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Evaluation Of Antimicrobial Effect Of Licorice Extrcat Agiasnt Oral Microorganisms

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EVALUATION OF ANTIMICROBIAL EFFECT OF LICORICE EXTRCAT AGIASNT ORAL MICROORGANISMS

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ABSTRACT

Introduction: The causative factors of dental caries have been studied for many years, however, preventing the formation of dental caries remains an elusive task. Microorganisms have been the focal point of numerous studies as a prime cariogenic factor. *Streptococcus mutans* and *Candida albicans* have been frequently co-isolated from dental biofilm in patients with early childhood caries (ECC) and studies have demonstrated the synergistic effects of these two microorganisms in promoting tooth demineralization. Acid and glucan production, which are two virulence factors produced by *S. mutans* that mediate biofilm formation and tooth decay, have been shown to be promoted during co-colonization with *C. albicans*. Interestingly, published reports have demonstrated that licorice extract exhibits both antibacterial and antifungal properties, particularly against *S. mutans* biofilms. However, no studies have tested the effect of licorice extract on *S. mutans* and *C. albicans* synergism.

Purpose: The goal of this study was to evaluate the antimicrobial effect of Chinese licorice (*Glycyrrhiza uralensis*) extract against oral microorganisms and to investigate whether licorice extract inhibits biofilm synergy between *S. mutans* and *C. albcians,* as well as test its effect on acid and glucan production by these oral microorganisms.

Materials and Methods: Minimal inhibitory and bactericidal concentration was determined utilizing disk diffusion assays on Todd-Hewitt broth (THB) agar plates. Biofilm assays were performed to assess the effect of licorice extract on *S. mutans* and

C. albicans, single and dual species biofilms formation in THB containing 1% sucrose in order to simulate ECC patients that consume high concentrations of dietary sugars. Biofilm biomass was quantified using crystal violet. Fluorescence microscopy was utilized to measure acid and glucan production with pHRodo red and Cascade blue stains, respectively. Furthermore, a *Drosophila melanogaster* sucrose-dependent model was utilized to evaluate the effect of licorice extract on the colonization of *S. mutans* and *C. albicans* in single and dual infection.

Results: *G. uralensis* extract significantly inhibited biofilm formation by *S. mutans* and *C. albicans* in a dose dependent manner. Microscopic analysis showed the reduction of acid and glucan production in the presence of 2.5% licorice extract. Less inhibition was observed in *S. mutans* and *C. albicans* co-cultures compared to *S. mutans* single cultures. Lastly, licorice extract reduced the colonization of *S. mutans* and *C. albicans* in single and dual infections.

Conclusion: *Glycyrrhiza uralensis* extract displayed significant antimicrobial and antibiofilm activity against *S. mutans* and *C. albicans* in both single and co-species cultures. Taken together, this study provides evidence that licorice extract may be an effective and natural therapeutic for the prevention of dental caries.

Keywords: Dental Caries, *Streptococcus mutans, Candida albicans*, Licorice extract, *Glycyrrhiza uralensis*

DEDICATION

"And put your trust in God, and sufficient is God as a trustee affairs"

This dissertation is lovingly dedicated to the precious gifts I have in my life:

My Father, Mother and Brothers

For the endless love, support and encouragement that have sustained me throughout my life

And my best friend, my amazing partner, Amin,

For always loving and supporting me and my dreams

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I am truly grateful for everything my Professors and mentors at UAB have done for me and hope that I can pay it forward by helping other people.

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LIST OF ABBREVIATIONS

ADI	Acceptable Daily Intake
C. albicans	*Candida albicans*
CFU	Colony Forming Units
DGL	De-glycyrrhizinated Licorice
ECC	Early Childhood Caries
EPS	Extracellular Polysaccharide
FDA	Food and Drug Administration
GRAS	Generally Recognized as Safe
Gtf	Glycosyltransferase
G. glabra	*Glycyrrhiza glabra*
G. uralensis.	*Glycyrrhiza uralensis*
Gfp	Green Fluorescent Protein
JECFA	Joint Expert Committee on Food Additives
LE	Licorice Extract

MBC	Minimal Bactericidal Concentration
MIC	Minimal Inhibitory Concentration
NS	Not Significant
OD	Optical Density
PBS	Phosphate-buffered saline
SCF	Scientific Committee on Food
Spp.	Species
SD	Standard Deviation
S. mutans	*Streptococcus mutans*
THB	Todd- Hewitt Broth
TYE	Tryptone Yeast Extract Broth
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

Dental Caries

Dental caries is one of the most prevalent yet preventable chronic diseases, which continues to be a major oral health problem affecting more than 600 million children under six years of age worldwide.[1] This complex, multifactorial, disease can cause discomfort, pain, poor nutrition and altered sleeping habits, and as a result, it can have negative impact on a child's overall health and well-being. Additionally, the early childhood caries (ECC, caries in children under age 6 years)[2] epidemic has placed significant health and economic burdens on patients and health care systems. According to the World Health Organization (WHO), the cost for treatment and prevention of oral disease, predominated by dental caries is estimated to be more than $120 billion per year in the US, as the fourth most expensive condition to treat (2014).[1,3]

Tooth decay is characterized by an ecological imbalance in physiological equilibrium between 2 factors; the "pathological factors" (i.e. cariogenic bacteria and high frequency of fermentable carbohydrates consumption) which result in acid production and demineralization, and "protective factors" (i.e. saliva and fluoride) that inhibits cariogenic activities and enhance remineralization. Therefore, caries prevention can be achieved either by reducing pathological factor or enhancing protective factors.[4]

Cariogenic Factors

A better understanding of dental caries activity has led to an accepted proposed etiology that is based on a four-factor theory including oral cariogenic microorganisms, diet, susceptible tooth and host factors, and time. Cariogenic microorganisms are the main causal factor and excessive exposure to sugars in diet leads to the accumulation of acid-producing and acid-resistant microorganisms in the oral cavity which over time can result in dental caries.[5,6] (Fig 1)

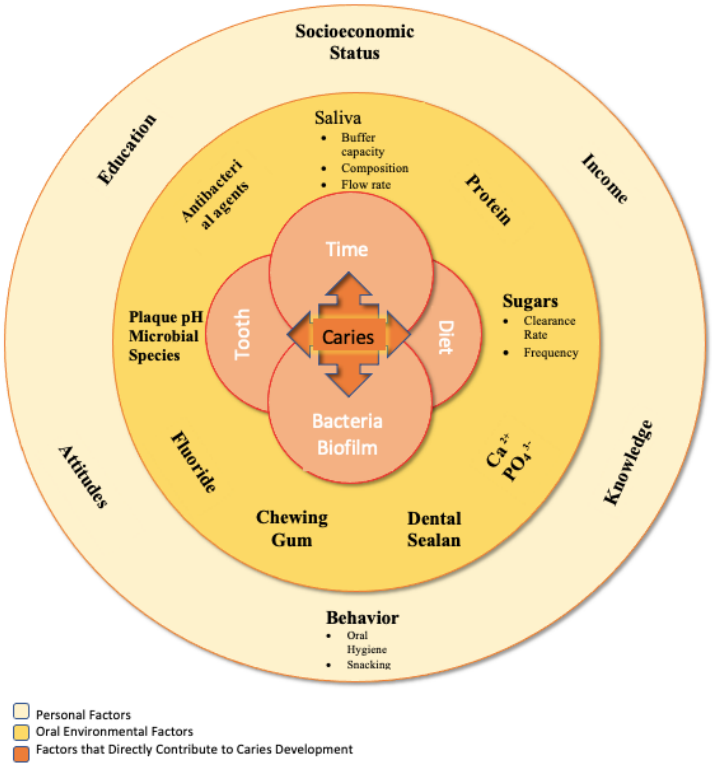

Figure 1. **Risk Factors of Dental Caries.**
Reproduced by permission from John Wiley & Sons for Fejerskov and Kidd, 2008.[79]

However, dental caries is a biofilm-dependent disease which consists of a polymicrobial environment, encapsulated in a self-produced extra cellular polymeric

matrix that adheres to the tooth surface.[7] In general, within the biofilm environment, microbial species are highly incorporate social interaction and multicellular behavior via quorum-sensing and coaggregation systems.[8,9] These unique survival strategies of biofilm help single and multi-species to access the optimal nutrients and niches to survive and protect them with a collective defense against other competitor organisms and host defense system.[7,8]

The oral cavity harbors diverse biofilms, supporting up to 1000 different species of microorganisms which in normal condition, live in symbiosis and plays an essential role in maintaining oral homeostasis and preventing oral diseases.[10] However, a change in environmental or stress signal, can tip the equilibrium toward pathogenic bacteria leading to oral diseases, such as dental caries.[11,12]

The major contributors to the initiation of dental caries are the mutans streptococci (i.e. *Streptococcus mutans* and *Streptococcus sobrinus*).[11,6] *S. mutans* which is a facultative anaerobic gram-positive bacterium is the most common caries-associated bacteria and is one of the most acidogenic species identified in oral biofilms that metabolizes dietary sucrose and produce lactic acid as byproduct[15]. This acid production can lead to a change in the biofilm ecology by giving the bacteria one of its most important cariogenic properties by enhancing the presence of other acid-producing and acid-tolerant species such as the lactobacilli and increases the proportion of *S. mutans* in the dental biofilm.[16] This acidogenic flora may result in a drop in dental plaque pH below the "critical decalcifying pH level" [17] and a prolonged recovery to the neutral pH that favor enamel demineralization and dental caries.[18] (Fig 2)

