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Research Article

GLYCYRRHIZA GLABRA EXTRACT ATTENUATES POLYMICROBIAL INTERACTIONS AND DISRUPTS BIOFILM FORMATION BY CARIOGENIC MICROBES – AN *IN-VITRO* STUDY

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ABSTRACT

Objectives: Polymicrobial interactions among oral microflora accelerate biofilm mediated oro-dental infections. Phytocomponents are gaining pre eminence in dental therapeutics owing to their multi factorial activity. *Glycyrrhiza glabra* (GG) due to abundant phytochemicals shows excellent bioactivity. Current study aims to evaluate the anticariogenic activity of ethanolic extract of *Glycyrrhiza glabra* and to elucidate possible mechanisms of action.

Materials and methods: *Glycrrhiza glabra* was subjected to cold extraction and phytochemical analysis was performed. Extract was screened for its antimicrobial activity by well diffusion method and MIC by twofold serial dilution method. Extract was checked for its anti-adhesion, anti-biofilm activity by anti-adherence assay and crystal violet staining method respectively. Inhibitory effects of *Glycyrrhiza glabra* against extra cellular polysaccharide (EPS) formation and amylase enzyme activity were determined spectrophotometricaly. Polymicrobial interaction studies among the selected cariogens (*Streptococcus mutans, Lactobacillus acidophilus, Enterococcus faecalis and Candida albicans*) were assessed individually and in combination by co-culture methods. All experiments were performed in triplicates and expressed as mean +/- standard deviation.

Results: *Glycyrrhiza glabra* extract showed potent antimicrobial activity and effectively reduced adhesion of *S. mutans*. Extract exhibited increased biofilm depletion and inhibited biofilm formation. Antibiofilm response was mediated directly by extra cellular polysaccharide inhibition and indirectly by inhibiting alpha-amylase activity. *Glycyrrhiza glabra* also reduced polymicrobial interactions among coaggregating microbes in the biofilms and was more effective than the conventional irrigant.

Conclusions: *Glycyrhiza glabra* can be ideal as therapeutic agent in biofilm mediated oro-dental infections due to its multiple modes of actions against cariogens.

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INTRODUCTION

Dental caries is one of the major microbial derived oral health problems and affects mankind from all age groups globally. Oral micro-flora, includes more than 750 species, of which several cariogenic organisms have been identified (Jenkinson and Lamont, 2005). Dental biofilm and plaque have been considered as major etiologic factors in the initiation and progression of dental caries. Involvement of acidogenic and aciduric Gram positive bacterial species like *Streptococcus mutans, Lactobacillus sp*, and *Actinomycetes sp.,* has been well established in caries formation (Chenicheri *et al*, 2017). Cariogenic microbes metabolize the fermentable dietary carbohydrate residues in oral cavity. The acidic metabolites of such microbes, induce decalcification and dissolution of calcium phosphate from the teeth leading to continuous demineralization and tooth decay (Loesche, 2007). Moreover, inflammatory responses and associated collagen degradation aggravate the situation and ultimately end up in loosening the supportive structures of tooth. Both intra and inter species interactions play a crucial role in community dynamics and contribute to plaque formation, leading to polymicrobial diseases like caries and periodontitis (Wen *et al*, 2010). Polymicrobial biofilm formation begins with adhesion of initial colonizers followed by sequential aggregation by secondary colonizers and tertiary colonizers (Peters *et al*, 2012). Process of co-aggregation, facilitates communication and interaction between and/among the micro biomes in the biofilm (Huang *et al*, 2011). Strategies that interferes and/disrupts these microbial interactions among the biofilm communities can pave way for a new treatment regime and thereby prevention of oral diseases.

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Anti-cariogenic agents that arrest the formation and progression of caries are well appreciated. Still, the quest for an effective agent continues due to the side effects of conventional therapeutics. Fluoride (popular cariostatic agent) and chlorhexidine (routine anti-plaque agent) are being used by dentists all over the globe. Toxicity and fluorosis associated with the former and genotoxicity associated with the later limits their long term and excessive applications (Hasan *et al*, 2015). Furthermore, the development of resistance by pathogenic bacteria to current antibiotic regimen (penicillins, cephalosporins, erythromycin and tetracycline) is another hurdle (Palombo, 2011). Prolonged use of these chemicals also alters the normal oral micro biota and cause undesirable effects like vomiting, irritation and local ulcerations (Chung *et al*, 2006; Park *et al, 2003)*. An alternative anti-cariogenic agent with minimal side effects is still wanted.

Owing to the diversity and abundance of bioactive molecules, medicinal plants have been hailed as a major source for therapeutics. More than 70% of the therapeutic agents developed during past four decades were based on plant sources (Newman and Cragg, 2007). Certain drugs like quinine, chloroquine, mefloquine and artemisinin (anti-malarial) (Onguene *et al*, 2013), camptothecin, paclitaxel, podophyllotoxin and vinblastine (anticancer) (Efferth *et al*, 2008), acarbose, miglitol and voglibose (anti-diabetic) (Bedekar *et al*, 2010), statins (hypolilidemic) (Heber *et al*, 1999), coumarol (anti-cloting) and reserpine and ruinine (psychiatric)(Taur and Patil, 2011) are well established phyto-based therapeutic agent in current scenario. In addition, phytomedicine has gained significance in health care and therapeutics as it is cheaper, safer and easily available (*Wilt et al, 2000).* Still, attempts of using such phyto-products in dentistry are minimal when compared with other medical applications (Jeon *et al*, 2011).

Glycyrrhiza glabra L. (GG) (Licorice) has been considered as one of the oldest and most common herbal medicine for respiratory, cardiovascular, gastrointestinal and skin deformities. *Glycyrrhiza glabra* is well known for antioxidant and anti-inflammatory potential (Kaur *et al*, 2012) and has been approved by Food and Drug Administration (FDA) and has given GRAS (Generally Regarded As Safe) status (Isbrucker and Burdock, 2006). Even though *Glycyrrhiza glabra* bears abundant bioactive components, the majority of its activity relies on flavonoids and saponins like isoliquiritigenin, glycyrrhizin, and glabridin (Kamei *et al*, 2003) .

