen found in animal and human guts and is known to cause a variety of illnesses and disorders(Price, Zeyniyev et al. 2012)

E. faecalis is a natural component of human and animal intestinal flora that is discharged in vast amounts into the environment through feces(Rince, Flahaut et al. 2000)

This bacterium is used to identify harmful bacteria in water contaminated with feces (Noskin, Till et al. 1991)This pathogenic bacteria is the second most hazardous agent in producing nosocomial infections and has been linked to a variety of different diseases such as Urinary Tract Infections, Intraabdominal, Pelvic, Soft Tissue Infections, Bacteremia, and Endocarditis(Rince, Flahaut et al. 2000).Three prophages were also identified which were involved in *E. faecalis* V583 adherence to human platelets, which is thought to be a precursor to the development of infective endocarditis. Overall, the interaction of these prophages enhances their mobility and biological functions(Matos, Lapaque et al. 2013).

In many settings, *E. faecalis* has been proven to impede the development of other bacteria. Streptococcus gordonii was totally suppressed in root canals when co-cultured with *E. faecalis.*

Another research found that the presence of *E. faecalis* in polymicrobial biofilms at 39 C restricted the presence of Listeria monocytogenes owing to competition for resources and the generation of toxic metabolites (da Silva Fernandes, Kabuki et al. 2015)

1.2 Virulence of Enterococcus faecalis

The ability of an organism to cause illness is typically characterized as virulence(Casadevall and Pirofski 2001)Previous research has linked virulence factors to biofilm formation, such as the esp., geIE and asa1 genes, which promote cell adhesion, surface colonization, aggregation, and urinary tract persistence(Thrall and Burdon 2003, Kubinak and Potts 2013)
Extracellular matrix proteins such as aggregation substance (AS), enterococcal surface protein (Esp.), *E. faecalis* antigene (EfaA), adhesion to collagen (Ace), endocarditis and biofilm associated pili), and cell and tissue damaging activities such as bacteriocin cytolysin (Cyl) and protease gelatinase are major virulence factors (GelE)(Tiller and Garsin 2014)

1.3 Resistance of *Enterococcus faecalis*

Due to the thick cell wall, *E. faecalis* may modify the way cells divide and proliferate, as well as acquire inherent resistance to several clinical antibiotics such as vancomycin, linezolid, and daptomycin(Papp and Lázár 2016)

A significant criterion by which bacteria and other disease-causing organisms exhibit the physiology of cells is their ability to tolerate changes in environmental conditions.(Csonka and Hanson 1991).

A lot of studies have been done over the last two to three decades to show how these pathogens acquire resistance to various stress situations, which largely involves the production of different genes that change the metabolism of bacteria to create energy(Jett, Huycke et al. 1994)

E. faecalis has adapted to the nutrient-rich, low-oxygen environment of the gastrointestinal, mouth, and vaginal cavities, producing a variety of infectious illnesses (Hartke, Bouche et al. 1995)

Enterococci is a metabolically flexible bacteria that can withstand a wide range of circumstances, including severe temperatures, pH, ionizing radiation, high metal concentrations, and certain medications (Paulsen, Banerjei et al. 2003).

E. faecalis can withstand temperature of up to 60 degrees Fahrenheit and a pH range of 3.5-11.1 when exposed to high salt concentrations. This tolerance is especially important when testing for foodborne pathogens, and because *E. faecalis* causes so many illnesses, its virulence and resistance have gotten a lot of attention(Demple 1991, Olson 1993).

Enterococci may also live in a dried form on surfaces for months (Kramer, Schwebke et al. 2006)Because these bacteria have innate resistance to stress, they can endure in healthcare settings. In the 1950s and 1960s, these bacteria gained resistance to strong antibiotics like tetracycline and chloramphenicol, and in the 1970s, gentamycin and erythromycin developed resistance, while ampicillin and vancomycin developed resistance in the 1980s and 1990s.The short palindromic (CRISPR)/Cas locus, which is present repeatedly and appropriately spaced, is the cause of antibiotic resistance in *E. faecalis*. The CRISPR/Cas system in *E. faecalis* uses mobile genetic fragments as a source of threat, and because of a flaw in the system, it has also gained antibiotic resistance. Hospital-adapted *E. faecalis* strains may have become more vulnerable to phages and other mobile elements due to antibiotic selection for CRISPR/Cas loss.(Palmer and Gilmore 2010, Gilmore, Lebreton et al. 2013)

1.4 *Enterococcus faecalis* Metabolism:

It is a homofermentative lactic acid bacteria that catabolizes glucose or similar carbohydrates mostly through the Embden-Meyerhof-Parnas (glycolysis) route (Price, Zeyniyev et al. 2012).

Lactate is the most common end product of fermentation under these circumstances (Noskin, Till et al. 1991, Rince, Flahaut et al. 2000).

Lactate dehydrogenases (LDH) reduce the glycolytic end product pyruvate to lactate, allowing the NADH produced during glycolysis to be regenerated into NAD, which keeps glycolysis continuing (Rana, Sauvageot et al. 2013)

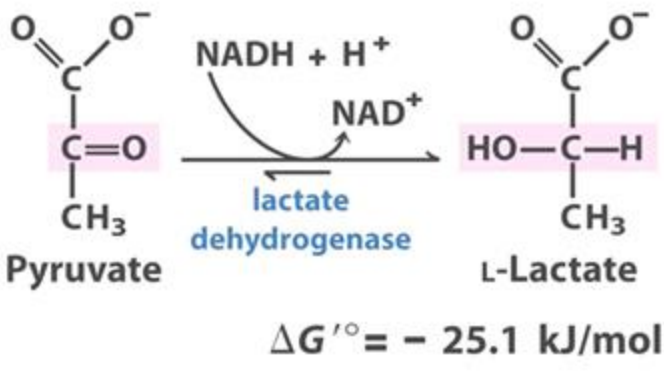


Figure 1: Anaerobic homolactic fermentation {Read, 2001 #63} {McClendon, 2005 #64}

During its development, *E. faecalis* produces homo lactic acid fermentation of various carbohydrates and glycerol, resulting in the production of lactate as an end product(Feldman-Salit, Hering et al. 2013)During glycolysis, NAD+ is synthesized from NADH by reducing pyruvate in the presence of the lactate dehydrogenase enzyme (LDH).

LDH1 and LDH2 are two distinct isoforms of LDH produced by the *E. faecalis* genome(Paulsen, Banerjei et al. 2003) However, depending on culture growth, LDH1 is more actively transcribed in these two and is responsible for the majority of lactate generation.

Lactate production in *E. faecalis* is reduced by almost 25% when the LDH gene is mutated, according to several studies, with the LDH1 mutation accounting for the majority of the reduction. This 25% lactate production showed that there are other pathways for energy production in *E. faecalis* such as shifting homolactic fermentation to heterolactic fermentation by upregulating some genes and downregulating others, resulting in the formation of different acids, but they weaken the bacteria and have an impact on its pathogenicity and virulence.

These bacteria are extremely effective in sugar metabolism, metabolizing approximately 30 different sugars(JT 1957) *E. faecalis* can also metabolize glycerol, which is involved in an essential route for lipid production and might be utilized as a source of energy by this pathogen(Bizzini, Zhao et al. 2009)

1.5 LDH AS AN EFFECTIVE DRUG TARGET

The genes ldh-1 and ldh2 encode two cytosolic L - () - lactate dehydrogenases in *E. faecalis*. LDH-1 is responsible for the majority of the activity, whereas LDH-2 plays a small part, In

E. faecalis, general resistance to very different environmental stresses depends on the ability of *E. faecalis* to maintain redox balance via lactate dehydrogenase (LDH) making it an effective drug target(Rana, Sauvageot et al. 2013)

Under conditions of high glucose and low oxygen concentration, the primary result of Enterococcus fermentation is lactic acid. Enterococcus is the most significant of all lactic acid bacteria, and it is created by the reduction of pyruvate for the regeneration of NAD for continuing glycolysis(Ramsey, Hartke et al. 2014)

The idea of inhibition mediated by lactic acid generation is further supported by *E. faecalis* V583 wt. and V583 mutant strains with deletions in ldh-1, ldh-2, or both genes. When polymicrobial biofilms of *E. faecalis* V583 wt. or *E. faecalis* V583 Dldh2 strains were produced, the inhibition was discovered. When *E. faecalis* V583 Dldh1 or Dldh-1/ldh-2 mutant strains were used, however, there was no statistically significant decrease in colony counts of K.pneumoniae.(Jönsson, Saleihan et al. 2009, Rana, Sauvageot et al. 2013)

E. faecalis inhibited K. pneumoniae development and changed the pH as a result of lactic acid generation, which is detrimental to K. pneumoniae but has no effect on its own growth. *E. faecalis* antibiofilm action on K. pneumoniae is mostly related to the generation of lactic acid and the subsequent pH drop (Ballén, Ratia et al. 2020)Lactic acid production may provide *E. faecalis* an edge over K.pneumoniae or other species, as certain *E. faecalis* strains are resistant to acidic environments and can adapt to varied pH ranges (pH 2.9)(Rince, Flahaut et al. 2000, Mubarak and Soraya 2018)