Figure 2. **Stephan Curve.**
Adapted from data provided in: Stephan, R. Intro-oral hydrogen-ion concentrations associated with dental activity [18]

Interestingly, the critical pH varies over a wide range from individual to individual and from tooth to tooth, depending on multiple factors such as presence or absence of caries and the level of calcium, phosphate and fluoride in dental plaque and saliva and it can be as high as 6.5 and as low as 5.1.[18,19]

Additionally, *S. mutans* has the unique ability of adhering within dental plaque which is mediated via sucrose- independent and sucrose- dependent pathways.[15] The antigen I/II surface protein influence the sucrose-independent adhesin by initiation of attachment process of *S. mutans* to salivary coated hydroxyapatite as well as inter-bacterial adhesion and secondary colonization.[20,15] On the other hand, sucrose- dependent adhesion is thought to be responsible for colonies establishment on the tooth surface and initiating changes in dental plaque ecology that can result in dental caries.[15] One of the key contributor factors in sucrose- dependent attachment is converting sucrose to extracellular

polysaccharide (EPS) matrix such as glucan which is synthetized by streptococcal glycosyltransferases (Gtfs)[21]. Studies showed that up to 40% of the dry weight of dental plaque is constituted of polysaccharides, mostly glucans.[21]

S. mutans produces at least three Gtfs encoded by *gtfB*, *gtfC*, and *gtfD* which synthetize both water-soluble and water-insoluble glucans.[15] Although both types of glucans are thought to play roles in *S. mutans* colonization, the water-insoluble polymer may have the major contribution in smooth surface caries.[15] The Gtfs secreted by this bacterium, which is enzymatically active, can adhere to the pellicle and produce glucan within minutes when exposed to a source of sucrose.[22]

The biofilm extracellular matrix rich in insoluble EPS alter the diffusion properties of biofilm matrix by acting as a barrier to the buffering effects of saliva on surfaces while conferring protection to microorganisms from antimicrobial agents by limiting their diffusion. It can also increase the porosity in the biofilm, resulting in sugar penetration into deepest part of biofilm structure to promote the acidic microenvironments and thereby the formation of acidic niches that exhibit an increased demineralization potential.[23]

Other than *S. mutans, Candida albicans*, a common opportunistic pathogenic fungus in humans,[24] is also found to be a colonizer in oral biofilms and has been associated with carious lesions mainly in children with severe ECC.[14,25,26] Although, *C. albicans* wan initially known to have minimal to no physical adhesion with cocci in the absence of sucrose resources[27], it was determined that, when sucrose is available, there are some evidence of coadherence between these 2 microorganisms via the glucans forming between the cocci and yeast cells.[28] This cross-kingdom biofilm contains an extensive extracellular matrix that is secreted by *S. mutans*.[29] *In vitro* studies showed *S. mutans gtfB*, *gtfC*, and

gtfD exoenzymes adsorbed onto *C. albicans* cells produced large quantity of extracellular matrix which enhance the *S. mutans* binding as well as more fungal adhesion to the tooth surfaces. [30] In fact, this symbiotic relationship, allows them to create an enhanced biofilm in oral cavity and increase cariogenic potential compared to single-species biofilms.[25,27,31] Therefore, the survival of *C. albicans* is largely depends on this multi-species communication and coaggregation between *C. albicans* and *S.mutans*.[9]

Furthermore, the biofilm growth and virulence of *C. albicans* are linked to its morphology transition between yeast and hyphae form, which signifies a fundamental step towards its pathogenicity.[32] It can also ferment some dietary sugars, produce organic acids and secrete dentine-degrading enzymes, which contribute to the pathogenicity of *C.albicans* of dental caries [33,34]

Some *in vivo* studies also confirmed that animals infected by both *C. albicans* and *S. mutans* showed higher levels of infection and microbial carriage in biofilms, compared to animals infected with either species alone, therefore, co-infection was found to augment the virulence of the biofilm.[27,35]

Licorice Extract

There are different approaches to manage dental caries disease, including surgical intervention, arresting the carious lesions and chemical preventive treatment. However, the surgical treatment and arresting the lesions is not always feasible mainly in young patients and individuals with special health care needs, hence, dental treatment, in these groups of patients, frequently requires some kinds of advanced behavior management such as sedation or general anesthesia. Further, these approaches are after the disease has occurred, therefore, emphasis on prevention is a more optimal approach aimed to develop and use various materials to control and inhibit the formation of cariogenic biofilm.[36]

In search for effective, efficient, safe and economical alternatives substance, use of natural phytochemicals derived from plant extracts is gaining more popularity in modern medicine. Clinical trials and various *in vitro* and *in vivo* studies have proved the effectiveness of several natural compounds in the treatment of dental caries with fewer side effects and more cost efficiency in comparison with synthetic antimicrobials.[5,37]

Liquorice (British English) or Licorice (American English)[38] is one such herbal remedy which have been used for decades for its potentials to treat various types of health conditions such as inflammatory diseases, gastrointestinal disorder, bacterial infection, atherosclerosis, and skin conditions.[39-40] The name licorice given to the roots of Glycyrrhiza spp. from Fabaceae family which is native to Mediterranean, Asian and European countries. The genus name *Glycyrrhiza* derived from a Greek word , meaning sweet root, mainly due to the sweet product that is extracted from unpeeled, dried root of this perennial plant.[41,42] This plant has an extensive root system including a taproot and numerous stolons. The soft, fibrous taproot is mainly harvested for medicinal uses, while

the stolons, which can reach up to 8 meters in length, together with the taproot are the source of commercial licorice.[42](Fig 3).

Figure 3. **Dried Licorice Root.**

Although, the genus *Glycyrrhiza* consists of about 30 species, the roots of *Glycyrrhiza glabra* L. and *Glycyrrhiza uralensis* Fisch. are the most common sources of licorice root for traditional medicine, as well as cosmetics and tobacco industries and most importantly as additive and sweetener agent in food industry.[43] The chemical properties of licorice species vary due to different plant species, geographic area, growth environment, harvesting and extracting process that may affect its quality and therapeutic activities.[44]

The active constituents of licorice are saponins, flavonoids, chalcones, isoflavones and coumarins, as well as miscellaneous compounds such as fatty acids and phenols.[40] More than 20 triterpenoids and nearly 300 flavonoids have been isolated from licorice which are responsible for most of its pharmacological effects such as anti-inflammatory and antimicrobial activities.[45]

The main sweetener of the licorice extract is glycyrrhizin, which is its major triterpenoid saponin and used as a tool to recognize the herb.[43] Glycyrrhizin constitutes about 2–25% of licorice root extract and is considered the primary active ingredient.[46,47] Interestingly, the sweetness of this component is known to be 50 times sweeter than sucrose, with different characteristics, being less instant, tart, and lasting longer.[40,42,47]

Although the details of exact mechanism of antimicrobial activities of licorice extract are still in the initial stage, there are several compounds isolated from licorice, such as glycyrrhetinic acid, glabridin and licochalcones which are found to have antimicrobial effects by inhibiting the formation of bacteria and biofilm, the production of exotoxins, preventing yeast-hyphal transition and activating the immune response.[48]

Licorice and its derivatives are listed as "Generally Recognized as Safe (GRAS)" by the Food and Drug Administration (FDA)[49] It's available in various forms such as liquid extract, powder, capsules, etc. and it's assumed to be a safe over-the-counter remedy that do not pose any health hazard when used appropriately and by individuals who are not sensitive to glycyrrhizin (i.e. old age, female sex and hypertension).[42,47] However, excessive use of licorice, more particularly glycyrrhizin, may cause some side effects such as severe hypertension, hypokalemia, hypermineralocorticoid-like effect headache, premature birth, muscle weakness, and paralysis. [42,46,47,50]

Although there is no clear recommendation on acceptable daily intake (ADI) for licorice, Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the European Community's Scientific Committee on Food (SCF) considered 100mg/day use of glycyrrhizinic acid (approximately the amount found in 60–70 g licorice) to be a reasonable upper limit for the majority of the population, However, an individual's baseline health status may influence the susceptibility to glycyrrhizin. [51,52] The content of glycyrrhizin varies in different forms of licorice. For instance, licorice fluid extracts contain approximately 10%–20% glycyrrhizin and typical doses of 2– 4 mL deliver 200 – 800 mg , whereas powdered licorice root is 4%–9% glycyrrhizin and daily doses of 1–4 g contain 40–360 mg.[52]

Due to the possible adverse effect of glycyrrhizin, de-glycyrrhizinated licorice (DGL) has also been manufactured by removing the active compound glycyrrhizin in licorice and made available in different forms (Liquid, lozenges, wafers, etc.)[47]. Considering the possible complication related to excess consumption of the licorice-containing compounds, public awareness is mandatory to avoid the inadvertent use of such product.