Based on this background, the current study aims in evaluation of ethanolic crude extracts of *Glycyrrhiza glabra* against cariogenic organisms like *Streptococcus mutans, Lactobacillus acidophilus, Candida albicans* and endodontic bacteria *Enterococcus faecalis* followed by elucidation of its possible mechanisms for the prevention of biofilm formation.

MATERIALS AND METHODS

Preparation of Glycyrrhiza glabra ethanolic crude extract

Glycyrrhiza glabra stem were collected from the Herbal medicine vendors in Thiruvananthapuram, India and washed in tap water, shade dried and powdered. 100 g powder was subjected to cold extraction in 70% ethanol for 72 h. The extracts were recovered by filtration and concentrated under pressure. The extract was dissolved in sterile distilled water (200mg/ml), filter sterilized and stored in amber colored containers under aseptic condition for further studies.

Phytochemical analysis

Presence of major phytochemical compounds like alkaloids, flavonoids, phenols, steroids and glycosides in the extracts (100mg/ml) were evaluated using previously reported protocols (Singh *et al*, 2012; Ali Hassan and Abu Bakar, 2013) (Table 1).

Microbicidal effect of Glycyrrhiza glabra against cariogens Microbial culture and maintenance

Bacterial strains, *Streptococcus mutans* (MTCC 890), *Lactobacillus acidophilus* (MTCC 10307), *Enterococcus faecalis* (MTCC 1059), and *Candida albicans* (MTCC 227) were procured from MTCC, Chandigarh, India. Lyophilized cultures were revived by suspending them in BHI (Brain Heart Infusion) broth followed by subculturing them on Mitis Salivarius Agar (MSA) for *E. faecalis*, Trypticase Soy Agar (TSA) for *S. mutans*, de Man, Rogosa Sharpe medium (MRS) for *Lactobacillus acidophilus* and Potato Dextrose Agar (PDA) for *Candida albicans.* The organisms except *Candida albicans* were incubated in candle jar at 37^oC for 24h to 48h (Nelson-Filho *et al*, 2013).

Antimicrobial activity

The extract was checked for its antibacterial activity against selected oral pathogens, *Streptococcus mutans, Lactobacillus acidophilus*, *Enterococcus faecalis* on Muller Hinton Agar (MHA) and *Candida albicans* on PDA (Potato dextrose Agar) plates respectively, by agar well diffusion method. Plates were swabbed with over night grown cultures of test organisms to obtain a lawn culture and 25µl, 50µl and 100µl of the extract from a stock solution of 100mg/ml were added in each well, which corresponds to 2.5mg, 5mg and 10mg respectively. Gentamycin (10µl from 10mg/ml stock) was used as a positive control for bacteria and Clotrimazole (10µl from 10mg/ml stock) for *Candida albicans*. Zone of inhibition of the test organisms was measured in millimetres (Bonev *et al*, 2008) (Table 2).

Minimal inhibitory concentration (MIC)

MIC was determined by agar well diffusion method (as mentioned above) and tube dilution method. In tube dilution method the extract was serially diluted to a concentration of 50, 100, 250, 500 and 1000µg/ml in BHI broth and organism was inoculated as per 0.5 OD Mc Farland standards and incubated overnight at 37^oC. Optical density of the culture was measured using a spectrophotometer (Agilent Cary 60) at 600nm. MIC of extract is the lowest concentration showing no turbidity in culture broth. All the tests were done in triplicates and results were expressed as mean average with SD and significance was accepted at $p< 0.05$(Wiegand *et al*, 2008).

Anti-cariogenic effects of Glycyrrhiza glabra

Anti-adherence assay

The test organisms were grown in BHI broth supplemented with 5% sucrose in 5ml screw capped glass tubes. 25µl, 50µl and 100µl GG extract was added (from 100mg/ml stock) to the culture and were grown for 24h with tubes inclined at 45°. The adhered cells were scored from +1 to +4 depending on visual observation after removing the supernatant medium. 2ml 0.5N NaOH was added to the adhered cells, vortexed, spun at

2000rpm and OD was read at 600nm against supernatant media (Islam *et al, 2008)*. Percentage adherence was calculated using the below relation;

$$\% \text{ Adherence } = \frac{\text{OD adhered cells}}{\text{OD of adhered cells} - \text{ OD of supernatent}} X\,100$$

All the experiments were done in triplicates and results were expressed as mean average with SD and significance was accepted at $p < 0.05$

Anti-Biofilm assay

Anti-biofilm efficiency of Glycyrrhiza extract was determined by biofilm inhibition assay and biofilm depletion assay, with regular media replenishment on every 24h. In biofilm depletion assay, 7 days aged biofilm in culture was treated with 0.5mg/ml extract and was grown for another 48 h in a 24 well culture plate. For biofilm inhibition assay, the above concentration of extract was added to 24 h culture and allowed to secrete constituent biofilm for another 7 days. The biofilm retained after GG treatment was quantified and compared by crystal violet assay in both cases(O'Toole, 2011). The culture medium was removed and detached cells were washed with sterile distilled water for three times and 300 µl, crystal violet in 0.5% ethanol was added to the wells and was incubated at room temperature for 30 mins. The excess stain was removed by extensive washing with distilled water and the stain retained in biofilm was dissolved in 500 µl DMSO. Absorbance of the solution was read at 600 nm in a UV double beam spectrophotometer (Agilent Cary 60, USA). A control without GG treatment and a standard treated with 50µl/ml 5% hypochlorite was also treated as above for all the four organisms. The results were expressed as percentage inhibition. All the experiments were repeated 3 times independently and results were expressed as mean average with SD and significance was accepted at $p < 0.05$