1.6 AIMS AND OBJECTIVES

1. To Evaluate Antibacterial Activity of Gossypol against *E. faecalis*
2. Optimization of Gossypol dose as inhibitor against *E. faecalis*
3. Evaluation of Gossypols Antibacterial Activity with different stress factors against *E. faecalis*

Chapter 2

2. LITERATURE REVIEW:

2.1. GOSSYPOL:

In 1899, gossypol was discovered as a phenolic chemical. The name is a combination of the scientific name for the plant species (Gossypium) and the phenolic ending "ol." (Soto-Blanco 2008)Adams et al. described Gossypol for the first time in a series of classic studies in 1938(Heinstein, Smith et al. 1962)It is a polyphenolic aldehyde that accounts for 20–40% of the pigment glands weight and 0.4–1.7% of the total cottonseed kernel weight. Gossypol is a phytoalexin that confers both constitutive and inducible resistance to pests and diseases.(Carrière, Ellers-Kirk et al. 2004, Wang, Cai et al. 2004, Stipanovic, Lopez et al. 2006, Mao, Cai et al. 2007, Stipanovic, López Jr et al. 2008, de la Paz Celorio-Mancera, Ahn et al. 2011)

Polyphenols are chemicals found naturally in fruits, vegetables, cereals, and drinks. Because of their pharmacological effects, polyphenols hold a special place in biological science. Gossypol is a versatile chemical with a wide range of biological activities. It is a non-selective competitive inhibitor of NADH binding with LDH.(Keshmiri-Neghab and Goliaei 2014)

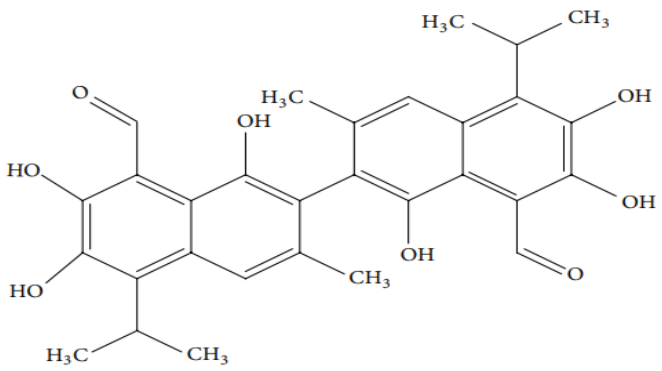
Gossypol is a polyphenolic sesquiterpene that has been isolated as a racemic combination from cottonseed. Because of the impeded rotation around the binaphthyl link, Gossypol exists in two enantiomers.(Freedman, Cao et al. 2003) Two distinct enantiomers are: (+)-gossypol and ()-gossypol. According to prior research, ()-gossypol has a higher biological activity than (+)-gossypol.(Puckhaber, Dowd et al. 2002, Wolter, Wang et al. 2006, Kline, Rajkumar et al. 2008)

The researchers attempted to employ gossypol as a dye but discovered that it was unstable when exposed to light. Because it came from the genus Gossypium, the chemical was given the name gossypol. Gossypol is a dimeric-sesquiterpenoid that is formed in the plant by the dimerization of two molecules of hemi gossypol. Sesquiterpenoids are terpenes that have three isoprene units and protect plants from infections and insects.(Stipanovic, Stoessl et al. 1986)It has six phenolic hydroxyl groups and two aldehydic groups; making it chemically reactive.(Kenar 2006)In comparison to ()-gossypol or racemic gossypol, ()-gossypol had a more effective effect on cells at lower doses. Pigment glands in cotton stems, leaves, seeds, and flower buds produce gossypol, a

phenolic substance (Gossypium spp.). Cottonseed meal is a by-product of the cotton industry that is utilized in animal feed due to its high oil and protein content. Cottonseed's usage in animal feed is limited, however, due to gossypol toxicity.(Gadelha, Fonseca et al. 2014)

Gossypium hirsutum L., Gossypium barbadense L., Gossypium arboreum L., and Gossypium herbaceum L. are the only four species used for cotton fiber in the genus Gossypium, spp.G.hirsutum is the most economically significant cotton species, accounting for 90% of global cotton production.(Borém, Freire et al. 2003)

Gossypol has a molecular weight of 518.55 Dalton, is crystalline, has a yellow color, is insoluble in water and hexane, soluble in acetone, chloroform, ether, and methyl ethyl ketone (butanone), and is somewhat soluble in crude vegetable oils. C30H30O8 is the chemical formula, whereas 2,2 -bis is the chemical structural formula (8-formyl-1,6,7-trihydroxy5-isopropyl-3-methylnaphthalene)(Abou-Donia 1976, Rogers, Poore et al. 2002, Soto-Blanco 2008)



Chemical formula: $C_{30}H_{30}O_8$

Chemical structural formula: 2,2′-bis(8-formyl-1,6,7-trihydroxy-5-isopropyl-3-methylnaphthalene)

Figure 2: Chemical structure, formula, and structural formula of gossypol {Gadelha, 2014 #62}

Pigment glands are microscopic black dots found all over the cotton plant, but the seeds have the highest concentration.(Rogers, Poore et al. 2002, Alexander 2008, Soto-Blanco 2008)

Cottonseed may have total gossypol concentrations of more than 14,000 mg/kg and free gossypol values of more than 7,000 mg/kg.(Alexander 2008)

The release of gossypol from layer-by-layer gossypol/PEG films in vitro follows a flawless zero-order kinetics' film made of gossypol and PEG as a drug carrier for gossypol was developed. As a result of the films' slow breakdown, the film releases gossypol. The release of Gossypol follows a flawless zero-order kinetics.(Wen, Dong et al. 2018)

Cotyledon was revealed to be the predominant source of gossypol during seed germination in this investigation. However, it was primarily derived from the development of roots after that. The polyphenolic chemical Gossypol ($C_{30}H_{30}O_8$) is generated from the cotton plant (genus Gossypium, family Malvaceae).

Chemically, gossypol is reactive due to the presence of six phenolic hydroxyl groups and two aldehydic groups. Schiff base generation, ozonolysis, oxidation, and methylation can all be used to create gossypol derivatives. (Wang, Howell et al. 2009)

The increasing prevalence of microbial resistance to currently available antimicrobials necessitates the development of novel agents, such as biologically active compounds derived from plants, that can serve as a viable alternative. As a result, tests that consistently indicate the real antimicrobial activity of selected compounds must be developed and optimized.(Kolarević, Milovanović et al. 2016)

MIC readings can be used to track the progression of antibiotic resistance.(Wiegand, Hilpert et al. 2008)In theory, MICs could be a useful tool for comparing various susceptibility levels.

The preparation of the bacterial inoculum employed in the microdilution method is one of the elements that can have a big impact on MIC values.
Because the microdilution method is currently the most appropriate approach for determining MIC values (in terms of speed of execution and cost), it is critical to standardize methods that will allow data from different laboratories to be compared.(Kolarević, Milovanović et al. 2016)

Because of these factors, gossypol's medicinal efficacy often appears to necessitate a larger dose, but at the risk of increased toxicity. As a result, researchers have attempted to create gossypol derivatives in order to boost efficacy and/or reduce toxicity.(Wang, Beckham et al. 2008).

2.2 PHARMACOKINETICS OF GOSSYPOL:

Gossypol has a molecular weight of 518.55 Dalton, is crystalline, has a yellow color, is insoluble in water and hexane, soluble in acetone, chloroform, ether, and methyl ethyl ketone (butanone), and is partially soluble in crude vegetable oils.

2.3 THERAPEUTIC EFFECTS OF GOSSYPOL:

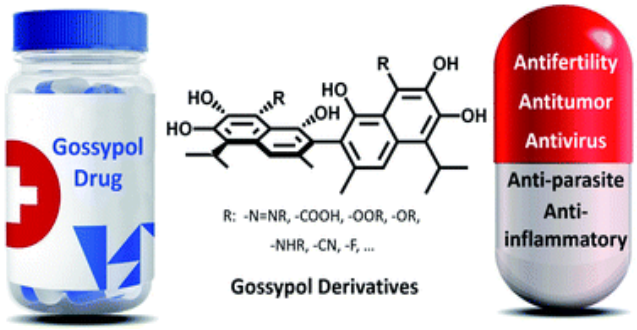


Figure 3: Therapeutic Effects of Gossypol Derivatives {Liu, 2017 #8}

2.3.1. ANTIOXIDANT:

Methylation of even one of the phenolic hydroxyl groups drastically reduced gossypol's antioxidant activity, indicating that the hydroxyl group of gossypols is important for quenching free radicals.(Nishida and Kawabata 2006)

According to Dodou et al. (2005), gossypol's antioxidant qualities may be beneficial in disorders characterized by lipid oxidative damage, such as psoriasis.(Li, Bandy et al. 2000)

2.3.2. ANTIFERTILITY:

Antifertility, antioxidant, anticancer, antiviral, antiparasitic, and antibacterial effects of gossypol have been documented, as well as a reduction in plasma cholesterol.(Keshmiri-Neghab and Goliaei 2014)During the 1950s, a study of couples who cooked with crude cottonseed oil revealed low birth rates, with men having very low sperm counts and women experiencing amenorrhea.