Literature Review

Multiple studies have evaluated and documented that licorice extract and licorice bioactive ingredients such as glycyrrhizin, glycyrrhizol-A, glabridin, licoricidin, licorisoflavan A and licochalcone have beneficial effects in inhibiting cariogenic microorganisms and preventing oral diseases such as dental caries.[46,53,54]

By contract, there are some studies, such as Söderling's experiment, that showed no differences in the oral microbial counts after using licorice product. In this study, sixteen healthy volunteers were instructed to use a gel containing 2.5% licorice three times a day for 2 weeks and no difference was found between the experiment and control groups.[55]

In separate studies, He, Villinski, Gafner and Sedighinia *et al*. investigated the effect of Glycyrrhiza spp. and the extracted metabolites from the roots, using a set of different approaches, and concluded that those metabolites show activity against *S. mutans*. [54,56-57] Liu *et al*. have also conducted an *in vitro* study to evaluate the effect of glycyrrhizic acid on *S. mutans* growth and acid production via minimal inhibitory concentration (MIC), the minimal bactericidal concentration (MBC) and pH value and reported the glycyrrhizic acid can inhibit bacterial growth and acid production.[58]

Recently, Vaillancourt *et al*. studies the effect of Isoflavans Licoricidin and Glabridin, two major isoflavans of licorice, on growth, acid and dextran production and adhesion of *S. mutans,* as well as the biocompatibility of those components. The author reported that licoricidin and glabridin exhibited antibacterial activity against *S. mutans* cells and decreased the acid production and bacterial adherence to hydroxylapatite with low cytotoxicity for oral keratinocytes. In this study, only the glabridin showed some decrease in dextran production. [59]

The efficacy of licorice extract as a cleaning agent against *S. mutans* in the cavities in deciduous molars was also investigated by Godbole *et al*. and the results indicated the significant antibacterial activity against this species.[60]

Although the number of human experiments is limited, a few clinical studies evaluated the potential of licorice lollipops consumption for caries control in pre-school children and other populations which showed a decline in the number of *S. mutans*. [61,62,63]

Some studies have also investigated the antimicrobial effects of licorice on *C. albicans* demonstrating the antifungal effect of Glycyrrhiza spp.[64,65] In their studies, Roque, Fatima, Seleem, and Grenier investigated the *Glycyrrhiza glabra* and its bioactive constituents, such as glabridin, lichochalcone-A and glycyrrhizic acid and concluded antifungal activities against *C. albicans.* [64-66]

In another study, Sharma *et al*. evaluated the antifungal activities of three medicinal plants including *Glycyrrhiza glabra* and confirmed the antifungal properties of *G. glabra* against infectious fungal diseases caused by *C. albicans* and this antifungal activity is largely dependent on the extract used, due to the possible loss of some of the volatile components of *G. glabra* during drying and evaporating process.[67]

In an *in vivo* study, Utsunomiya *et al*. reported that, compared to normal mice, MAIDS mice (mice infected with LPBM5 murine leukemia virus) are 100 times more susceptible to be infected with *C. albicans* and administration of glycyrrhizin improved the resistance of MAIDS mice to *C. albicans* infection.[68]

There are limited studies evaluating the antimicrobial activities of Glycyrrhiza spp against both *S. mutans* and *C. albicans*. In Brazil, de Oliveira *et al*. studied cytotoxicity of four Brazilian plant extracts (*Equisetum arvense* L., *Glycyrrhiza glabra* L., *Punica granatum* L. and *Stryphnodendron barbatimam* Mart.) against oral microorganisms including *S. mutans* and *C. albicans* and concluded all 4 extracts were effective against those microorganisms with *G. glabra* exhibiting the least cytotoxicity.[69]

In a more recent study, Yang *et al.* looked at the antimicrobial effect of *G. uralensis* extract on *S. mutans* and *C. albicans* and its biocompatibility for dental applications. In this study, different concentrations of licorice extract were used on single cultures of microorganisms and tested for inhibition and cytotoxicity and the results showed a significant antimicrobial activities in *G. uralensis* groups compared to the control group regardless of the concentration.[70]

CHAPTER 2

PURPOSE OF THE STUDY

The aim of this study was to evaluate the antimicrobial effect of licorice extract on *Streptococcus mutans* and *Candida albicans* as well as its inhibitory effect on these microorganisms' synergism**.**

Hypothesis

Glycyrrhiza uralensis extract has inhibitory activity against biofilm formation in *Streptococcus mutans* and *Candida albicans* dual culture.

CHAPTER 3

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Wild-type *Streptococcus mutans* UA159 and *Candida albicans* SC5314 strains used in this study (Table 1) were cultured in Todd- Hewitt Broth (THB) (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) agar plate and incubated in an atmosphere of 5% CO_2 at 37°C.

Table 1. **Strains used in this study**

Strain	Description	Source
UA159	Wild-type *Streptococcus mutans*	71
S. mutans-Gfp	Gfp-labeled *S. mutans*, Kan^R	71, 72
SC5314	Wild-type *Candida albicans*	73

Chinese licorice extract (*Glycyrrhiza uralensis)* was purchased from an FDA registered pharmaceutical laboratory (Hawaii Pharm, Honolulu, HI, USA). This product is distributed as an herbal dietary supplement containing organic Chinese licorice dried root powder in a base of glycerin and water, 60% and 40% respectively. According to the

supplement facts issued by manufacturer, the concentration of the licorice extract was 330 mg/mL of the suspension. The product was stored in an airtight, amber glass container in refrigerator at 4°C and was shaken well prior to the daily fresh preparation for each experiment. Tryptone Yeast Extract Broth (TYE) (Thermo Fisher Scientific, Waltham, MA, USA) and 1% sucrose was utilized to dilute the original extract to the required concertation.

In all the assays described below, the corresponding concentrations of TYE medium and 1% sucrose and 1.5% glycerin were used as control groups. One percent sucrose was used to simulate ECC patients challenged with high dietary sugars and the glycerin concentration was equal to the glycerin content in 2.5% of licorice extract.

Disk Diffusion Test

The growth inhibitory potential of the extract was determined using the agar disk diffusion method. Twenty microliters of licorice extract at concentrations of 12, 25,50 and 100% were dispensed on a 6mm sterile filter disk. One hundred microliters of diluted microbial suspension (OD_{600} of 0.1) were spread on a THB agar plate and filter disks were transferred to the media. Subsequently, the plates were incubated under 5% CO_2 at 37°C overnight. A disk soaked in TYE, sucrose and glycerin were used as negative control group in each plate. The zone of inhibition around each sample was measured and recorded. Subsequent to the dose dependent biofilm formation result, disk diffusion experiment was also performed on 2, 2.25, 2.5, 2.75 and 3% of licorice extract using the previously

described protocol. The assays were performed in triplicate in three independent experiments, and a representative set of data is presented.

Biofilm Formation Assay

The biofilm formation assay was conducted based on the disk diffusion results, to investigate the ability of licorice extract to eradicate *S. mutans* and *C. albicans* single and dual-culture biofilms formation. *Streptococcus mutans* and *Candida albicans* were grown in TYE broth overnight and the microbial culture was subsequently diluted 1:100 into fresh medium for the biofilm assays. Single and two-species groups were prepared by adding 1µL of diluted bacteria to 1mL of either control or experiment solutions. Two-hundred microliters of each sample was aliquoted into a 96-well microtiter plate (Thermo Fisher Scientific, Waltham, MA, USA) and incubated for 16 h at 5% CO_2 at 37˚C under static conditions. Dose dependent biofilm assays were performed using 2, 2.25 ,2.5 ,2.75 and 3% licorice extract.

After 16 h, the plate reader (Synergy HTX, Multi-Mode Reader, BioTek, Winooski, VT, USA) was used to quantify the microbial growth at OD_{600}. Each experiment was repeated three times on the same sample for technical replicate and in triplicate in three independent experiments for biological replicate.

Biofilm biomass was quantified as previously described.[74] Following incubation, the biofilms were rinsed three times with distilled water and stained with 0.1% crystal violet and incubating at room temperature for 15 minutes. Following crystal violet staining, microtiter wells were then rinsed three times, shaken out and blotted on paper towels to

remove the excess cells, unbound dye and water. The biofilms were dissolved with 30% acetic acid. Biofilm biomass was measured at an absorbance of 562 nm using the plate reader.

Fluorescence Microscopy and Acid and Glucan Production

S. mutans-Green fluorescent protein (Gfp-labelled) (Table 1) and *C. albicans* were prepared and single and dual microorganism were grown with 1μM dextran-conjugated pHRodo red or dextran-conjugated Cascade Blue (Molecular Probes, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) in two separate 8 well microscope slides (ibidi, Martinsried, Planegg, Germany) and incubated under 5% CO_2 at 37˚C for 16 h. The wells were gently washed with Phosphate-buffered saline (PBS) (Sigma-Aldrich, St Louis, MO, USA) three times to remove any unbound cells and stained with calcofluor white (Sigma-Aldrich, St Louis, MO, USA) to label *C. albicans.* Subsequently, the stained biofilms were examined using fluorescence microscopy. Images were used to quantify fluorescence of acid production in pHRodo red and glucan production in cascade blue groups. Three independent experiments were performed.