Extracellular Polysaccharide (EPS) - anti-biofilm response
EPS inhibitory effect

Extracellular polysaccharide constitutes the basic unit of biofilm matrix. EPS in biofilm was determined by phenol sulphuric acid method (Darveau and Hancock, 1983). EPS on the surface of culture plate was quantified by growing test organisms individually in BHI broth for 10 days in 24 well culture plates. Extract (2.5, 5 and 10mg/ml) was then added and incubated for another 3 days. The culture medium was removed, washed in PBS and treated with 0.5ml 5% phenol (in 0.1N HCl), followed by 2.5ml conc. H_2SO_4 at room temperature. OD was read at 490nm after10 mints and % inhibition was calculated. A control without extract and a blank without organisms were also maintained. All the experiments were repeated 3 times independently and results were expressed as mean average with SD and significance was accepted at $p < 0.05$

Amylase inhibition assay

As alpha amylase activity contributes to biofilm formation, effect of *Glycyrrhiza glabra* to inhibit amylase was evaluated by 3, 5-dinitrosalicylic acid method (Oboh *et al*,2012). 1ml porcine α-amylase (in 25mM phosphate buffer, pH 6.9) was added to the extract (2.5, 5 and 10mg/ml) and incubated for 10min at room temperature. After incubation 500µl, 0.5% starch solution was added and incubated for 10min. 500µl, 96mM 3,5-dinitrosalicylic acid was added, boiled for 5min, cooled to room temperature and OD was read at 540nm. The results were represented as % inhibition with respect to OD values. A control without extract and a reagent blank without the enzyme were also maintained.

Glycyrrhiza glabra attenuated polymicrobial interactions
Co-culture viability

Monoculture of test organisms and combination cultures comprising dual, triple and quadruple microbes per batch were maintained in BHI. Microbial inoculums were adjusted to OD of 0.5 Mc Farland standards. Inoculum for combination culture was prepared by dilution with equal volumes of individual inoculum also of 0.5 Mc Farland standard OD. 100µl test inoculum was seeded on to 5ml screw cap tubes made up to 1ml and incubated for 48hrs. 50µl extract (100mg/ml stock) was added and incubated for 48 hrs. To the cell pellets, 40µl MTT solution (5mg/ml stock in PBS) was added and incubated at 37°C for 30min by keeping the tubes open. After incubation 300µl DMSO was added, spun at 1500rpm for 5min and OD was read at 540nm. Viability of monocultures and co-cultures were expressed as relative variations in OD units and correlated to viability of microbes after treatment with respect to corresponding controls constituted by the preceding combinations (Hengwei Wang, 2010; Finosh and Jayabalan, 2015). The experiment was repeated 4 times independently and results were expressed as mean average with SD and significance was accepted at $p < 0.05$.

Co-aggregation mediated biofilms

Combinations cultures as mentioned above were prepared and grown for 24hrs. 50µl *Glycyrrhiza glabra* extracts (from 100mg/ml stock) was added and continued up to 7 days with media supplementation. Biofilm inhibition was determined by crystal violet assay as mentioned above and compared with respect to appropriate control combinations. Experiment was repeated 4 times independently and results were expressed as mean average with SD and significance was accepted at $p < 0.05$

Statistical analysis

All experiments contained three or four replicates from each group. The values were represented as mean ± standard deviation. Statistical analysis was done with one way ANOVA using online calculator, statistics calculator version 3 beta. $P < 0.05$ was accepted as level of significance for all calculations (Finosh *et al*, 2015; Montelongo-Jauregui *et al*, 2016).

RESULTS

Extraction and phytochemical analysis

Glycyrrhiza glabra extract obtained by cold extraction was dried, filtered and stored aseptically for further studies (100mg/ml). Yield of extract was found to be 14%. Phytochemical analysis confirmed the presence of phenols, terpenoids, flavonoids and steroids, which may contribute to proposed activities (Table 1).

Table 1 Phytochemical analysis of *Glycyrrhiza glabra*

Parameters	Results
Alkaloids	-
Phenols	++
Glycosides	-
Terpenoids	+++
Flavanoids	++
Saponins	-
Steroids	++
Tanins	-

Anti-Cariogenic Activities of Glycyrrhiza Glabra

Antimicrobial Activity

Ethanolic extract of *Glycyrrhiza glabra* showed distinguishable anti-microbial activity against the selected oral pathogens as done by agar well diffusion method. Among the four oral pathogens studied, the extract showed excellent antimicrobial activity for *S. mutans* followed by *Lactobacillus acidophilus and E. faecalis* and a comparable activity for *Candida albicans*.

The zone of inhibition at different concentrations is displayed in Table 2. Results revealed that MIC value for *S. mutans* was 0.25mg/ml. MIC for *Lactobacillus acidophilus and E. fa*ecalis was 0.5mg/ml and that of *Candida albicans* was1mg/ml (Table 2).

Table 2 Antimicrobial effects of *Glycyrrhiza glabra*

S. mutans (n=3, P<0.001)		
Concentration (mg)	**Zone (mm)**	**MIC (mg/ml)**
2.5	26 ± 1	
5	28.67 ± 1.5	0.25
10	29.33 ± 2.3	
Gentamycin (10 µg)	38 ± 1	
L. acidophilis (n=3, P<0.001)		
Concentration (mg)	**Zone (mm)**	**MIC (mg/ml)**
2.5	14.33 ± 0.58	
5	18.33 ± 2.08	0.5
10	20.67 ± 1.15	
Gentamycin (10 µg)	27.67 ± 1.53	
E. faecalis (n=3, P<0.01)		
Concentration (mg)	**Zone (mm)**	**MIC (mg/ml)**
2.5	18 ± 2.65	
5	21 ± 2	0.5
10	23.33 ± 1.15	
Gentamycin (10 µg)	28 ± 0	
C. albicans (n=3, P<0.001)		
Concentration (mg)	**Zone (mm)**	**MIC (mg/ml)**
2.5	0 ± 0	
5	13 ± 0.57	1
10	17.77 ± 1.15	
Clotrimazole (10 µg)	26.67 ± 1.53	

Anti-cariogenic response

Anti-cariogenic effect of *Glycyrrhiza glabra* extract was assessed by prevention of microbial adhesion and inhibition of biofilm formation. GG extract effectively reduced the adhesion of *S. mutans* and *Candida albicans* (Table 3, Fig. 2). This effect was prominent in *S. mutans* at a concentration of 2.5mg/ml and 10mg/ml in *Candida albicans* (Fig. 2). *Lactobacillus acidophilus and E. faecalis* showed comparatively higher adhesion to the substratum (Fig. 2).