Gossypol's antifertility effect in mammals and humans has been validated in subsequent studies. Gossypol is a non-steroidal substance that has no effect on hormone levels but inhibits sperm production and motility in both animals and humans.(Coutinho 2002)

It works as a contraceptive by blocking enzyme systems that affect sperm and spermatogenic cell energy consumption. The male antifertility impact is dose and time dependent; at effective doses, gossypol inhibits sperm motility, lowers sperm concentrations, causes specific mitochondrial injury to the sperm tail, and damages the germinal epithelium, resulting in infertility.(Randel, Chase Jr et al. 1992)

2.3.3. GOSSYPOL AS ANTIVIRAL:

Antiviral properties of gossypol have been documented against enveloped viruses such

as HIV-1, HSV-2, influenza, and parainfluenza.(Tai-Shun, Schinazi et al. 1993)

2.3.4 ANTIBACTERIAL ACTIVITY:

Gossypol shows more efficacy as antibacterial against gram positive species than gram negative bacteria. It shows more potency against Staphylococcus aureus, Bacillus species etc. than Pseudomonas aeruginosa, E. coli etc. This could be due to structural differences between Gram-positive and Gram-negative bacteria's cell walls and cell membranes.

Gram positive bacteria have a higher peptidoglycan content in their cell walls and do not have the outer membrane that Gram negative bacteria possess. This may have an impact on how gossypol gets to its goal.(Vadehra, Kalla et al. 1985) Antibiotic action was found in Gossypol against aerobic spore formers and lactobacilli, as well as antagonistic activity against some of the more oxidative yeasts. Later, Vadehra et al. (1985) looked into the effects of gossypol on bacterial growth as well as spore formation and germination in Bacillus cereus.(Kolarević, Milovanović et al. 2016)

2.3.5 ANTIMICROBIAL ACTIVITY:

Margalith (1967) tested gossypol's antibacterial activities against spore formers and lactobacilli in cottonseed meal-fed animals and found that it inhibited microorganisms.

The findings revealed that gossypol altered the microbiota of the gastrointestinal system in a significant way.(Margalith 1967) Gossypol exhibits antifungal properties, with LD50 values ranging from 20 to 100 ppm for pure gossypol (Bell, 1967)

2.3.6 ANTIHYPERLIPIDIMIC:

When gossypol was given orally to adult male Cynomolgus monkeys at a dose of 10 mg/kg/day for 6 months, it reduced total plasma cholesterol levels as well as bad cholesterol LDL levels while having little effect on high density lipoprotein levels.(Shandilya, Clarkson et al. 1982)

2.3.7 EFFECTS ON HEART:

Gossypol also reduces the heart's contraction force and the extent of cardiac fiber contraction.(Wei-Min and Urthaler 1986)

2.3.8 GOSSYPOL AS ANTITRYPANSOMAL:

One investigation found that gossypol has antitrypanosomal action at a similar level, with an IC50 of 7.8 ppm following a 24-hour exposure.(Wang, Beckham et al. 2008)

2.3.9 EFFECTS OF GOSSYPOL ON IMMUNE SYSTEM:

Gossypol may induce a decrease in the number of leukocytes, particularly lymphocytes, affecting the organism's immunocompetence.(Braga, Maciel et al. 2012)

Gossypol possesses immunosuppressive effect, which affects lymphocytes by reducing proliferation and promoting death, according to in vivo and in vitro mice tests.(Quintana, de Peyster et al. 2000, Xu, Xu et al. 2009)

Furthermore, mice receiving gossypol had lower CD4+ thymocyte numbers and higher CD8+ lymphocyte populations in their spleen and lymph nodes.(Sijun, Pawlak et al. 2012)

2.4. METABOLISM:

Although it is the more physiologically active form, the (-) gossypol enantiomer is removed more slowly. As a result, it is more poisonous than (+) gossypol.(Wu, Yu et al. 1986, Bailey, Stipanovic et al. 2000, Kakani, Gamboa et al. 2010)

Gossypol is absorbed and accumulates in the liver and kidneys. Bile is the primary excretion pathway for gossypol, which is then excreted in feces after conjugation with glucuronides and sulphates.(Abou-Donia, Othman et al. 1989, Kim, Calhoun et al. 1996)Gossypol has an effect on thyroid metabolism as well(Tang and Wong 1984, Rikihisa and Lin 1989) T4 and T3 blood

concentrations in male and female rats were shown to be lower after gossypol treatment in several investigations.(Lin, Chitcharoenthum et al. 1990)

2.5 CONCENTRATION OF GOSSYPOL:

At a dosage of 100 ppm, all Gram-positive organisms examined were fully suppressed. At 100 ppm of gossypol, none of the Gram-negative germs tested were inhibited, and only one-third of the organisms tested were inhibited at 200 ppm of gossypol. The antibacterial activity of gossypol, according to the authors, was linked to the Gram character of the bacterium.(Kolarević, Milovanović et al. 2016)

2.6 GOSSYPOL TOXICITY AND POISONING:

Acute clinical indicators of gossypol poisoning include respiratory distress, impaired body weight growth, anorexia, weakness, lethargy, and mortality within several days, which may be caused by high quantities of free gossypol.

The disruption of male and female reproduction is, nevertheless, the most common harmful impact. Another significant toxicity of gossypol is its interaction with immunological function, which lowers an animal's resistance to infection and reduces vaccine efficacy.

2.7 REDUCTION OF GOSSYPOL TOXICITY:

Treatment of the cottonseed product to reduce the concentration of free gossypol, with heat exposure being the most common treatment, is one of the most prevalent preventive treatments for limiting gossypol toxicity. Concentrations of gossypol range between 0.02 and 6.64 percent.(Price, Lovell et al. 1993)

2.8 NEED OF NATURAL DRUGS DUE TO MICROBIAL RESISTANCE:

The increasing prevalence of microbial resistance to currently available antimicrobials necessitates the development of novel agents, such as biologically active compounds derived from plants, that can serve as a viable alternative. As a result, tests that consistently indicate the real antimicrobial activity of selected compounds must be developed and optimized.(Kolarević, Milovanović et al. 2016)The determination of MIC values can be used to track the progression of antibiotic resistance.(Wiegand, Hilpert et al. 2008)MICs, in theory, might be a useful tool for comparing different susceptibility levels. The preparation of the bacterial inoculum employed in

the microdilution method is one of the elements that could have a major impact on the calculation of MIC values. Because the microdilution method is the most appropriate approach for determining MIC values at the moment.(Kolarević, Milovanović et al. 2016)

Chapter 3

3. Material and Methodology:

3.1. Materials:

E. faecalis Strain JH2-2 (derived from the parental strain JH2) Cultured on GM 17 media containing MOPS (3-(N-morpholino) propane sulfonic acid), MOPS was buffering agent in this media.M17 media supplemented with 0.5% (wt./vol) of 30 % Glucose at 37 Degrees. Gossypol 98+% (Alfa Aesar USA), Lauria Bertani Broth (CM1018 OXOID)

Constituents of GM 17 media were Universal Peptone 5g/L (HIMEDIA), Tryptic Soy Broth 5g/L (Merck Germany), Yeast Extract 2.5g/L (Sigma Aldrich),Beef Extract (Sigma Aldrich), Ascorbic Acid 0.5g/L (BDH AnalaR),Magnesium Sulphate 0.25g/L (GPR BDH),MOPS (3-(N-morpholino) propane sulfonic acid 42g/L (Sigma Aldrich), Sodium Hydroxide Pellets (GPR BDH) to maintain ph. 7.1 to 7.3,Glucose 0.5%(wt./vol) (AppliChem GmbH)

Glucose was added by making its separate solution and sterilizing by autoclave and then added into media by 0.5% w/v after cooling and its addition in media in sterilized environment.

Figure 4: GM17 Media in 10ml Test Tubes after autoclaving

3.2. Methodology:

3.2.1 Bacterial Isolation and culturing:

Bacterial isolation on Lauria Bertani Broth and LB Agar by spreading and streaking, Bacterial isolation on GM17 Agar by spreading and streaking. Bacterial Cultivation in GM17 Broth Media.