Colonization of *Drosophila melanogaster* and Quantification of Colony Forming Units (CFU)

Mid-log phase *S. mutans* and *C. albicans* cells were harvested and re-suspended in 5% sucrose as control group and 5% sucrose in addition to 2.5 % licorice extract as experiment group and adjusted to an OD_{600} of 2.5. One hundred microliters of the re-suspended cells were dispensed on large filter disks and transferred to *Drosophila* culture

vials to infect the flies. The samples were incubated in room temperature overnight. Flies were then transferred to an Eppendorf tube and ground in with 250μL of PBS with pipette tips and serial dilutions of the homogenates were carried out before plating the samples onto blood agar plates and incubating them at 37°C for CFU.

Statistical Analysis

All assays were performed in triplicate and repeated 3 times independently. Statistical analysis was performed using the *t*-test (GraphPad Software Inc, CA, USA). All data are expressed as mean ± standard deviation (SD). Differences were considered significant if p-value ≤ 0.05.

CHAPTER 4

RESULTS

In this study, the antimicrobial activity of the Chinese licorice extract was investigated by determining their inhibitory effects against *S. mutans* UA159 and *C. albicans* SC5314 single and co-culture growth, biofilm formation, acid and glucan production and colonization in a *Drosophila melanogaster* model.

Disk Diffusion Test

In vitro antimicrobial activity of *G. uralensis* was quantitatively and qualitatively assessed by determining the zone of inhibition for cells. Following 16-hour exposure with licorice-soaked filter disks, the zone of inhibition around each microbial species was carefully measured.

Inhibitory zones were observed and measured at licorice extract concentrations of 50 and 100% in *S. mutans*, *C. albicans* single and dual cultures. The 50 and 100 % licorice extract contained 150 mg/mL and 330 mg/mL of licorice respectively. The inhibitory zone increased in a dose dependent manner. However, there was no significant difference noted between 50 and 100% concentrations ($p > 0.05$). In contrast, there was no inhibition zone in lower concentrations of licorice extract as well as control groups (Fig 4, Table 2)

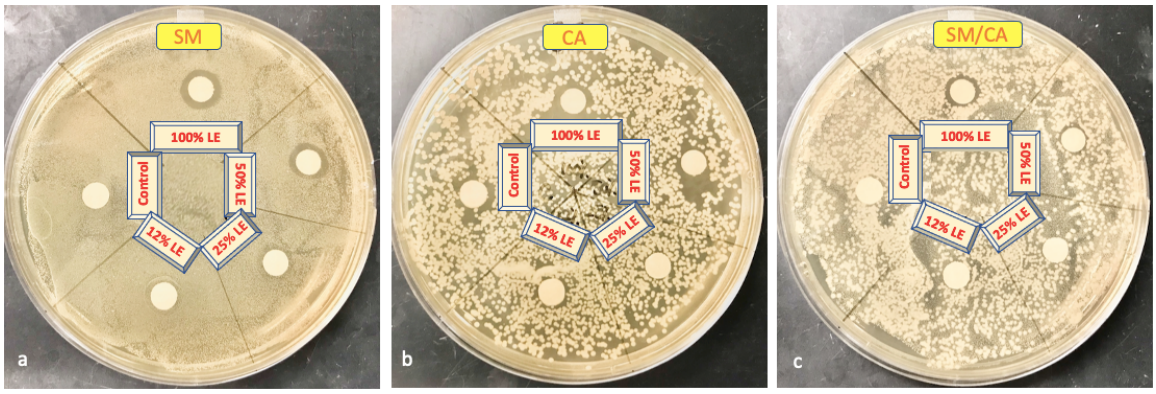

Figure 4. **Licorice Extract Disk Diffusion Assay in Single and Dual Culture.**
Inhibition zones of different concentrations (12,25,50,100 %) of *G. uralensis* extract and control group on THB agar plates containing (a) *S. mutans*, (b) *C. albicans* and *(c) S. mutans*/*C. albicans* dual culture. A significant growth inhibition was observed in 50 and 100% licorice extract. SM= *S. mutans,* CA= *C. albicans,* LE =licorice extract.

Table 2. **Zone of Inhibition Measurements. ***

Species	**Control**	**LE (12%)**	***P*-value ****	**LE (25%)**	***P*-value**	**LE (50%)**	***P*-value**	**LE (100%)**	***P*-value**
S. mutans	0	0	----	0.5±2.5	$P \geq 0.05$	0.3±0.33	**$P \leq 0.001$**	0.33±0.33	**$P \leq 0.001$**
C. albicans	0	0.3±2.5	$P \geq 0.05$	0	-----	0.3±0.33	**$P \leq 0.001$**	0.33±0.88	**$P \leq 0.001$**
S. mutans/ C.albicans	0	0	-----	0	-----	7±0	**$P \leq 0.001$**	0.33±0.33	**$P \leq 0.001$**

* Mean of inhibition zone measurement and standard deviation of *S. mutans* and *C. albicans* in control and experimental groups in millimeters. Significant inhibition noted in 50 and 100 % licorice extract in all 3 groups of microorganisms.
** *P*- value was obtained based on *t*-test. $P \leq 0.001$= significant difference, $P \geq 0.05$ = no significant difference, LE =licorice extract.

Biofilm Formation Assay

The formation of dental caries is initiated by the adhesion of cariogenic microorganisms to the tooth surface to form biofilm in a carbohydrate-rich environment.[10] To investigate the effect of licorice extract on biofilm formation, a sucrose-dependent biofilm model in microtiter plates was utilized to mimic the cariogenic conditions associated with the oral cavity of children with ECC. Dose dependent biofilm assays were performed using 2, 2.25, 2.5, 2.75 and 3% licorice extract and the assays were performed in triplicate in three independent experiments.

The minimum concentration of licorice extract with statistically significant reduction effect on biofilm formation was 2.5% (8.35 mg/mL of licorice extract) for all three groups (p <0.01). (Fig 5)

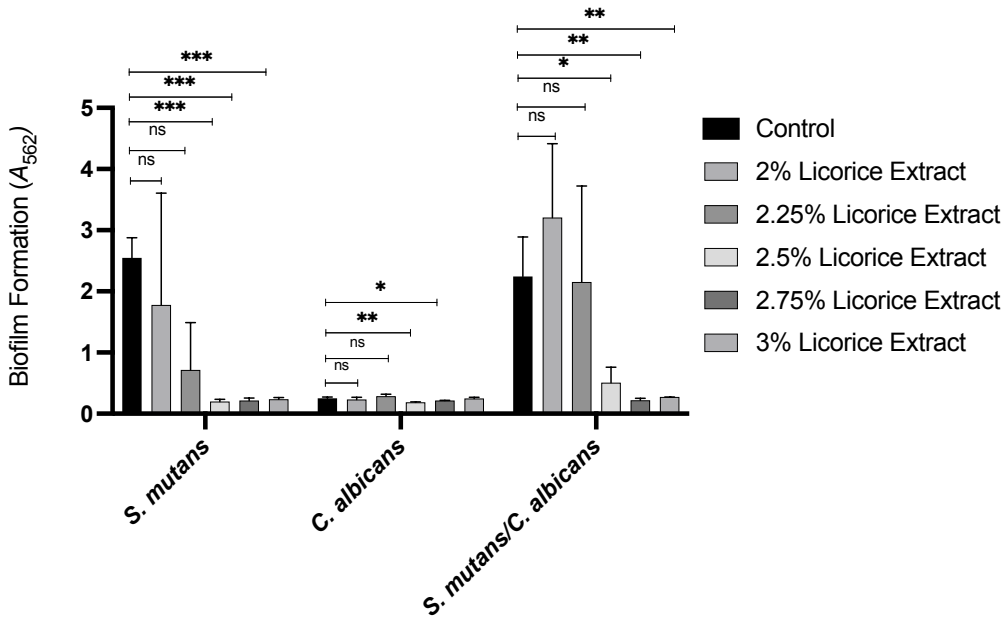

Figure 5. **Dose Dependent Effect of Licorice Extract on Biofilm Formation.**
Dose dependent biofilm assay on 2, 2.25, 2.5, 2.75 and 3% licorice extract, demonstrating licorice extract with concentration ≥ 2.5%. inhibits biofilm formation in *S. mutans*, *C. albicans* and *S. mutans*/*C. albicans* co-culture groups significantly.
*($P \leq 0.05$), ** ($P \leq 0.01$), *** ($P \leq 0.001$), ns= not significant ($P > 0.05$).

Licorice extract (2.5%) significantly inhibited single species biofilms of *S. mutans* and *C. albicans*, and the dual *S. mutans* and *C. albicans* biofilm. Licorice extract inhibited the single species *S. mutans* biofilm to a higher degree than the dual species biofilm (Fig 6).