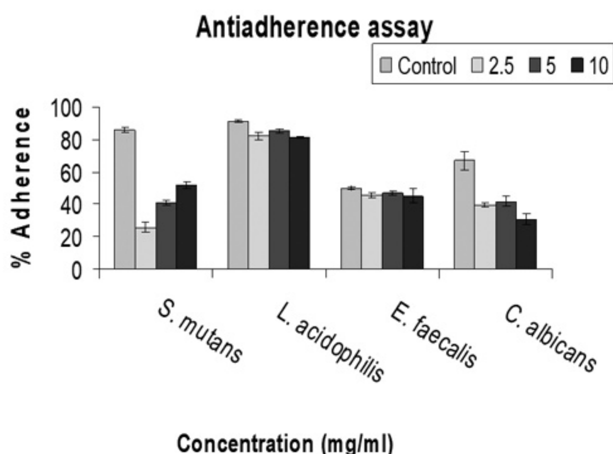


Fig. 2: Antiadherence effects of GG extract determined by Tube inclination method showing prevention of adhesion of cariogenic microbes to the substratum. Along X axis – microorganisms used for the study, along Y axis % adherence. GG exhibited maximum inhibition of adhesion to *S mutans* and *C albicans*. Extracts were added in different volumes such as 25, 50 and 100 µl from a stock of 100 mg.ml-1.All the experiments were done in triplicates and results were expressed as mean average with SD and significance was accepted at $p < 0.05$

Table 3 Anti-adherence effects of *Glycyrrhiza glabra* against the cariogenic microbes

Organisms	Scoring
Streptococcus mutans	+++
Lactobacillusacidophilus	-
Enterococcus faecalis	-
Candida albicans	++

Furthermore, extract showed increased biofilm depletion and inhibited biofilm formation more efficiently than sodium hypochlorite. Results showed that lower concentrations of extract were effective against *S. mutans* in depleting preformed biofilms. But concentrations of 5 and 10mg/ml showed a reverse trend. *E. faecalis* also showed a drop to about 30% in biofilm formation and depletion at a concentration of 5mg/ml and increased the same at a concentration of 10mg/ml (Fig. 3). Extract also exhibited better anti-biofilm activity than sodium hypochlorite for *Candida albicans* and *Lactobacillus acidophilus* (Fig. 3).

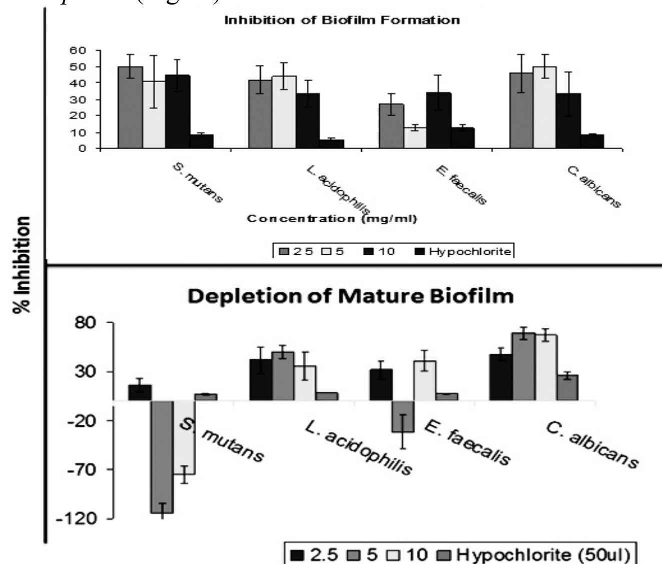


Fig. 3: Anti-biofilm effects of *GG* extract showing the prevention of biofilm formation and depletion of preformed biofilm. All experiments were done in triplicates and results were expressed as mean average with SD and significance was accepted at $p < 0.05$

EPS – anti-biofilm response

EPS assay

EPS constitutes major building blocks for biofilm matrix. The ability of *Glycyrrhiza glabra* extract to inhibit EPS formation was found to be concentration dependent for all the test organisms with a highest percentage of inhibition at a concentration of 2.5 $mg.ml^{-1}$. Also effect of *Glycyrrhiza glabra* was highly appreciable than conventional irrigant hypochlorite (Fig. 4a).

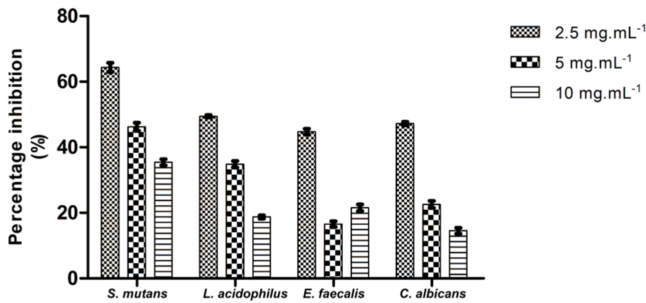


Fig. 4 a: EPS inhibition assay depicting inhibition of extra polysaccarhide synthesis of cariogenic bacteria upon treatment with GG extracts, Along X axis – microorganisms used for the study, along Y axis- Percentage inhibition in EPS production. Microorganisms were treated with different volumes such as 25, 50 and 100 µl from a stock of 100 mg.ml-1 GG extracts. All experiments were done in triplicates and results were expressed as mean average with SD and significance was accepted at $p< 0.05$

Amylase inhibition

Effect of *Glycyrrhiza glabra* to inhibit amylase enzyme, by spectrophotometric method, showed, the lower concentrations (2mg/ml) of *GG* extract have higher percentage inhibition of amylase enzyme followed by 5mg/ml and 10mg/ml (Fig. 4b).