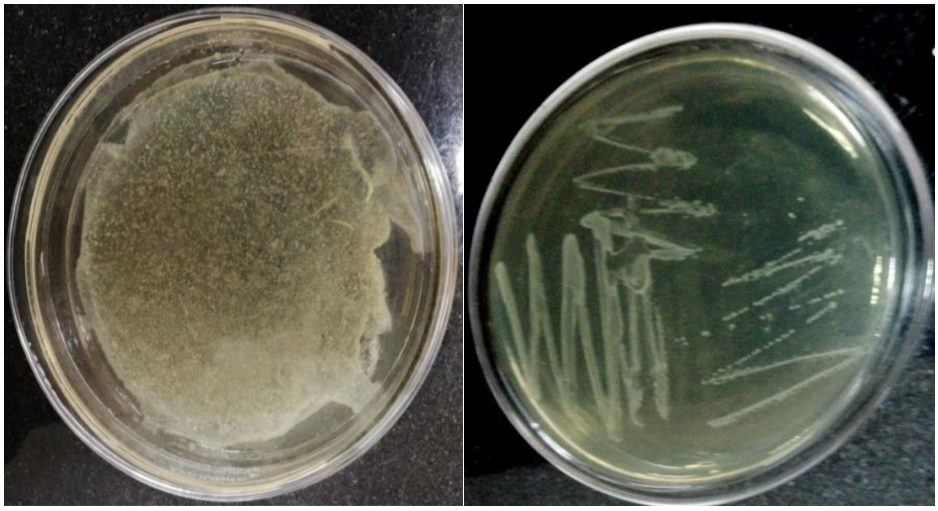


Figure 5: Spreading and streaking on LB Agar

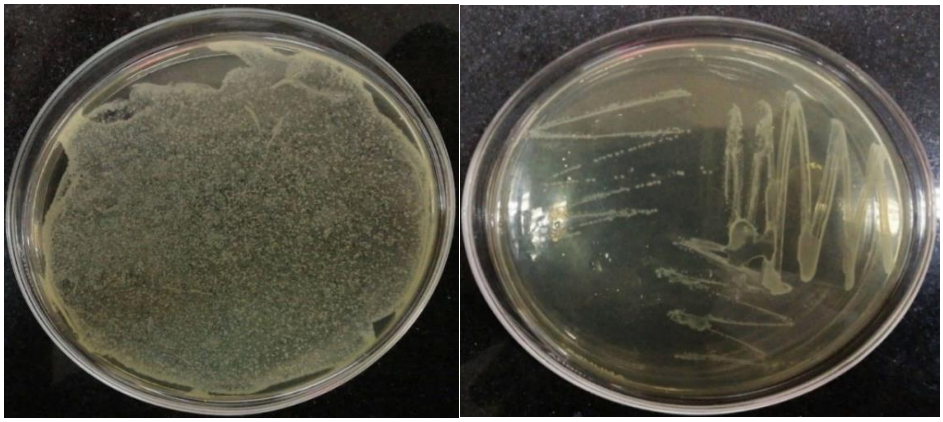


Figure 6: Spreading and streaking on GM17 Agar

Glycerol Tube prepared for daily tests from overnight fresh preculture in 10ml GM17 Media in 25ml test tube from pure isolated colony of E. faecalis obtained after spreading and streaking on GM17 Agar. To prepare the glycerol tube for daily experiments, 1ml of fresh bacterial preculture and 1ml of 0.5 percent glycerol solution were added to a 10ml GM 17 Medi in a 25ml test tube overnight. 50 microliters were drawn from the glycerol tube, inoculated in a 10 ml test tube containing GM17 medium, and then the mixture was incubated at 37 °C and 120 rpm overnight.

3.2.2. Preliminary Evaluation using Large Test tubes:

200 microliters of a fresh preculture were taken and inoculated into 10 test tubes containing 10 ml of GM17 Broth media. The test tubes were then incubated at 37 °C and 120 rpm for 45

minutes in a shaking water bath after the OD at 600 nm was measured and recorded for each tube. The OD at 600 nm of each test tube was determined after an hour of incubation at 37 °C and 120 rpm. Until the OD at 600nm reached 0.8, the procedure was repeated. A 5mM stock solution of gossypol was made, and samples of the drug (Gossypol) at various concentrations were obtained to test for inhibition.

3.2.3 Concentrations of Gossypol for Evaluation:

Eight different concentrations (5, 10, 25, 50, 75, 100, 150, and 200 micrograms per millimeter) were used to test the growth inhibitory impact.

Figure 7: Stock Solution of Gossypol

The first tube containing the bacterial preculture with media was utilized as a control, while the second tube was inoculated with 100 microliters of DMSO, which acts as the control's solvent. The third tube was filled with 5 micrograms of the drug, the fourth with 10 micrograms of the drug, the fifth with 25 micrograms of the drug, the sixth with 50 micrograms of the drug, the seventh with 75 micrograms of the drug, the eighth with 100 micrograms of the drug, the ninth with 150 micrograms of the Gossypol, and the tenth with 200 micrograms of the Gossypol. The tubes were incubated in a shaking water bath at 37 degrees Celsius and 120 revolutions per minute. The OD at 600nm was once more measured after an hour, and the results for each test tube were recorded. The test tubes were then incubated in a water bath that was shaking at 37 degrees Celsius and 120 rpm. Till 7 hours, absorbance at 600 nm was measured every hour under the same incubation conditions.

3.2.4 Serial Dilution:

Serial dilution was then performed after 3 hours, when 0.8 concentration was reached, and after 7 hours, or 4 hours after drug, for controls with preculture and media only, and for two different drug concentrations, namely 75 and 100 micrograms per milliliter, to determine the minimum inhibitory concentration. Inhibition at 100 micrograms milliliter was well-defined and yielded positive outcomes.

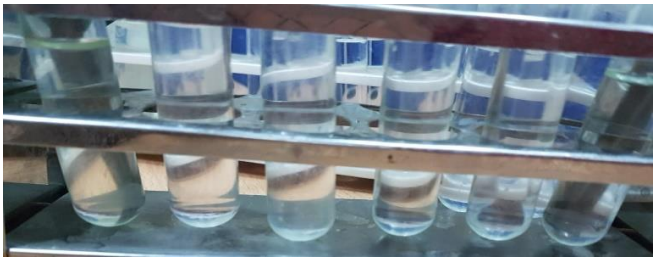


Figure 8: Serial Dilution

3.2.5 Bacterial Cell Lysis and Lactate Dehydrogenase Activity Determination:

After an 8-hour experiment, 1ml was removed from each test tube and centrifuged at 4 degrees Celsius for 5–10 minutes at 4500 rpm. Pellets remains on former Eppendorf's, and supernatant eliminated in ten different Eppendorf's. Using Cell lysis buffer (100 microliters) and 200 microliters of autoclaved distilled water, bacterial cells in the pellets were first vortex-lysed, then supernatant was centrifuged at 1500 rpm for ten minutes. Supernatant was taken for performing LDH Assay, followed by the LDH Assay kit Protocol of (LDH-P FLUID 4+1) Kinetic Assay for the determination of Lactate Dehydrogenase as per manufacturer's instructions.

Figure 9: Supernatant Collection

3.3 Absorbance on Microscale (Broth Dilution Method)

Fresh Bacterial Culturing from Overnight Preculture in Shaking Water Bath at 37 Degrees incubation in 11 wells of 96 Microwell Plate, First Well Serves as Blank, Second as Control using GM17 medium and Preculture, Third as Vehicle Control containing DMSO as solvent for Gossypol along with Culture media. From fourth to eleventh well culture media.

Plated was placed in a microplate reader with an incubation temperature of 37 degrees Celsius, a kinetic loop of 3 hours with a kinetic interval of 1 hour, and an absorbance setting of 600nm wavelength with continuous shaking. The initial absorbance result was calculated in the first minute, and three further absorbance values were calculated after a one-hour interval.

After 3 hours, the session was terminated, and the OD hit 0.8. The plate was ejected, and drug addition began. Nothing was added to the first well because it serves as a blank, nothing was added to the second well because it serves as a control,100 microliters of DMSO was applied to the third well. In the fourth well, 5 micrograms of Gossypol were added, in the fifth well, 10 micrograms of Gossypol were added, in the sixth, 25 micrograms of Gossypol were added, in the seventh, 50 micrograms of Gossypol were added, in the eighth, 75 micrograms of Gossypol were added, in the ninth, 100 micrograms of Gossypol were added, and in the tenth, 150 micrograms of Gossypol were added, eleventh well with 200 micrograms of Gossypol.

The plate was put into the microplate reader once more, and the incubation time was set to 5 hours with a kinetic interval of one hour and absorbance at 600 nm wavelength.

The first reading was recorded in the first minute, and the subsequent five readings were recorded at one-hour intervals. This experiment was repeated thrice, and mean results were interperated.

Figure 10: Microplate Reader in working and 96 well Flat Bottom Plate with Samples

3.3.1. Addition Of Stress Factors along with MIC Value of Gossypol that was 100µg/ml

There were Six Stress factors used along with MIC Value of Gossypol that were such as Sodium Dodecyl Sulphate (SDS, 0.01% w/v), Hydrogen Peroxide (H_2O_2), Ethanol (8% w/v) and Dimethyl Sulfoxide (DMSO, 10% w/v), 10% Glucose, 0.25% HOCl.