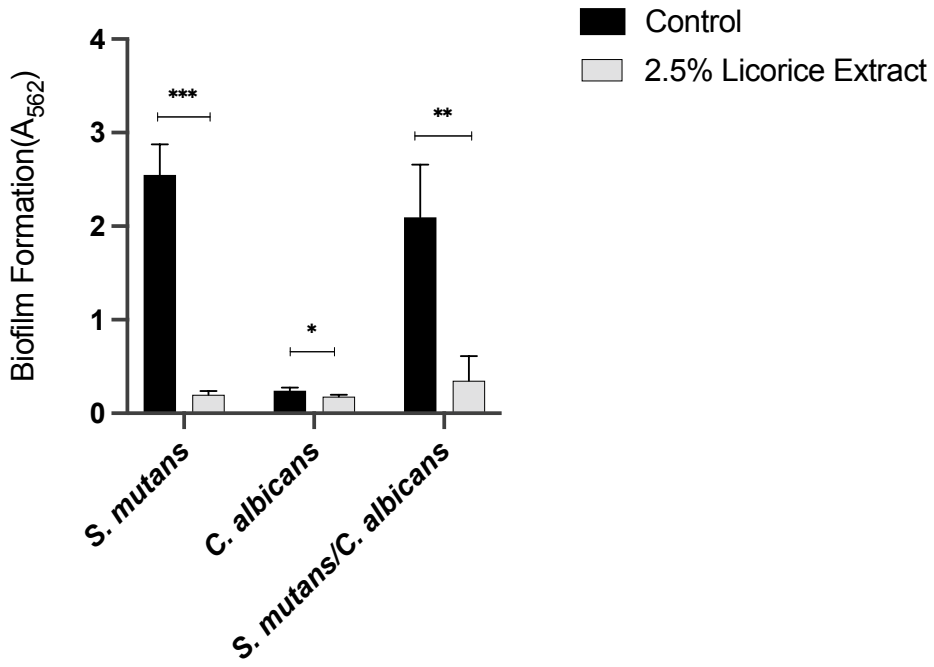

Figure 6. **Effect of 2.5% Licorice Extract on Biofilm Formation.**
Effects of 2.5% licorice extract on biofilm formation. The extract was most efficient to eradicate biofilm in *S. mutans* group, followed by *S. mutans/ C. albicans* co-culture and *C. albicans group.* *P*- value was obtained based on *t*-test. *($P \leq 0.05$), ** ($P \leq 0.01$), *** ($P \leq 0.001$)

To determine whether the decrease in biofilm formation was caused by a reduction in cell viability, planktonic cell growth was measured. As shown in Fig 7, licorice extract did not significantly inhibit single or dual species cell growth of *S. mutans* and *C. albicans*. Taken together, these data demonstrate that licorice extract solely acts on the ability of microbes to adhere but does not inhibit cell growth at 2.5%.

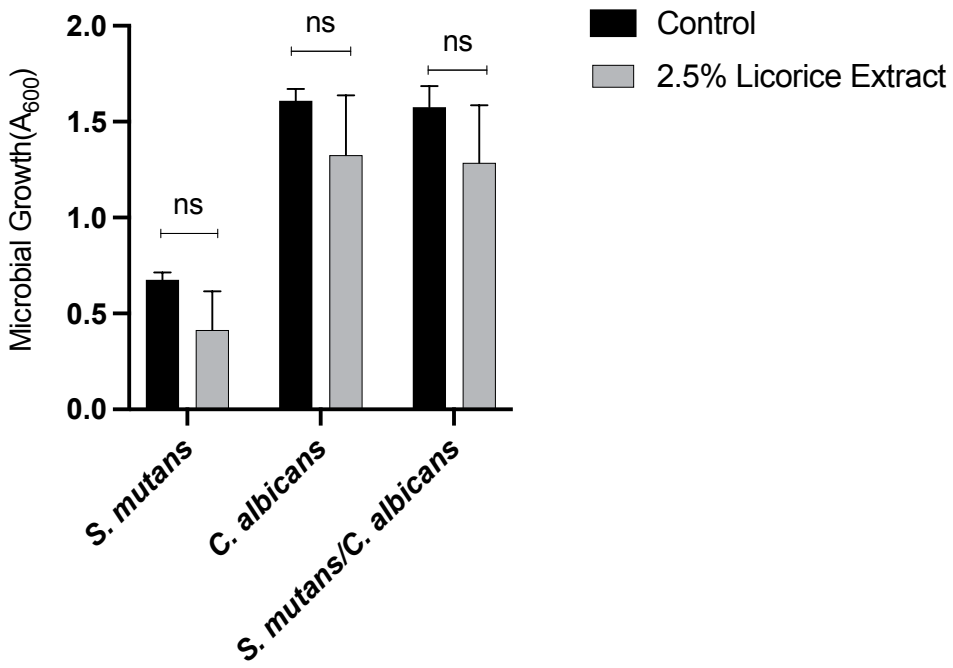

Figure 7. **Microbial Growth in Control and Licorice Extract Groups.**
Effects of 2.5% licorice extract on microbial growth in 96-well microtiter plate. No significant difference ($P > 0.05$ by *t*-test) between control and experimental groups was noted in *S. mutans, C. albicans* single and co-culture growth. ns= not significant ($P>0.05$).

Fluorescence Microscopy and Acid and Glucan Production

Acid and glucan productions are two important virulence factors used by cariogenic microorganisms, which are critical in caries pathogenesis.[15,21] To determine if the presence of licorice extract influences acid and glucan production, dextran-conjugated pHRodo red and dextran-conjugated Cascade Blue were added to preformed *S. mutans* and *C. albicans* single and two-species biofilm to monitor pH and glucan changes respectively. *S. mutans* was labeled with green fluorescent protein (GFP) and *C. albicans* was stained with calcofluor white. In agreement with the crystal violet findings, fluorescence microscopy

showed a decrease in attachment for single and dual *S. mutans* and *C. albicans* cells that were treated with 2.5 % Licorice extract (Fig 8).

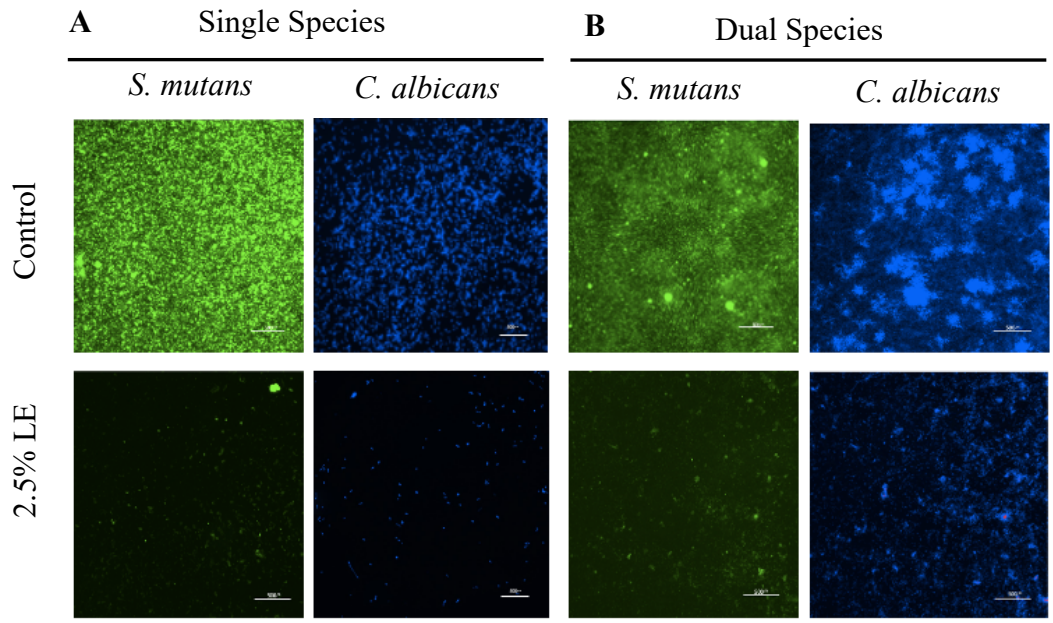

Figure 8. **Fluorescence Microscopic Images of Cell Reduction.**
Gfp labeled *S. mutans* and Calcofluor white *C. albicans* in control and 2.5% licorice extract groups. Figures 8A and 8B demonstrate the cell reduction in 2.5% LE groups in single species and dual species, respectively. LE =licorice extract

Similarly, licorice extract inhibited glucan (Fig 9) and acid production in the *S. mutans* and *C. albicans* single and two species biofilms. (Fig 10).

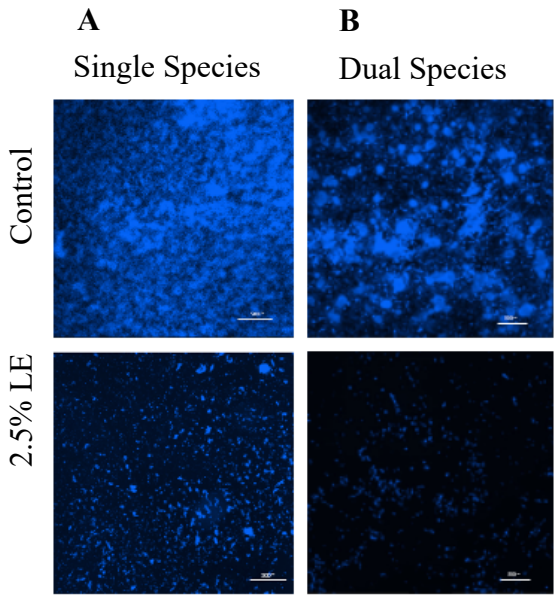

Figure 9. **Fluorescence Microscopic Images of Glucan Production**
The analysis demonstrating a significant reduction in glucan production in 2.5 % LE in single (Fig 9A) and co-species (Fig 9B) biofilms at 60X magnification. LE =licorice extract.