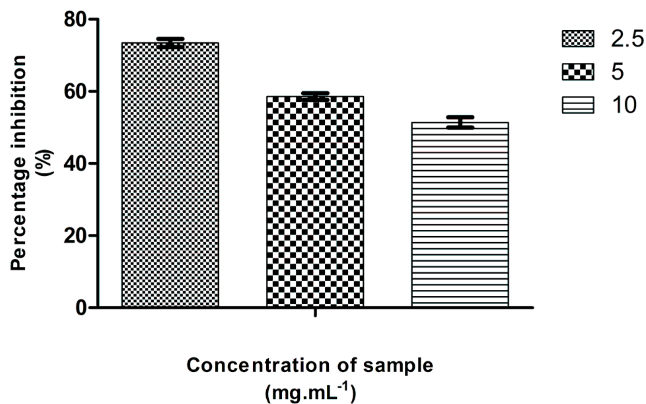


Fig. 4 b: Amylase inhibitory effects of GG extracts- Along X axis concentration of extracts 2.5, 5, 10 mg.ml-1, along Y axis – percentage inhibition of amylase activity.All experiments were done in triplicates and results were expressed as mean average with SD and significance was accepted at $p< 0.05$

Polymicrobial interaction attenuation by Glycyrrhiza glabra extract

Polymicrobial interactions among the test organisms in monoculture and coculture with respect to viability and biofilm formation, were quantified. *Glycyrrhiza glabra* extract significantly reduced the viability of co-cultured organisms, quantified by spectrophotometric MTT assay and expressed in terms of optical density (OD). On comparing monocultures treated with hypochlorite and GG extract, a significant reduction in microbial viability was observed in GG extract treated cultures(Fig. 5a). OD value was significantly ($p<0.01$) reduced by GG extract treatment of dual culture of *Lactobacillus acidophilus* and *Enterococcus faecalis* (LE) and also reduced ($p<0.05$) the growth of coculture of dual cultures namely, *Streptococcus mutans* and *Lactobacillus acidophilus* (SL), *Streptococcus mutans* and *E. faecalis* (SE), *Lactobacillus acidophilus* and *Candida albicans* (LC), *Candida albicans* and *Enterococcus faecalis* (CE)(Fig. 5b). Significant reduction in viability ($p<0.5$) was noted in coculture of triple microbes viz *S. mutans, L. acidophilus* and *E. faecalis* (SLE) and *L. acidophilus*, *E. faecalis* and *C. albicans* (LEC) upon GG extract treatment when compared with the commonly used irrigant sodium hypochlorite. GG extract treatment significantly reduced ($p<0.5$) the growth of co-culture of quadruple microorganisms (SLEC), than hypochlorite treatment.

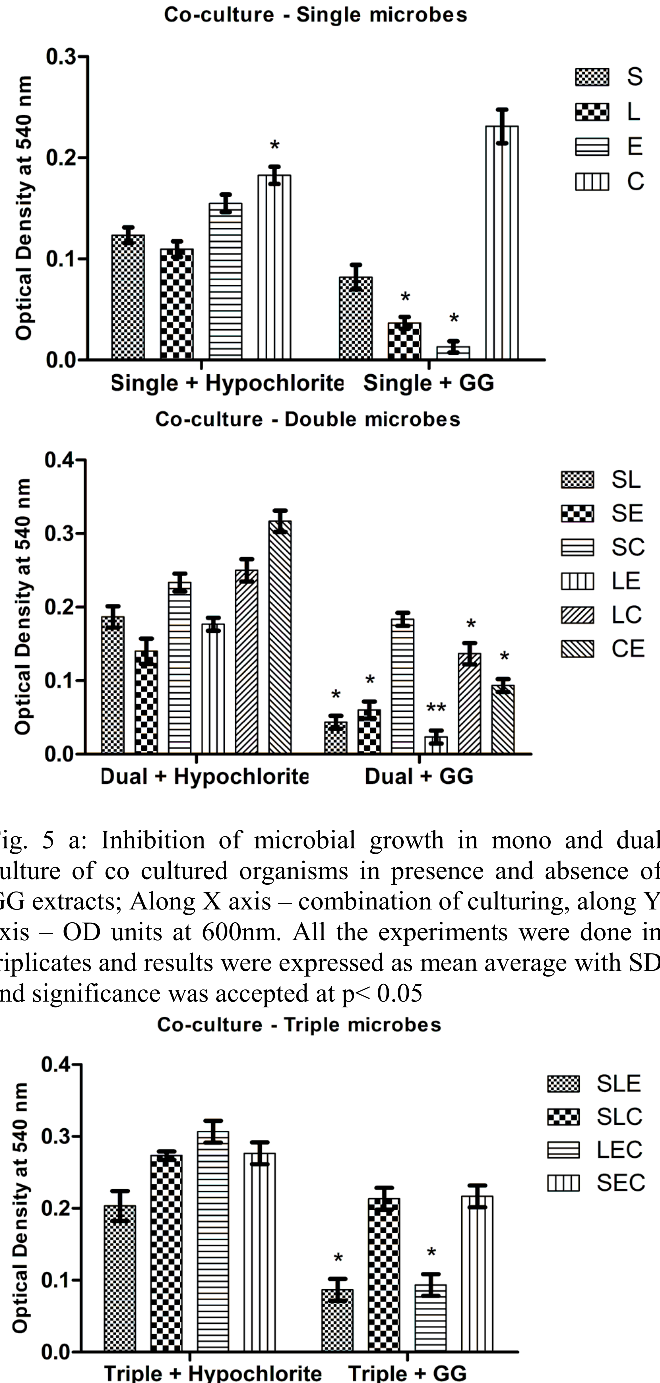


Fig. 5 a: Inhibition of microbial growth in mono and dual culture of co cultured organisms in presence and absence of GG extracts; Along X axis – combination of culturing, along Y axis – OD units at 600nm. All the experiments were done in triplicates and results were expressed as mean average with SD and significance was accepted at $p< 0.05$