The combined inhibitory effect of six stress factors, including 0.01% SDS, 2.5 mM H2O2, 8% ethanol, 10% DMSO, 10% glucose, and 0.25% HOCl, was measured for Gossypol at doses of 100 micrograms per milliliter. The first well was blank only GM17 Media, the second was the control (Culture+ Media), the third was for the Culture Media+ DMSO (vehicle control), the fourth was with 0.01%SDS plus 100 micrograms per milliliter of Gossypol, the fifth was with 2.5mM H2O2 and 100 micrograms per milliliter of Gossypol, the sixth contained 8% ethanol and 100 micrograms per milliliter of Gossypol. The 7th well has 10% DMSO plus 100 micrograms of Gossypol per milliliter; the 8th well with 10% Glucose and 100 micrograms per milliliter, the 9th well contained 0.25% HOCl and 100 micrograms per milliliter of Gossypol.

Before adding Gossypol and stress factors, fresh overnight preculture in wells 2 through 9^{th} well produced an OD of 0.8; well 1 was used just for a blank with GM17 media. Each well was filled with 200 microliters of GM 17 media, 4 microliters of preculture, and was shaken continuously throughout a kinetic loop of three hours. After stressors and inhibitors were applied to the wells as previously mentioned. The effect of stressors on OD at 600nm was assessed and recorded. Three distinct experiments were used to test the inhibitory impact in conjunction with six different stressors and Gossypol.

Chapter 4

4. Results and Discussion

4.1 Effect of different concentrations of Gossypol on *E. faecalis* growth (Large Test Tubes)

Control and vehicle control are not significantly different from one another. Additionally, there is no discernible difference in growth inhibition at doses of 5, 10, 25, 50, 75, and 100 micrograms per milliliter; nevertheless, there is inhibition at higher doses of Gossypol, at 150 and 200 micrograms per milliliter, primarily during hours 6 and 7, as hours 3 and 4 of Drug addition as shown in figure 11.

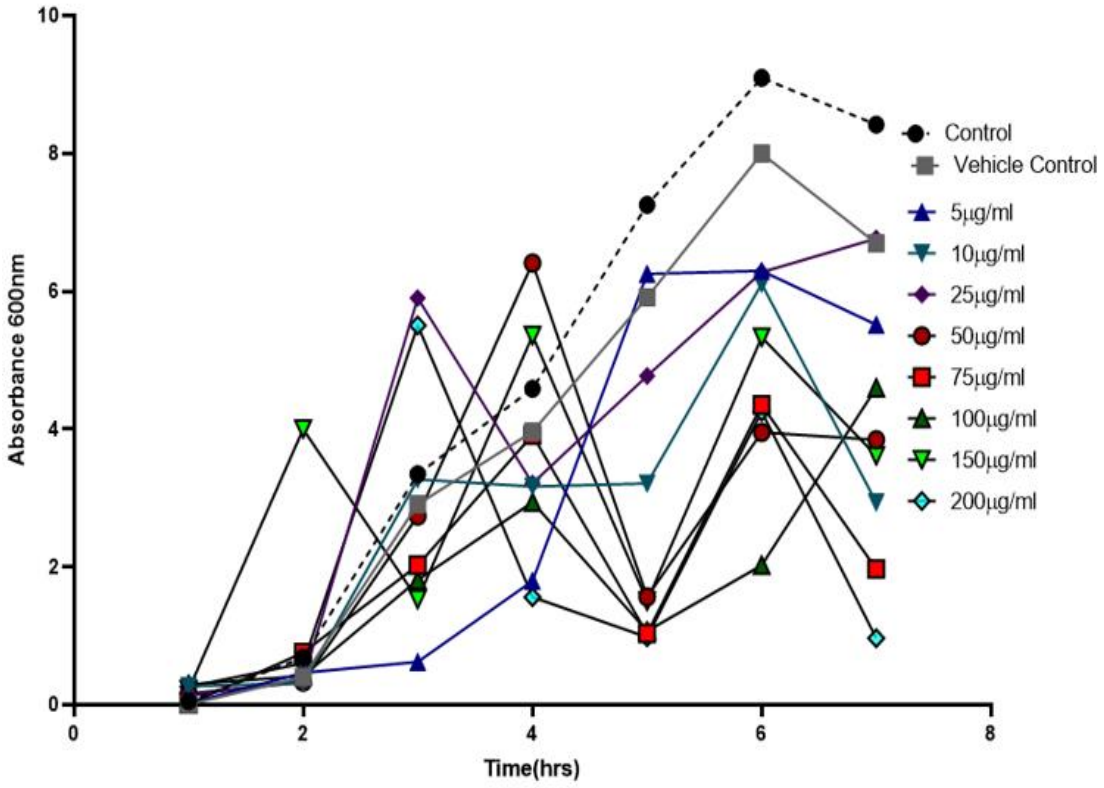


Figure 11: Effect of different concentrations of Gossypol on *E. faecalis* growth

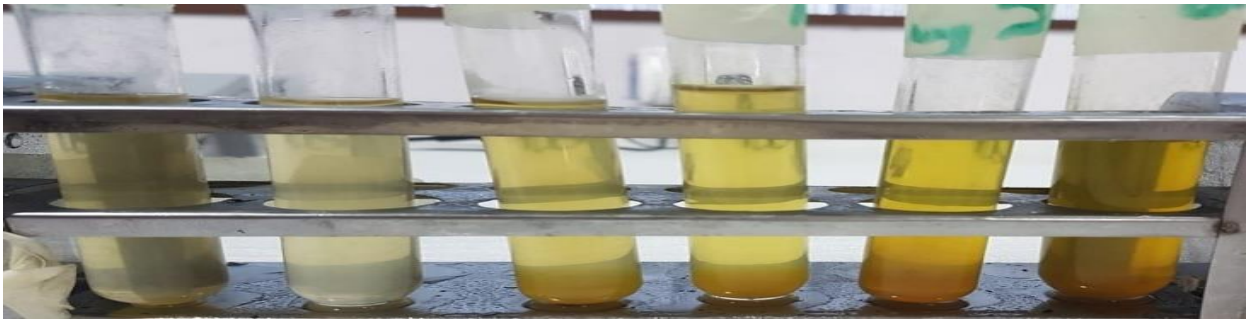


Figure 12: The first two test tubes show turbidity as a sign of distinct bacterial development, while the others show reduced growth as a result of an inhibitor (Gossypol)

4.2 Effect of different concentrations of Gossypol on *E. faecalis* growth (Microscale)

There is no significant difference between control and vehicle control. There is no significant growth inhibition at 5,10,25,50,75 and 100 microgram per milliliter. There is significant growth inhibition at 150 and 200 microgram per milliliter.

Different concentrations of Gossypol such as 5, 10, 25, 50, 75, 100, 150 and 200 µg/mL were added to the *E. faecalis* culture after 3 hours (hrs.) of preincubation and later on incubated for 5 hrs. There was no significant difference between control and vehicle control (DMSO) (Fig. 13). Similarly, no significant difference was observed between control and Gossypol at 5, 10, 25, 50, 75 and 100 µg/mL concentrations (Fig. 13). However, there was a significant difference between control and Gossypol at 150 µg/mL concentration during 4^{th} hr. and 5^{th} hr. of incubation with Gossypol (Fig. 13). Similarly, Gossypol at 200 µg/mL concentration showed significant difference compared to control during 4^{th} hr. and 5^{th} hr. of incubation (Fig. 13). Interestingly, compared to vehicle control, Gossypol at 150 µg/mL and 200 µg/mL concentrations significantly inhibited the growth of *E. faecalis* after 2 hrs. and 3 hrs. of post-Gossypol incubation, respectively (Fig. 13).

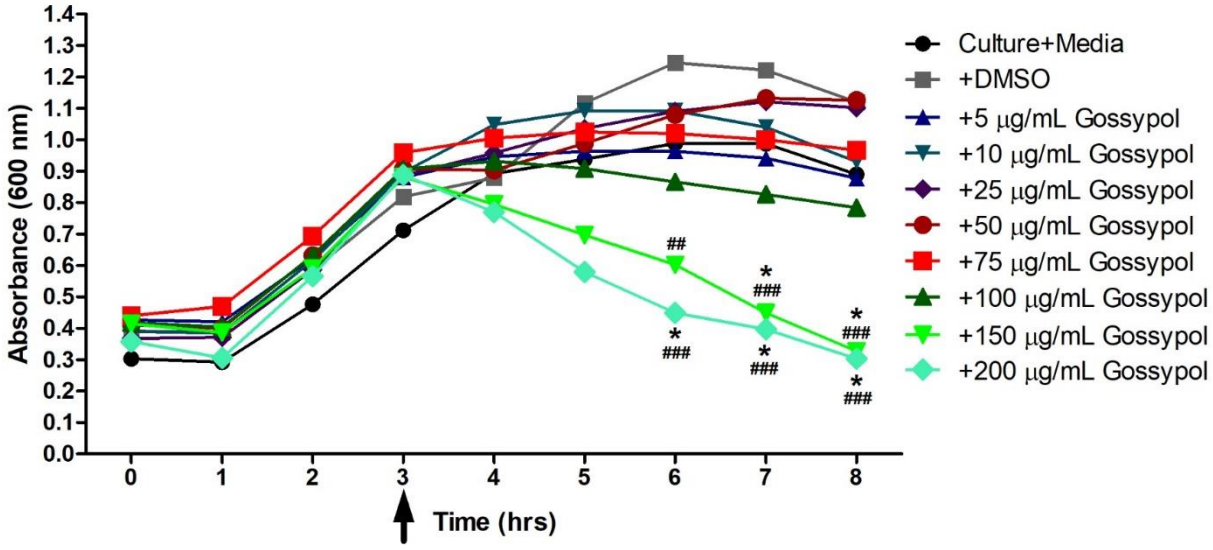


Figure 13: Effect of different concentrations of Gossypol on the growth of *E. faecalis*.