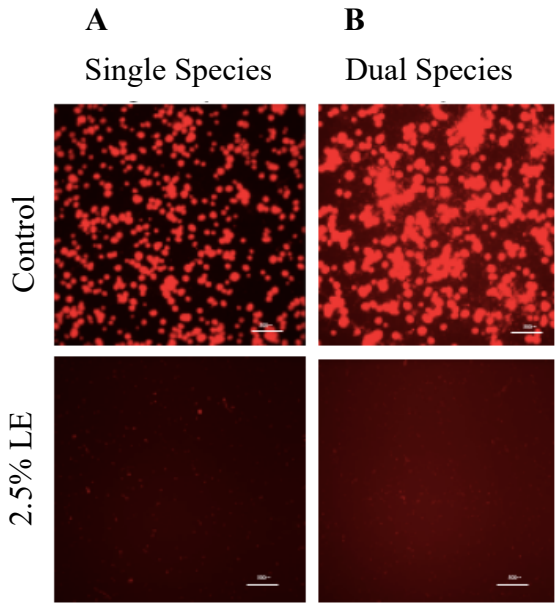

Figure 10. **Fluorescence Microscopic Images for Acid Production.**
The analysis demonstrating a significant reduction in the acidity in 2.5 % LE in single (Fig 10A) and co-species (Fig 10B) biofilms at 60X magnification. LE =licorice extract.

Colonization of Drosophila melanogaster

To determine if licorice extract influences the colonization of *S. mutans* and *C. albicans* single and co-species *in vivo*, a widely used *Drosophila* sucrose-dependent colonization model was employed. Flies were infected with mid-log phase of *S. mutans*, *C. albicans,* and *C. albicans* with *S. mutans*. Licorice extract decreased *S. mutans* colonization in single species infection, although this reduction was not statistically significant. There was no difference in *C. albicans* colonization with or without licorice supplementation, however the dual *C. albicans* and *S. mutans* biofilm was inhibited. While not significantly different, licorice extract inhibited colonization of both *S. mutans* and *C. albicans* in single and dual infections in *Drosophila* (Fig 11)

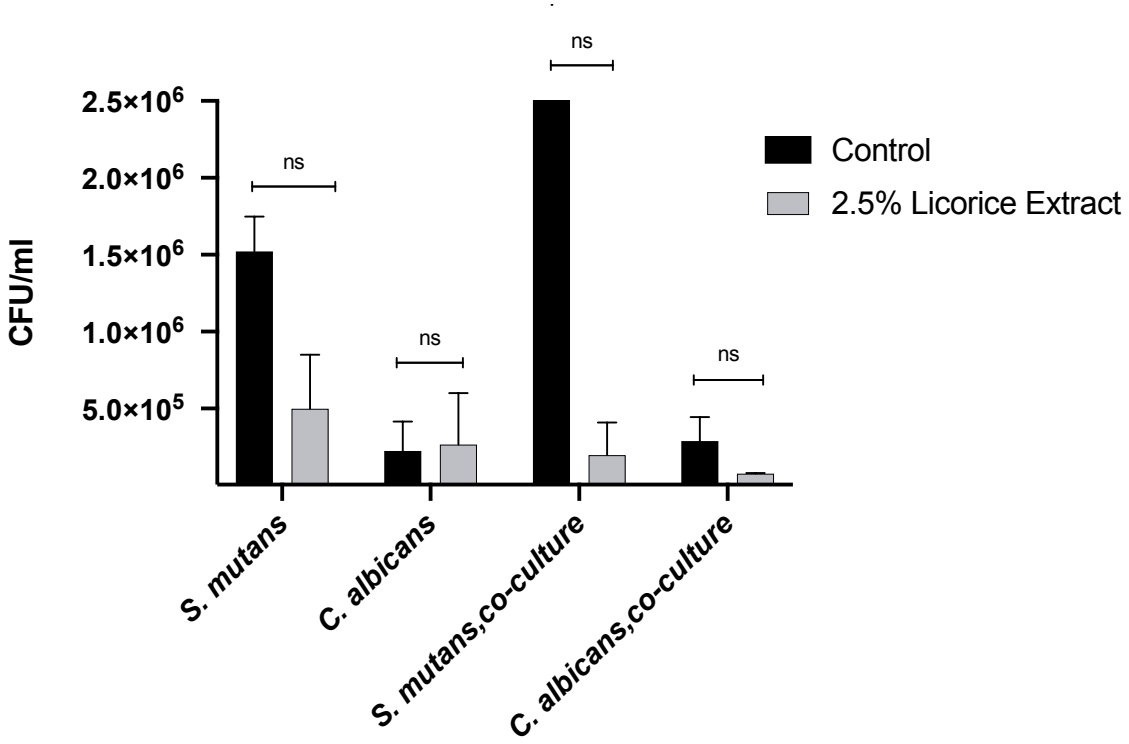

Figure 11. **CFU of Single and Dual Infection in *Drosophila* Model.**
Licorice extract (2.5%) reduces microbial colonization in *S. mutans, C. albicans* and *S. mutans*/*C. albicans* groups in *Drosophila melanogaster*, however the difference was not statistically significant ($P > 0.05$ by *t*-test).

CHAPTER 5

DISCUSSION

It is increasingly becoming more evident that dental caries is a polymicrobial phenomenon and that coadherence between *S. mutans* and *C. albicans* present in oral biofilms may play a role in caries progression. This symbiotic relationship that occurs through the extracellular signaling molecules or physical interactions allows these microorganisms to create an enhanced biofilm in the oral cavity and increase cariogenic potential compared to single-species biofilms.[25,27,31]

Despite attempts to control dental caries caused by these microorganisms, this chronic disease continues to be the major health issues worldwide[1] and efforts to develop efficient, safe and economical alternatives to prevent dental caries, remains a major challenge. Although licorice extract has been studied in the past in regard to its antimicrobial properties,[45,48,75] very little is known about its exact effect on *S. mutans* and *C. albicans* virulence properties and there are no studies on its effect on the synergism between these two microorganisms. Contrarily, few studies have reported that licorice extract has no significant effect on the oral microbiome. [55] The present study is among the first to assess the antimicrobial activity of *Glycyrrhiza uralensis* (Chinese licorice) against *S. mutans* and *C. albicans* synergism. In an attempt to understand this activity, the effect of licorice extract on the growth, virulence, and colonization of *S. mutans* and *C. albicans*

using *in vitro* biofilm assays and a *Drosophila melanogaster* oral infection model, were assessed.

In this study, licorice extract was effective in inhibiting both single and dual species *S. mutans* and *C. albicans* growth and biofilms formation and displayed the highest degree of inhibition against the single species *S. mutans* biofilm. These results are in agreement with another study performed by Yang *et al.* who investigated *S. mutans* and *C. albicans* in single groups.[70] Unlike planktonic cell growth, biofilm formation contributes to antimicrobial resistance, therefore it is considered a critical virulence property of oral microorganisms.[7,8] Prior studies have also reported that licorice bioactive components [59,65,66,76] and also deglycyrrhizinated licorice [77] inhibit biofilm formation by *S. mutans* and *C. albicans*. Taken together, these findings demonstrate that bioactive components in low concentration of licorice extract directly inhibit biofilm formation or adherence, but do not kill microbes, which could be beneficial for the development of new anti-biofilm therapeutics that do not disturb the oral microbiota.

S. mutans is among the most acidogenic species identified in oral biofilms [15] and *C. albicans* has the ability to ferment some dietary sugars and produce organic acids [33,34]. The acidogenicity property of these microorganisms can drop the pH below the critical value and promote tooth demineralization.[18,19] Hence, reducing acidic conditions in the oral cavity may prevent tooth decalcification. In our study, a pH probe used in combination with fluorescence microscopy revealed a significant reduction in acid production in the presence of licorice extract in both single and dual species cultures. This result is in agreement with previous studies that reported a less acidic environment in the presence of licorice components. In their study, Vaillancourt *et al.* evaluated the effect of licoricidin

and glabridin (two major licorice isoflavans) on *S. mutans* glycolysis leading to a pH drop and concluded that those compounds decreased acid production from glucose.[59] In another study, Yamashita *et al*. reported disodium succinoyl glycyrrhetinate (a derivative of glycyrrhetinic acid) inhibited the drop in pH in a dose dependent manner.[78] However, in contrast to the co-cultures studies reported herein, all of those studies were performed on single species *S. mutans* cultures. However, *S. mutans* and *C. albicans* produce more acid in dual species biofilms as previously reported [31] Overall, our data demonstrate that licorice extract has the ability to restrict acid production in two species biofilms, which is an important factor that contributes to tooth decay in caries development.

S. mutans ability to convert dietary sucrose to extracellular polysaccharide matrix such as glucan, is essential for the establishment of biofilms on the tooth surface. Additionally, glucan acts as a barrier to protect the microorganisms from environmental stress and enhances the acidic microenvironments.[21,23] Moreover, there is some evidence that shows the symbiotic interaction between *S. mutans* and *C. albicans* is largely dependent on the glucans formation, which enhances the strong binding of the microorganisms to tooth surface. [28,30] In this study, less glucan was produced in single and dual species biofilms that were treated with licorice extract. Vaillancourt *et al.* has also reported a reduction in dextran produced by *S. mutans* in the presence of glabridin.[59]

In order to validate the findings in an *in vivo* model, a sucrose-feeding co-infection model of *Drosophila melanogaster* was employed. In agreement with the *in- vitro* results, licorice extract decreased the colonization of both *C. albicans* and *S. mutans*.