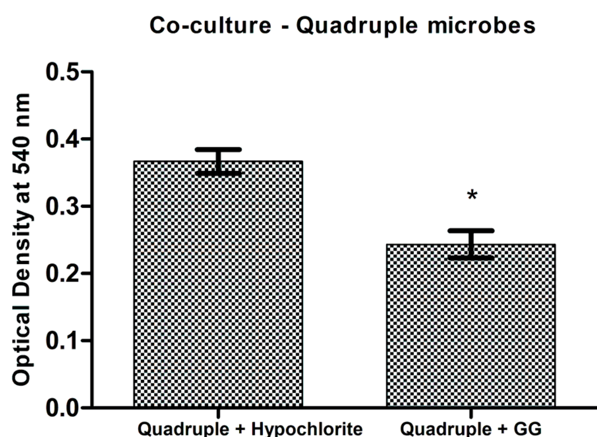
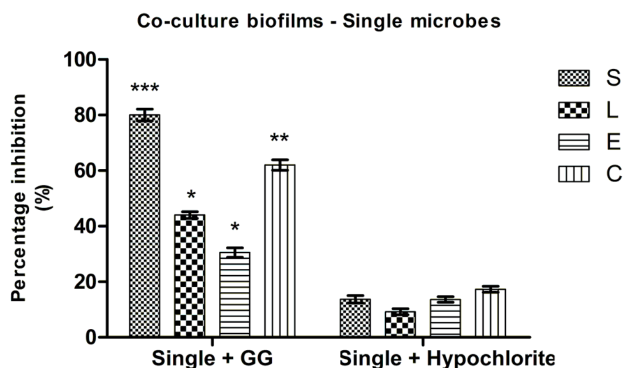
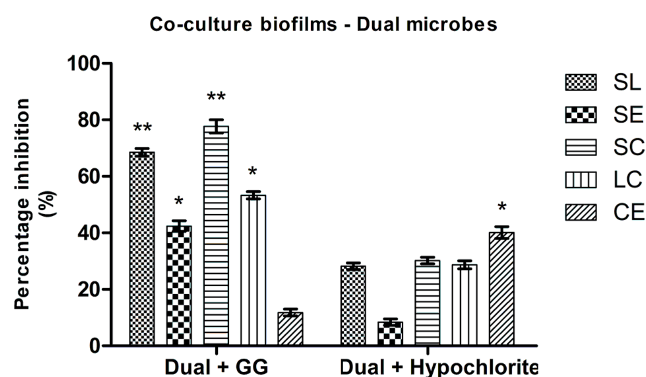


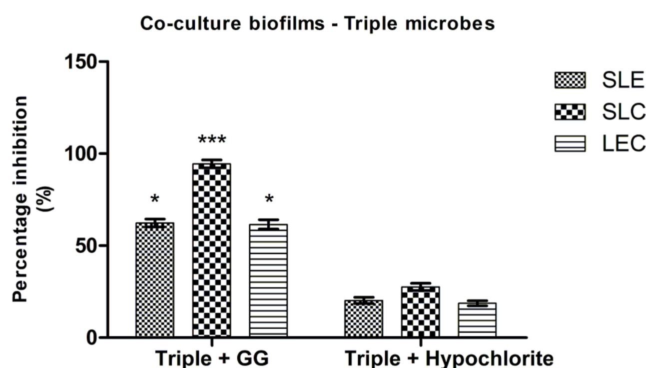
Fig. 5b: Inhibition of microbial growth in triple and quadruple cultures of microorganisms in presence of GG extracts. Along X axis – combination of culturing, along Y axis – OD units at 600nm. All experiments were done in triplicates and results were expressed as mean average with SD and significance was accepted at $p < 0.05$

Ability of the extract to prevent polymicrobial colonization and biofilm formations was also compared. In monocultures a comparative analysis was performed between hypochlorite treated organisms and GG treated test organisms. When compared with hypochlorite treated group, GG extract produced a significant increase ($p < 0.001$) in % biofilm inhibition, which was similar in *Candida albicans* group. GG extract treatment produced significant inhibition ($p<0.01$) of biofilm formation in *Lactobacillus acidophilus* and *E. faecalis* when compared with hypochlorite treatment (Fig .6a).

In case of dual culture MOs, GG treatment effectively inhibited biofilm formation ($p <0.01$) in the dual culture of *Lactobacillus acidophilus* with *S. mutans(SL)* and *Candida albicans* with *S. mutans (SC)*. Hypochlorite treatment was more effective when compared with GG extract treatment in groups having *Candida albicans* and *Enterococcus feacalis* (Fig. 6b). In all triple cultures GG treatment significantly inhibited ($p<0.001$) biofilm formation on experimental surface when compared with hypochlorite treatment (Fig. 6c). Quadruple culture of microorganism was significantly inhibited ($p< 0.001$) by GG treatment when compared with hypochlorite treatment (Fig. 6d).