* Compared to Culture+Media # Compared to Culture+Media+DMSO

* P value <0.05; ** P value <0.01; *** P <0.001 ; # P value <0.05; ## P value <0.01; ### P <0.001

4.2.1 Minimum Inhibitory Concentration of Gossypol:

Minimum inhibitory concentrations (MICs) are defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation {Andrews, #62} The Minimum Inhibitory Concentration of Gossypol is 100 because this is the lowest dose at which a notable inhibition in bacterial growth can be observed. As a result, we will compare the MIC value of Gossypol with the different Six Stress variables and found combined results of inhibition.

4.3 Effect of different concentrations of Gossypol in adjunct to different stress factors on the *E. faecalis* growth

E. faecalis is known to survive harsh environments and resist various types of different stress factors (Solheim et al., 2014). Therefore, we evaluated Minimum Inhibitory concentrations of Gossypol that showed significant inhibition (100 µg/mL) of the growth of *E. faecalis* in adjunct to

various stress factors such as Sodium Dodecyl Sulphate (SDS, 0.01% w/v), Hydrogen Peroxide (H_2O_2), Ethanol (8% w/v) and Dimethyl Sulfoxide (DMSO, 10% w/v),10% Glucose, 0.25% HOCl.

4.3.1 Effect of different concentrations of Gossypol in adjunct to 0.01% w/v Sodium Dodecyl Sulphate (SDS)

Sodium Dodecyl Sulphate (SDS) is a detergent and is used as a stress factor for the growth of *E. faecalis*. After 3 hrs. of preincubation, concomitant addition of the different concentrations of Gossypol with 0.01% SDS and incubation for further 5 hrs. did not show any significant inhibition of the growth of the *E. faecalis* (Fig. 14).

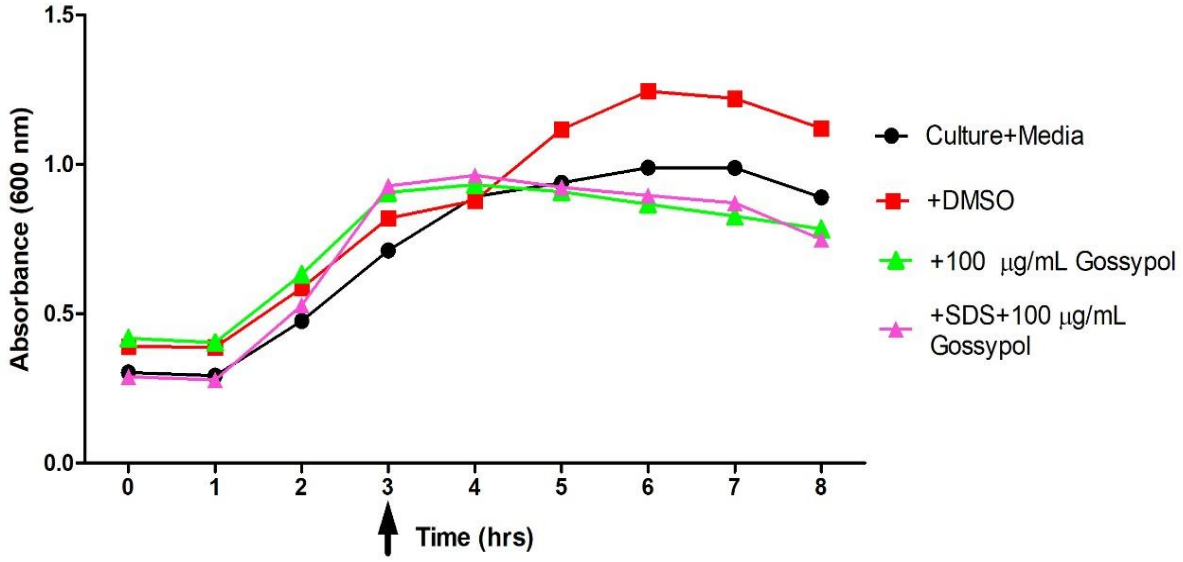


Figure 14: Effect of different concentrations of Gossypol in adjunct to 0.01% w/v Sodium Dodecyl Sulphate (SDS)

4.3.2 Effect of different concentrations of Gossypol in adjunct to 2.5 mM Hydrogen Peroxide (H_2O_2)

Similar to SDS stress, H_2O_2 was used as stress factor at a concentration of 2.5 mM to evaluate the increase in growth inhibition in adjunct to the Gossypol. Application of H_2O_2 did not significantly reduce the growth of *E. faecalis* when used in combination with different doses of the Gossypol (Fig. 15).

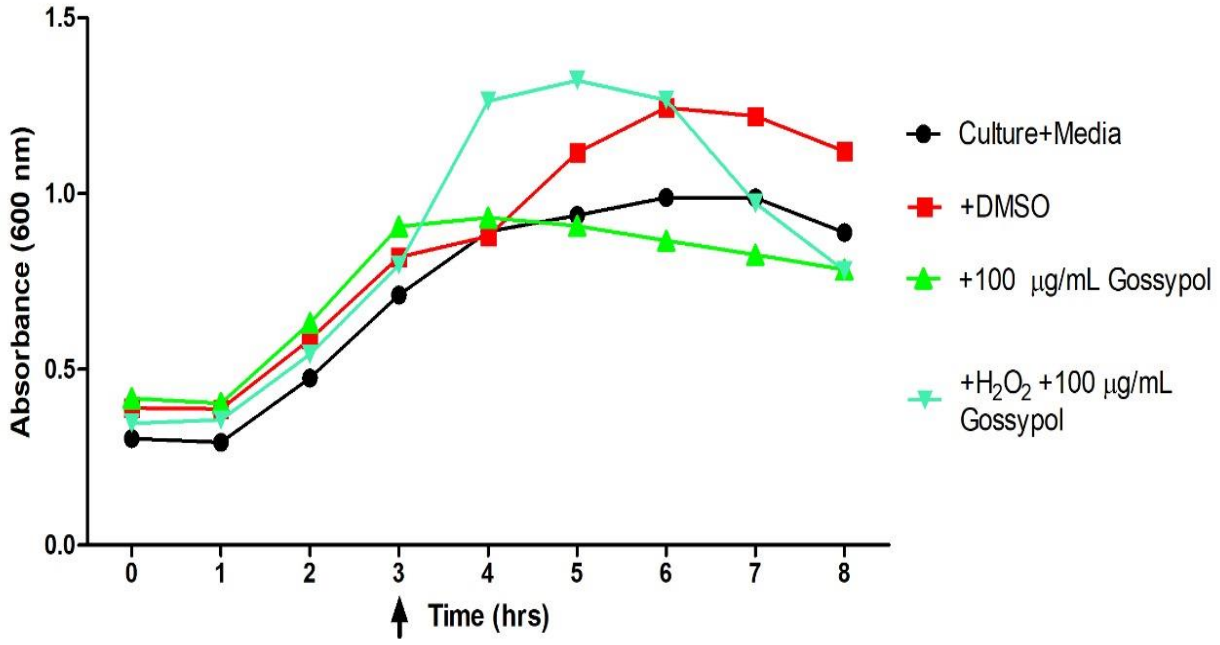


Figure 15: Effect of different concentrations of Gossypol in adjunct to the application of Hydrogen Peroxide (H_2O_2) stress.

4.3.3 Effect of different concentrations of Gossypol in adjunct to 8% Ethanol

Application of 8% Ethanol induced stress along with 100 µg/mL concentration significantly inhibited the growth of E. faecalis during 4th and 5th hrs. of incubation after the addition of Gossypol and 8% Ethanol in comparison to the control (Fig. 16). However, in comparison to the vehicle control, 100 µg/mL concentration of the Gossypol along with 8% Ethanol displayed significant inhibition during the 3rd, 4th, and 5th hr. of post-drug and stress incubation period. Although, the combination of Ethanol and Gossypol appeared to reduce the growth of *E. faecalis* more than when Gossypol was used alone at these concentrations, statistically significant difference was observed (Fig. 16).