Overall, this study demonstrates that licorice extract directly interferes with biofilm synergy displayed by *S. mutans* and *C. albicans* and has the ability to reduce colonization of these cariogenic pathogens in an *in vivo* model.

The findings suggest that Chinese licorice extract can be considered as an anticariogenic component as food additive or in dental products such as mouthwashes, toothpastes and wipes, mainly for young children and individuals with special healthcare needs with challenging oral hygiene routines. It can also be beneficial to contemplate licorice extract as a new non-cariogenic natural sweetener in pediatric oral medications to substitute the sweeteners with cariogenic potential.

The present study was conducted on two species of oral pathogens that does not mimic the diverse oral microbial community and limited to the verification of the antimicrobial activity of a pre-made *G. uralensis* extract in water and glycerin solution. To overcome the limitations, further *in vivo* investigation on scientifically identified licorice product and various oral pathogens species is required in future. Moreover, the antimicrobial potency, safety and biocompatibility of licorice extract when it's incorporated with other product such as toothpaste and mouthwashes should be assessed. Although, the accessible product, such as the licorice extract used in this study, or licorice candies, are usually well accepted by patients, it's not recommended to consume these products without a professional advice.

CHAPTER 6

CONCLUSION

In this study *G. uralensis* extract showed significant antimicrobial effects on *S. mutans* and *C. albicans* single and dual-species cultures compared to the control group. Low concentrations of licorice extract exhibited an excellent inhibitory effect on these microorganisms' biofilm formation, acidity and glucan production which are among their crucial cariogenic properties. Taken together, the results support the proposed hypothesis, and this naturally derived extract may have the potential for clinical use against cariogenic pathogens by facilitating the development of oral hygiene products that contain natural, anti-biofilm compounds

REFRENCES

1. Kassebaum NJ, Bernabé E, Dahiya M, Bhandari B, Murray CJL, Marcenes W. Global burden of untreated caries: A systematic review and metaregression. *J Dent Res*. 2015;94(5):650-658. doi:10.1177/0022034515573272
2. Drury TF, Horowitz AM, Ismail AI, Maertens MP, Rozier RG, Selwitz RH. Diagnosing and reporting early childhood caries for research purposes. *J Public Health Dent*. 1999;59(3):192-197. doi:10.1111/j.1752-7325.1999.tb03268.x
3. Fernando S, Tadakamadla SK, Bakr M, Scuffham PA, Johnson NW. Indicators of Risk for Dental Caries in Children: A Holistic Approach. *JDR Clin Transl Res*. 2019;4(4):333-341. doi:10.1177/2380084419834236
4. Featherstone J, B. The Continuum of Dental Caries — Evidence for a Dynamic. *J Dent Res*. 2004;83(3):C39-C42.
5. Oh DH, Chen X, Daliri EBM, Kim N, Kim JR, Yoo D. Microbial etiology and prevention of dental caries: Exploiting natural products to inhibit cariogenic biofilms. *Pathogens*. 2020;9(7):1-15. doi:10.3390/pathogens9070569
6. Robert H Selwitz, Amid I Ismail NBP. Dental Caries. *Mol Med Microbiol Second Ed*. 2007;2-3(4):945-955. doi:10.1016/B978-0-12-397169-2.00052-4
7. Donlan R. Biofilms: microbial life on surfaces. *Emerg Infect Dis*. 2002;8(9):881-890. doi:10.3201/eid0809.020063.
8. Williams P. Quorum sensing, communication and cross-kingdom signalling in the bacterial world. *Microbiology*. 2007;153(12):3923-3938. doi:10.1099/mic.0.2007/012856-0
9. Jenkinson HF, Lala HC, Shepherd MG. Coaggregation of *Streptococcus sanguis* and other streptococci with *Candida albicans*. *Infect Immun*. 1990;58(5):1429-1436. doi:10.1128/iai.58.5.1429-1436.1990
10. Berger D, Rakhamimova A, Pollack A, Loewy Z. Oral Biofilms: Development, Control, and Analysis. *High-throughput*. 2018;7(3):1-8. doi:10.3390/ht7030024
11. Avila M, Ojcius DM, Yilmaz Ö. The oral microbiota: Living with a permanent guest. *DNA Cell Biol*. 2009;28(8):405-411. doi:10.1089/dna.2009.0874
12. Kilian M, Chapple ILC, Hannig M, et al. The oral microbiome - An update for oral healthcare professionals. *Br Dent J*. 2016;221(10):657-666. doi:10.1038/sj.bdj.2016.865
13. Matsumoto-Nakano M. Role of *Streptococcus mutans* surface proteins for biofilm formation. *Jpn Dent Sci Rev*. 2018;54(1):22-29. doi:10.1016/j.jdsr.2017.08.002

14. Xiao J, Huang X, Alkhers N, et al. *Candida albicans* and Early Childhood Caries: A Systematic Review and Meta-analysis. *Caries Res*. 2018;52(585):102-112. doi:10.1159/000481833.Candida

15. Banas JA, Center. Virulence properties of *Streptococcus mutans. J Kyushu Dent Soc*. 1996;50(2):2. doi:10.2504/kds.50.2_1

16. Lemos JA, Quivey RG, Koo H, Abranches J. *Streptococcus mutans:* A new Gram-positive paradigm? *Microbiol (United Kingdom)*. 2013;159(PART3):436-445. doi:10.1099/mic.0.066134-0

17. Stephan R, Miller B. A Quantitative Method for Evaluating Physical and Chemical Agents which Modify Production of Acids in Bacterial Plaques on Human Teeth. *J Dent Res*. 1943;22(1):45-51.

18. Stephan RM. Intro-oral hydrogen-ion concentrations associated with dental activity. *J Dent Res*. 1944;23:257-266.

19. Dawes C. What is the critical pH and why does a tooth dissolve in acid? *J Can Dent Assoc*. 2003;69(11):722-724.

20. Brady LJ, Maddocks SE, Larson MR, et al. The changing faces of Streptococcus antigen I/II polypeptide family adhesins: MicroReview. *Mol Microbiol*. 2010;77(2):276-286. doi:10.1111/j.1365-2958.2010.07212.x

21. Koo H, Xiao J, Klein MI, Jeon JG. Exopolysaccharides produced by *Streptococcus mutans* glucosyltransferases modulate the establishment of microcolonies within multispecies biofilms. *J Bacteriol*. 2010;192(12):3024-3032. doi:10.1128/JB.01649-09

22. Hannig C, Ruggeri A, Al-Khayer B, et al. Electron microscopic detection and activity of glucosyltransferase B, C, and D in the in situ formed pellicle. *Arch Oral Biol*. 2008;53(11):1003-1010. doi:10.1016/j.archoralbio.2008.04.005

23. Leme AFP, Koo H, Bellato CM, Bedi G, Cury JA. Effecet of Sucrose on the Extracellular Matrix of Plaque-Like Biofilm Formed in vivo. *J Dent Res*. 2008;85(10):878-887.

24. Kim J, Sudbery P. *Candida albicans*, a major human fungal pathogen. *J Microbiol*. 2011;49(2):171-177. doi:10.1007/s12275-011-1064-7

25. de Carvalho FG, Silva DS, Hebling J, Spolidorio LC, Spolidorio DMP. Presence of mutans streptococci and Candida spp. in dental plaque/dentine of carious teeth and early childhood caries. *Arch Oral Biol*. 2006;51(11):1024-1028. doi:10.1016/j.archoralbio.2006.06.001

26. Raja M, Hannan A, Ali K. Association of oral candidal carriage with dental caries in children. *Caries Res*. 2010;44(3):272-276. doi:10.1159/000314675

27. Falsetta ML, Klein MI, Colonne PM, et al. Symbiotic relationship between *Streptococcus mutans* and *Candida albicans* synergizes virulence of plaque biofilms in vivo. *Infect Immun*. 2014;82(5):1968-1981. doi:10.1128/IAI.00087-14

28. Metwalli KH, Khan SA, Krom BP, Jabra-Rizk MA. *Streptococcus mutans, Candida albicans*, and the Human Mouth: A Sticky Situation. *PLoS Pathog*.