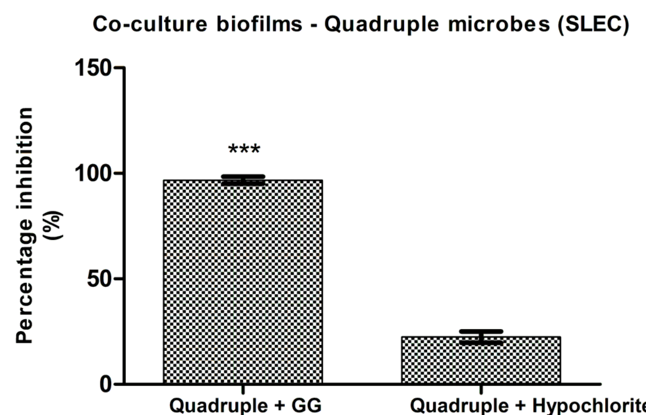


Fig. 6a, 6b,6c, 6d. The effect of *Glycyrrhiza glabra* extract in preventing polymicrobial biofilm among the individual and cocultured cariogens. All the experiments were done in triplicates and results were expressed as mean average with SD and significance was accepted at $p< 0.05$

DISCUSSION

Numerous studies have focused on antimicrobial activity of phytochemicals and phytoextracts against cariogenic bacteria. This owes to their negligible toxicity and preventing the emergence of drug resistant bacterial strains towards the modern pharmaceuticals. This study distinctively emphasises on systematic elucidation of mechanisms of anticariogenic activity of ethanolic extract of *Glycyrrhiza glabra* and its efficiency to arrest the progression of cariogenic and endodontic microbes like *Streptococcus mutans, Lactobacillus acidophilus, Candida albicans* and *Enterococcus faecalis* by virtue of its anti-cariogenic and anti-biofilm activity.

Various reports have shown that *Glycyrrhiza glabra* extracted in different solvent systems like ethyl acetate, n-Hexane, dichloromethane, ethanol & methanol possess appreciable outcomes against both Gram positive and Gram negative bacteria and also against fungi in higher concentrations

(Fatima *et al*, 2009; Ajagannanavar *et al*, 2014; Gaitry chopra, 2013). The use of crude ethanolic extract is advantageous as it overcomes the difficulty in purification /quantification process, ease of preparation, low cost and availability. Also, it avoids toxicity (acute and chronic) of the purified compound, which can overweigh the disadvantage of their use in higher concentrations. Hence we employed ethanolic extract of *Glycyrrhiza glabra* for further studies.

Incorporation of *Glycyrrhiza glabra* in dental practice is more advantageous due to the cumulative effects of its bioactivities like antibacterial, antifungal, anti-inflammatory, antioxidant and analgesic (Palombo, 2011). It has been postulated that pretreatment of oral cavity surfaces with the plant extracts creates an unfavorable film on the surfaces, which prevents microbial colonization. Snadasi et al has reported that plant extracts inhibit cell attachment and thereby reduce microbial colonization on surfaces and epithelial mucosa. The presence of terpenoids, phenols, flavonoids, steroids and saponins in our *Glycyrrhiza glabra* extracts suggests, antibacterial activity could be due to any of these mechanisms, even though the lesser content of saponins was obtained in this study. Still, the actual mechanism is not yet unveiled which requires further investigations. We focused to explain the possible anti-cariogenic mechanisms with respect to biofilm inhibition and antimicrobial activity of *G. glabra.*

Prevention of microbial adhesion, biofilm formation and destruction of pre-formed biofilms are the major concern to prevent carries progression (Pita *et al*, 2015). He et al., has showed that the inhibition of growth in a preformed biofilm as a successful approach. But, better rate of inhibition occurs in preventing the initial attachment (He *et al*, 2005). We believe that by choosing a polar solvent medium for the extraction, most of the hydrophilic components of *Glycyrrhiza glabra* are preserved and simultaneously, layer of unfavorable film formed by the extract on the oral surfaces prevents microbial adhesion. This may contribute for the anti-adherence property there by reducing the biofilm formation. More physiochemical and biological evaluations of both extract and the adhering surface are recommended for validation of this aspect. Moreover, many authors have reported that the phytochemicals particularly polyphenols have anti-adhesive properties against *Streptococcus mutans* and other oral microbes (Xiao *et al*, 2007; Furiga *et al*, 2008).

Dental plaque is a specialized bacterial biofilm and its higher resistance to antimicrobial agent and host immune mechanism is due to its extracellular polymeric matrix. Extracellular polymeric matrix of the biofilm hinders permeability of drugs and enhances the survival of microbial cells in the biofilm. Hence effective elimination of bacterial biofilm in the oral cavity and disruption of pre formed biofilms in the dentinal tubules are still a major hurdle in oral health scenario (Shrestha *et al*, 2010). Thickness of the biofilm also contributes to the impermeability of drug into the plaque biofilm consortium (Hoby *et al*, 2010). In this study, biofilm was quantified by growing test organisms in the well plate for 7 days with media replenishment after every 24hrs. Novelty of this study is, we investigated the anti-biofilm activity of *Glycyrrhiza glabra* extract by determining the depletion of preformed biofilm and also by the inhibition of biofilm formation. *Glycyrrhiza* extract showed excellent anti-biofilm activity against the studied oral pathogens at lower concentrations and appreciably higher than the commonly used irrigant in dentistry, sodium hypochlorite. Anti-biofilm mode of microbial removal and destruction will also limit the development of drug resistant mutans by preventing the selective mechanism by bacterial strains to suppress susceptibility in presence of the drug.

EPS components mediate most of the cell-to-cell and cell-to-surface interactions that are inevitable for the formation and stabilization of biofilm communities. On treatment with *Glycyrrhiza glabra* extract, the percentage EPS inhibition was highest for *Streptococcus mutans* at the lowest concentration of 2.5mg/ml, which further decreases at higher concentrations of 5 and 10mg/ml respectively. *Enterococcus faecalis* showed a slight lowering of percentage inhibition at 5mg/ml followed by increased inhibition at 10mg/ml. The standard irrigant sodium hypochlorite (5%) as control, showed negligible percentage inhibition of EPS compared to phyto extract (**Fig.** 4). This could be attributed to the bactericidal activity of sodium hypochlorite (Mohammadi, 2008) rather than inhibition of EPS formation in biofilm matrix. Hence dissociation of EPS moieties by *Glycyrrhiza* extract reduces load of microbial cells in the biofilm matrix, thereby lowering microbial adhesion. It also enhances penetration and buffering activity of saliva. It also favors drug penetration into the biofilm matrix.