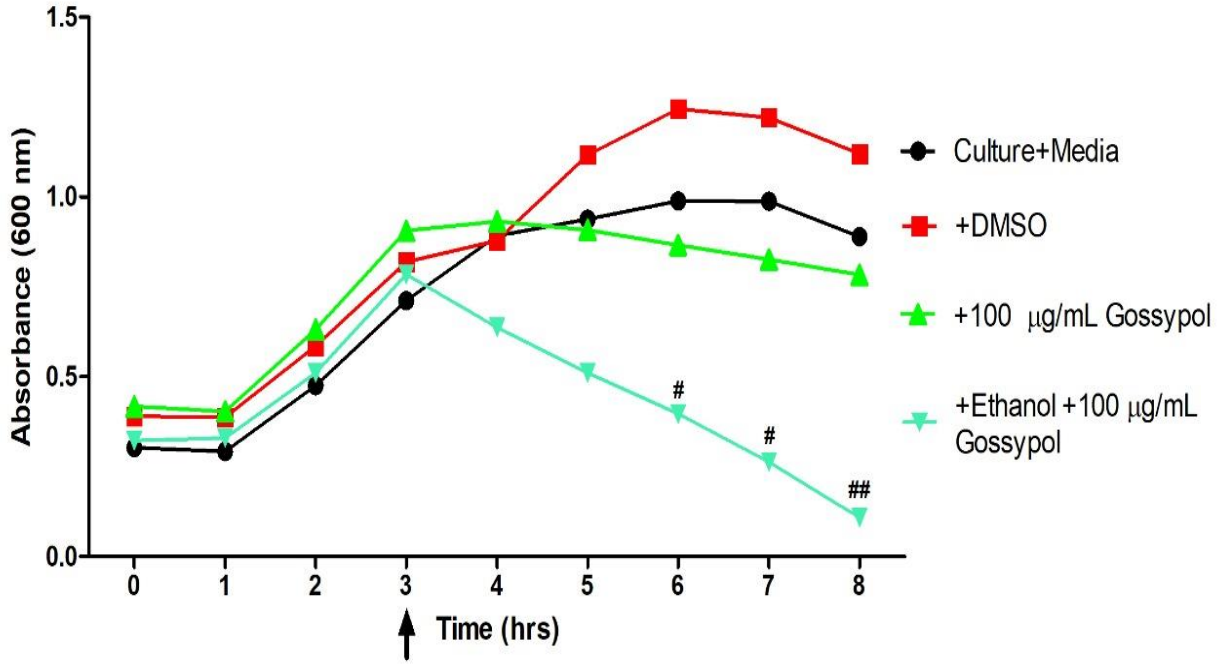


Figure 16: Effect of different concentrations of Gossypol in adjunct to the application of 8% Ethanol stress

* Compared to Culture+Media # Compared to Culture+Media+DMSO

* P value <0.05; ** P value <0.01; *** P <0.001 ; # P value <0.05; ## P value <0.01;

P <0.001

4.3.4 Effect of different concentrations of Gossypol in adjunct to Dimethyl Sulfoxide (DMSO)

Combination of 10% DMSO and 100 µg/mL concentration of Gossypol significantly inhibited the growth during the 3rd, 4th, and 5th hrs. of post-incubation period compared to the controls (Fig. 17). However, in comparison to the vehicle control (DMSO), Gossypol in combination with 10% DMSO showed significant growth reduction at 100 µg/mL concentration during the 4th and 5th hr. of the post-Gossypol and DMSO incubation (Fig. 17). At 5th hr. Significant Inhibition with Gossypol and 10% DMSO in Comparison 100 µg/mL when used lonely.

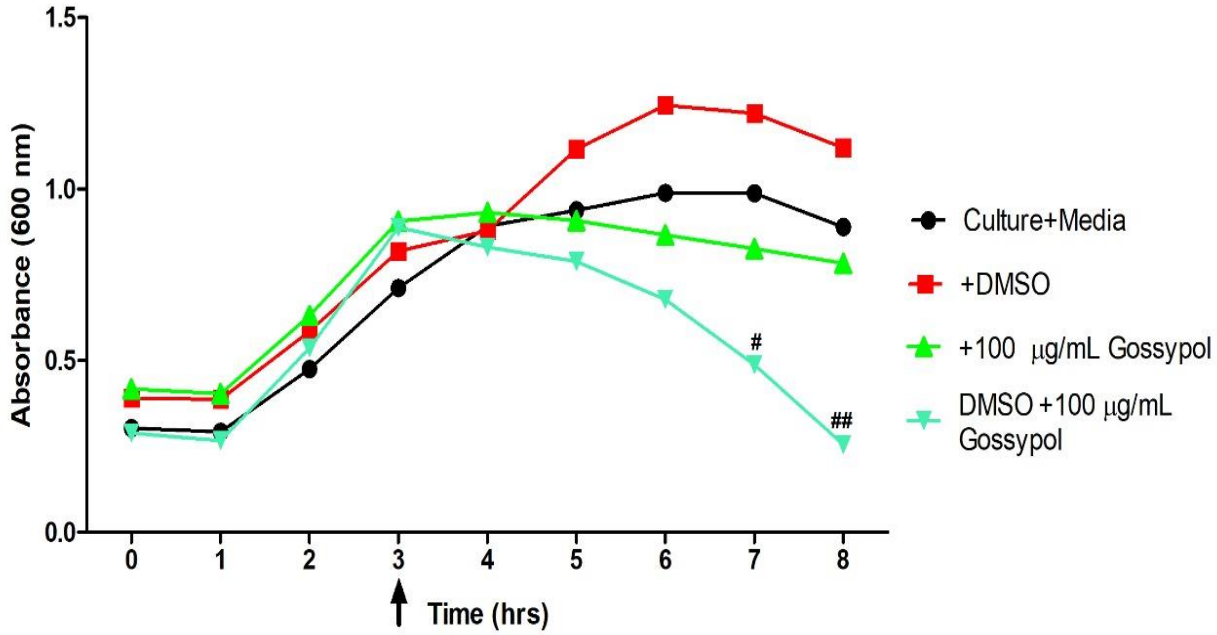


Figure 17: Effect of different concentrations of Gossypol in adjunct to the application of 10% DMSO stress.

*Compared to Culture+Media # Compared to Culture+Media+DMSO

P value <0.05; ## P value <0.01;

4.3.5 Effect of different concentrations of Gossypol in adjunct to 10% Glucose

Application of the Gossypol at 100 µg/mL concentrations along with 10% Glucose stress significantly inhibited the growth during 4th and 5th hrs. post-incubation period compared to controls. However, compared to vehicle control the inhibitory effect was evident one hour earlier i.e., during 3rd, 4th, and 5th hrs. of post-incubation period for the same concentrations of the Gossypol and 10% Glucose induced stress (Fig. 18).

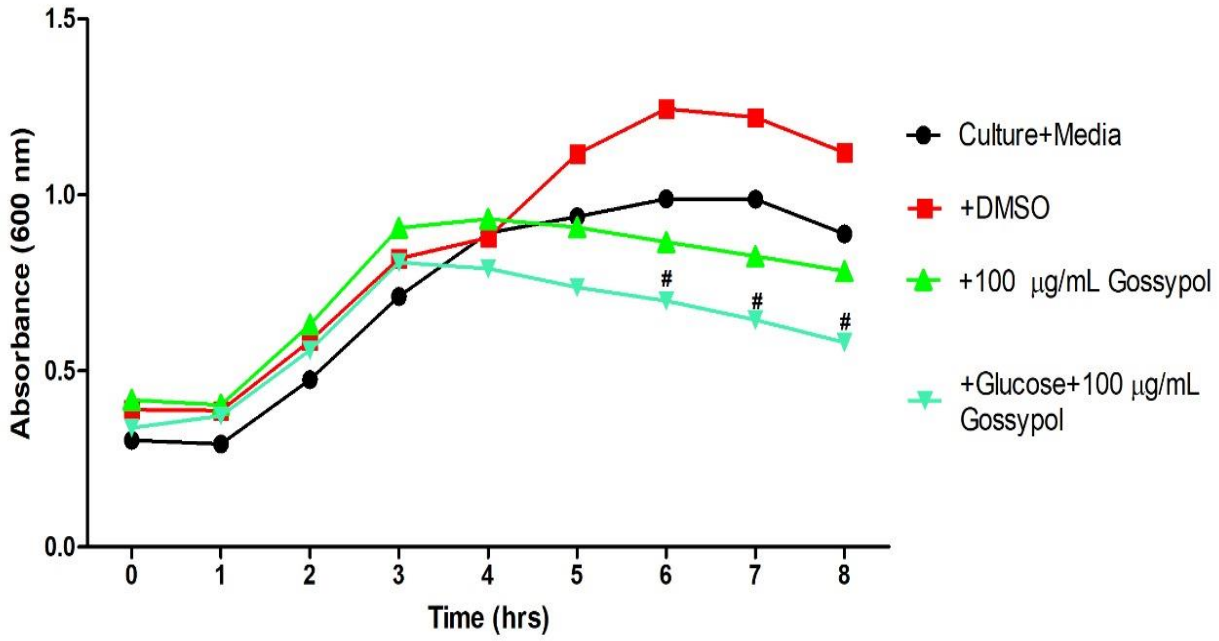


Figure 18: Effect of different concentrations of Gossypol in adjunct to the application of 10% Glucose stress

* Compared to Culture+Media # Compared to Culture+Media+DMSO

P value <0.05;

4.3.6 Effect of different concentrations of Gossypol in adjunct to HOCl

Combined application of the Gossypol at different concentrations such as 100 µg/mL with HOCl did not significantly inhibit the growth of *E. faecalis* In Comparison to DMSO 100µg/mL conc of Gossypol shows significant Inhibition (Fig. 18).