Anti-biofilm activity of *Glycyrrhiza glabra* can be indirectly facilitated by limiting the EPS availability via reducing the metabolic flux by inhibition of associated enzymes. Alpha-amylase in saliva hydrolyzes the residual dietary starch to provide additional glucose/sugar for metabolism by plaque microorganisms in the immediate vicinity of tooth surface. The resulting lactic acid produced may be added to the pool of acids in plaque and enhances tooth demineralization (Scannapieco *et al*, 1993). Scannapieco et al., has reviewed and pointed out a positive correlation between dental caries and high salivary amylase activity (Scannapieco *et al*, 1993). Actual mechanism of action of *G. glabra* on alpha amylase requires further investigations. From these results it is vivid that *G. glabra* extracts has a dual role to execute biofilm inhibition, directly by EPS inhibition and indirectly by curbing the monomer availability.

It has been estimated that in an adult individual, 1 gram of mature dental plaque may host around 10^9 bacterial cells from more than 200 species. Their successful co-existence is attributed to various physical, chemical and biological interactions between the individual species through various synergistic and antagonistic interspecies relationships(James, 2014). These relationships are driven by co-aggregation, cell-cell communication via quorum-sensing, colonization, virulence, immunomodulation or combinations of these events (Peters *et al*, 2012). Hence, the assessment of co-culture viability and co-culture biofilm formation can throw light on polymicrobial interactions and their behavior in oral biofilms. In this study increase in OD of cocultures with respect to monoculture confirms the co-existence of all the studied organisms. Spectrophotometric MTT assay was employed for quantification of microbial viability and was expressed in terms of optical density (OD). GG extract significantly reduced the viability of individual test organisms as well as coculture dual, triple and quadruple microbes when compared with hypochlorite treatment. This further supports the antimicrobial property of ethanolic extract of *G. glabra* against the studied oral pathogens. The same principle was exploited for co-culture biofilm assay. *Glycyrrhiza glabra* extract

treatment on co-cultured biofilm showed increased percentage inhibition compared to sodium hypochlorite as control. Activity on preformed biofilms shows *Glycyrrhiza glabra* to be more effective than conventional irrigant hypochlorite. Montelongo-Jauregui D et al., 2016 reported that compared to single species biofilms, higher level of resistance to antimicrobial treatment is displayed by mixed species biofilms. Ethanolic extract of *G. glabra* efficiently reduced the viability of coculture microbes and coculture biofilms than the conventional irrigant sodium hypochlorite, suggesting that the extract has antimicrobial as well as antibiofilm activity.

This effect was very much prominent in co-cultures with *Streptococcus mutans* combinations with *Candida albicans, Lactobacillus acidophilus* and *E. faecalis*. Similar trend was observed in co-culture biofilm inhibition in the presence of *Glycyrrhiza* extract. The adhesive interaction between *Candida albicans* and *Streptococcus mutans* is directly proportional to presence of sucrose in the oral cavity(Metwalli *et al*, 2013) . GG extract efficiently reduced the growth of co-culture of quadruple microorganisms, mimicking major initial oral biofilm population, than hypochlorite treatment. *In vitro* and *in vivo* studies have demonstrated that *C. albicans* also adheres to the major microbial constituents of early dental plaque such as *Streptococci* and *Actinomyces naeslundii*, as well as to late colonizers such as *Fusobacterium nucleatum* (Metwalli *et al*, 2013). Filoche et al., reported that *S. mutans* promoted the substantial biofilm growth of *Lactobacillus* species (Aas *et al*, 2005; Filoche and Anderson, 2004). Direct proportional relationship was reported between *Candida albicans* and *Lactobacillus* species on co-cultured biofilm as well (Tony Jose *et al*, 2014). But, the dual-species biofilm studies conducted by Fernández et al revealed minimal effects of *Lactobacillus* sp. on *S. mutans* cariogenicity without considerable changes in acidogenicity (Constanza E Fernandez, 2015). Deng et al., demonstrated that the presence of a *Streptococcal*-biofilm on hydroxyapatite significantly increases the biofilm formation of *E. faecalis* and can be strongly influenced by other species that are present in the root canal (Deng *et al*, 2009).

We are lacking sufficient literatures dealing with the mechanism of action of caries prevention by *Glycyrrhiza glabra* extracts as do for other extracts (Chaiya *et al*,) Current study throws light on various properties of *Glycyrrhiza glabra* extract, which explains and appreciates its bioactivities on cariogenic microbes. Antimicrobial activity enhances anti-biofilm effect (inhibition of biofilm formation and disruption of preformed biofilm) which explains versatile modes of operation *of Glycyrrhiza glabra*. Anti-adhesion activity of *Glycyrrhiza glabra* extract prevents microbial adherence on tooth surface; hindering the initial step in biofilm formation. Amylase inhibition activity and EPS dissolution activity of *Glycyrrhiza glabra* also augments the removal of cariogenic bacteria from oral cavity. Selection of ethanolic crude extracts can be appreciated for the abundance of phytochemical types and cumulative effects of these facilitates the prevention of caries progression. Non-toxic and lesser side effects of *Glycyrrhiza glabra* can be exploited for its safer use even in higher concentrations. Validating and identifying quorum sensing proteins and effect of the extract on expression of genes regulating quorum sensing process can aid in development of *Glycyrrhiza glabra* as a potent inhibitor of quorum sensing.

CONCLUSIONS

Ethanolic extract of *Glycyrrhiza glabra* showed excellent antimicrobial activity against the studied cariogenic microbes. The extract effectively inhibited adhesion of primary colonizing bacteria like *S. mutans* and inhibited biofilm formation by disrupting extracellular polymeric matrix in the biofilms and also by inhibiting alpha amylases. Extract was also efficient in disassembling pre formed biofilms. *Glycyrrhiza glabra* extract also reduced polymicrobial interactions among the biofilm communities. Hence, *Glycyrhiza glabra* extract can be projected as one of the best candidate for treatment of biofilm mediated oro-dental infections.

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