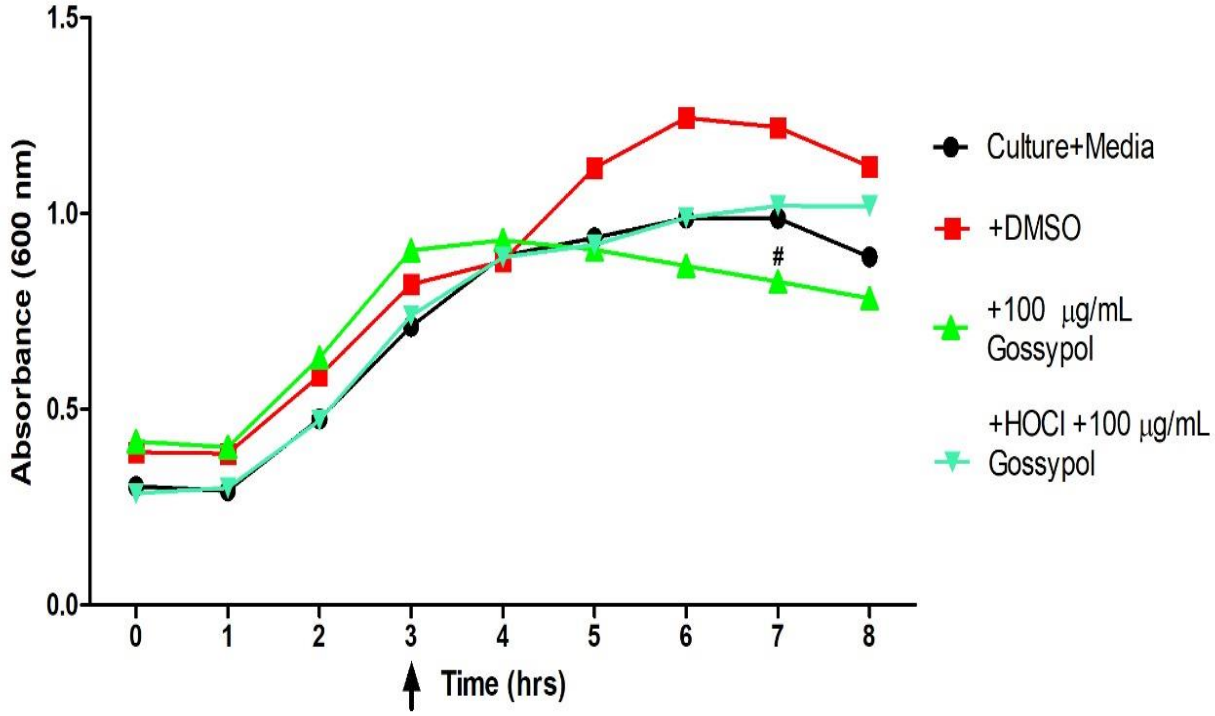


Figure 19: Effect of different concentrations of Gossypol in adjunct to the application of HOCl induced stress

* Compared to Culture+Media # Compared to Culture+Media+DMSO

P value <0.05

4.4 Kinetic Assay for the determination of Lactate Dehydrogenase Activity by DGKC Method:

Lactate Dehydrogenase (LDH) Enzyme activity was determined after incubation without and with Gossypol different doses as shown in fig. 20. The different doses of Gossypol did not show any impact on LDH activity except a slight increase in LDH activity that was seen after incubation with Gossypol 100 µg/mL. This slight increase in LDH activity could be speculated to stem from increased expression of LDH enzyme due to probably strong inhibition at this dose of Gossypol.

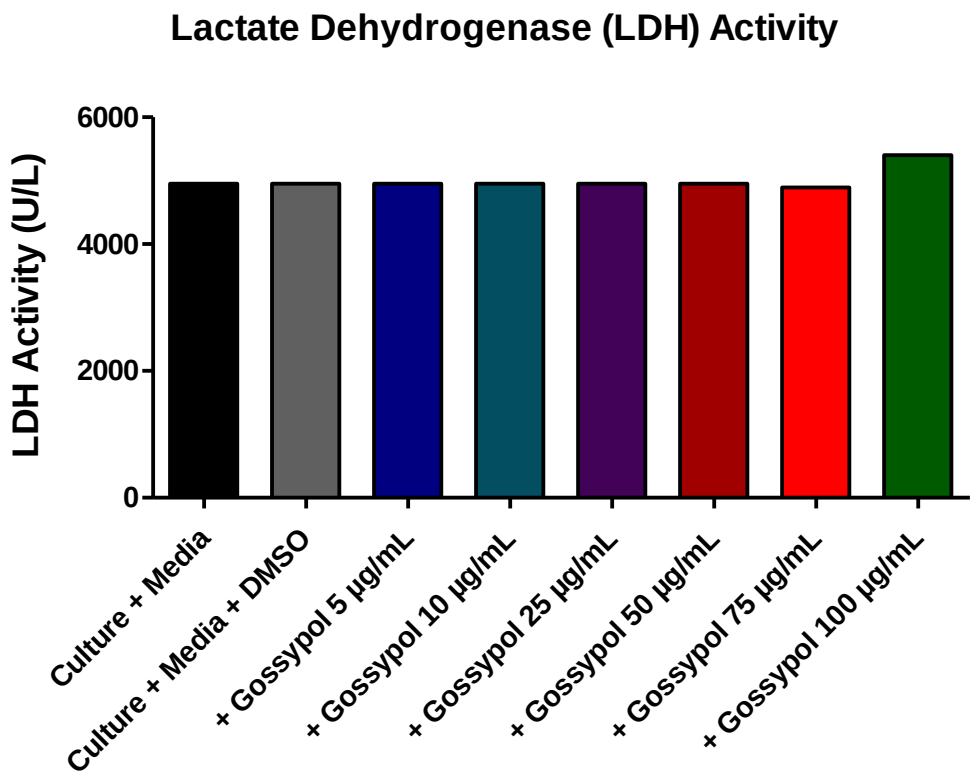


Figure 20: Determination of Lactate Dehydrogenase Activity at different concentrations of Gossypol

Conclusion:

A gram-positive bacteria called *E. faecalis* causes nosocomial infections and is also resistant to several powerful drugs. Therefore, the creation of innovative therapies is required to block it and lessen its pathogenicity. Lactate Dehydrogenase enzyme is primarily used by *E. faecalis* for growth, virulence, and resistance. There are two forms of lactate dehydrogenase enzyme in this bacterium: LDH1 and LDH2, with LDH1 playing a crucial part in how this bacterium acts. Gossypol, a polyphenol sesquiterpene with numerous medicinal benefits as well as an inhibitor of the Ldh enzyme in bacteria, is one of the many ligands for the inhibition of this important enzyme that have been found by computational approaches. We utilized one of Ligand Gossypol for evaluating its anti-bacterial activity. Optimized Gossypol concentrations for antibacterial activity and calculated its MIC value. We combined Minimum Inhibitory concentrations of Gossypol 100µg/ml with six stressors, including Sodium Dodecyl Sulfate (SDS, 0.01%w/v), Hydrogen Peroxide(H_2O_2), Ethanol (8%w/v), Dimethyl Sulfoxide(10%w/v),10%Glucose, and 0.25% HOCl. Despite being given at MIC of 100 micrograms per milliliter, SDS and H2O2 have similar effects and do not appreciably suppress the growth of Enterococcus faecalis. At MIC value of Gossypol dose of 100 microgram per milliliter at the fourth and fifth hours of the bacterial inhibitor interaction, ethanol at a concentration of 8% exhibits considerable inhibition. After the drug's post-incubation period with 10% DMSO, the addition of Gossypol at MIC of 100 micrograms per milliliter significantly inhibits the growth of *E. faecalis*. Additionally, glucose 10% significantly inhibits growth during hours four and five after drug incubation. While 0.25% HOCl did not significantly impede the growth of *E. faecalis*. A preliminary step towards evaluating Gossypol on animal models and toxicological assessments for preventing Enterococcus faecalis infections was demonstrated by an in vitro analysis against the pathogen. Gossypol is potent inhibitor of Lactate Dehydrogenase Enzyme in *E. faecalis*. Gossypol Minimum Inhibitory Concentration shows synergism with stress factors. In Future Gossypol Inhibition Effect should be evaluated with Different Antibiotics.

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