2013;9(10). doi:10.1371/journal.ppat.1003616

29. Hwang G, Liu Y, Kim D, Li Y, Krysan DJ, Koo H. *Candida albicans* mannans mediate *Streptococcus mutans* exoenzyme GtfB binding to modulate cross-kingdom biofilm development in vivo. *PLoS Pathog*. 2017;13(6):1-25. doi:10.1371/journal.ppat.1006407

30. Gregoire S, Xiao J, Silva BB, et al. Role of ghlucosyltransferase B in interactions of *Candida albicans* with *Streptococcus mutans* and with an experimental pellicle on hydroxyapatite surfaces. *Appl Environ Microbiol*. 2011;77(18):6357-6367. doi:10.1128/AEM.05203-11

31. Chan Yang, Jessica Scoffield, Ren Wu, Champion Deivanayagam, Jing Zou and HW. Antigen I/II mediates interactions between *Streptococcus mutans* and *Candida albicans*. *Physiol Behav*. 2019;176(3):139-148. doi:10.1016/j.physbeh.2017.03.040

32. Saville SP, Lazzell AL, Monteagudo C, Lopez-Ribot JL. Engineered control of cell morphology in vivo reveals distinct roles for yeast and filamentous forms of *Candida albicans* during infection. *Eukaryot Cell*. 2003;2(5):1053-1060. doi:10.1128/EC.2.5.1053-1060.2003

33. Klinke T, Kneist S, De Soet JJ, et al. Acid production by oral strains of *Candida albicans* and lactobacilli. *Caries Res*. 2009;43(2):83-91. doi:10.1159/000204911

34. Samaranayake LP, Hughes A, Weetman DA, MacFarlane TW. Growth and acid production of Candida species in human saliva supplemented with glucose. *J Oral Pathol Med*. 1986;15(5):251-254. doi:10.1111/j.1600-0714.1986.tb00617.x

35. Kim D, Sengupta A, Niepa THR, et al. Candida albicans stimulates *Streptococcus mutans* microcolony development via cross-kingdom biofilm-derived metabolites. *Sci Rep*. 2017;7(October 2016):1-14. doi:10.1038/srep41332

36. Kawashita, Yumiko and Kitamura, Masayasu , and Saito T. Early Childhood Caries. *Pediatr Clin North Am*. 2018;65(5):941-954. doi:10.1016/j.pcl.2018.05.004

37. Jeon JG, Rosalen PL, Falsetta ML, Koo H. Natural products in caries research: Current (limited) knowledge, challenges and future perspective. *Caries Res*. 2011;45(3):243-263. doi:10.1159/000327250

38. MERRIAM-WEBSTE. Liquorice. Merriam-Webster.com Dictionary, https://www.merriam-webster.com/dictionary/liquorice. Accessed 4/19/2021.

39. Mamedov NA, Egamberdieva D. Phytochemical constituents and pharmacological effects of licorice: A review. *Plant Hum Heal Pharmacol Ther Uses*. 2019;3:1-21. doi:10.1007/978-3-030-04408-4_1

40. Nassiri asl M, Hosseinzadeh H. Review of Pharmacological Effects ofGlycyrrhiza sp. and its Bioactive Compounds. *Phyther Res*. 2008;22:709–724.

41. Norsuzila Y, Mardina A, Mahamod M. Licorice as a Resource for Pharmacologically Active Phenolic Substances: Antioxidant and Antimicrobial Effects. *Intech*. 1989;32:137-144.

42. Messier C, Epifano F, Genovese S, Grenier D. Licorice and its potential beneficial

effects in common oro-dental diseases. *Oral Dis*. 2012;18(1):32-39. doi:10.1111/j.1601-0825.2011.01842.x

43. Nomura T, Fukai T, Akiyama T. Chemistry of phenolic compounds of licorice (Glycyrrhiza species) and their estrogenic and cytotoxic activities. *Pure Appl Chem*. 2002;74(7):1199-1206. doi:10.1351/pac200274071199

44. Zhang Y, Yang Y. A Systematic Review of the Comparison of Three Medicinal Licorices, Based on Differences of the Types and Contents about Their Bioactive Components. 2018;1(1):1-6.

45. Wang L, Yang R, Yuan B, Liu Y, Liu C. The antiviral and antimicrobial activities of licorice, a widely-used Chinese herb. *Acta Pharm Sin B*. 2015;5(4):310-315. doi:10.1016/j.apsb.2015.05.005

46. Isbrucker RA, Burdock GA. Risk and safety assessment on the consumption of Licorice root (Glycyrrhiza sp.), its extract and powder as a food ingredient, with emphasis on the pharmacology and toxicology of glycyrrhizin. *Regul Toxicol Pharmacol*. 2006;46(3):167-192. doi:10.1016/j.yrtph.2006.06.002

47. Omar HR, Komarova I, Abdelmalak HD, et al. Licorice abuse: Time to send a warning message. *Ther Adv Endocrinol Metab*. 2012;3(4):125-138. doi:10.1177/2042018812454322

48. Yang R, Wang LQ, Yuan BC, Liu Y. The Pharmacological Activities of Licorice. *Planta Med*. 2015;81(18):1654-1669. doi:10.1055/s-0035-1557893

49. U.S. Food and Drug Administration. Code of Federal Regulations Title 21. 21CFR201.306. 2020;3(2020):5-7.

50. Sigurjónsdóttir HÁ, Franzson L, Manhem K, Ragnarsson J, Sigurdsson G, Wallerstedt S. Liquorice-induced rise in blood pressure: A linear dose-response relationship. *J Hum Hypertens*. 2001;15(8):549-552. doi:10.1038/sj.jhh.1001215

51. SCF. Opinion of the Scientific Committee on Food on Capsaicin. *Sci Comm Food*. 2003;(April):1-41. http://ec.europa.eu/food/fs/sc/scf/out120_en.pdf.

52. Murphy SC, Agger S, Rainey PM. Too much of a good thing: A woman with hypertension and hypokalemia. *Clin Chem*. 2009;55(12):2093-2096. doi:10.1373/clinchem.2009.127506

53. Sidhu P, Shankargouda S, Rath A, Hesarghatta Ramamurthy P, Fernandes B, Kumar Singh A. Therapeutic benefits of liquorice in dentistry. *J Ayurveda Integr Med*. 2020;11(1):82-88. doi:10.1016/j.jaim.2017.12.004

54. He J, Chen L, Heber D, Shi W, Lu Q. Antibacterial Compounds from *Glycyrrhiza uralensis*. *J Nat Prod*. 2006:121-124.

55. Söderling E, Karjalainen S, Lille M, Maukonen J, Saarela M, Autio K. The effect of liquorice extract-containing starch gel on the amount and microbial composition of plaque. *Clin Oral Investig*. 2006;10(2):108-113. doi:10.1007/s00784-006-0040-9

56. Gafner S, Bergeron C, Villinski JR, et al. Isoflavonoids and coumarins from *Glycyrrhiza uralensis*: Antibacterial activity against oral pathogens and conversion

of isoflavans into isoflavan-quinones during purification. *J Nat Prod*. 2011;74(12):2514-2519. doi:10.1021/np2004775

57. Sedighinia F, Safipour Afshar A, Soleimanpour S, Zarif R, Asili J, Ghazvini K. Antibacterial activity of *Glycyrrhiza glabra* against oral pathogens: an in vitro study. *Avicenna J phytomedicine*. 2012;2(3):118-11824. doi:10.22038/ajp.2012.83

58. Liu G, He Y. Effects of glycyrrhizic acid on the growth and acid-producing of *Streptococcus mutans* in vitro. *Sichuan Da Xue Xue Bao Yi Xue Ban*. 2010;41(4):634-7.

59. Vaillancourt K, LeBel G, Pellerin G, Ben Lagha A, Grenier D. Effects of the Licorice Isoflavans Licoricidin and Glabridin on the Growth, Adherence Properties, and Acid Production of *Streptococcus mutans*, and Assessment of Their Biocompatibility. *Antibiotics*. 2021;10(2):163. doi:10.3390/antibiotics10020163

60. Singla S, Mali S, Tyagi S, Godbole E, Kulkarni P, Helge S. Efficacy of Liquorice and Propolis Extract Used as Cavity Cleaning Agents against *Streptococcus mutans* in Deciduous Molars Using Confocal Microscopy: An In Vitro Study. *Int J Clin Pediatr Dent*. 2019;12(3):194-200. doi:10.5005/jp-journals-10005-1620

61. Wang J, Chen Y, Agnello M, et al. Lollipop containing *Glycyrrhiza uralensis* extract reduces *Streptococcus mutans* colonization and maintains oral microbial diversity in Chinese preschool children. 2019:1-14.

62. Almaz ME, Ökte Z, Oba AA. Efficacy of a sugar-free herbal lollipop for reducing salivary *Streptococcus mutans* levels : a randomized controlled trial. 2017:839-845. doi:10.1007/s00784-016-1827-y

63. Peters MC, Tallman JA, Braun TM, Jacobson JJ. Clinical reduction of *S. mutans* in pre-school children using a novel liquorice root extract lollipop: a pilot study. *Eur Arch Paediatr Dent*. 2010;11(6):274-278. doi:10.1007/BF03262762

64. Roque L. Development of a bioadhesive nanoformulation with *Glycyrrhiza glabra* L . extract against *Candida albicans*. *Biofouling*. 2018;34(8):880-892.

65. Grenier D. Effect of licorice compounds licochalcone A, glabridin and glycyrrhizic acid on growth and virulence properties of *Candida albicans*. *Mycoses*. 2011:801-806. doi:10.1111/j.1439-0507.2011.02028.x

66. Seleem D, Benso B, Noguti J, Pardi V, Murata RM. In vitro and in vivo antifungal activity of lichochalcone-A against *candida albicans* biofilms. *PLoS One*. 2016;11(6):1-17. doi:10.1371/journal.pone.0157188

67. Sharma H, Yunus GY, Agrawal R, Kalra M, Verma S, Bhattar S. Antifungal efficacy of three medicinal plants *Glycyrrhiza glabra,* Ficus religiosa, and Plantago major against oral *Candida albicans*: A comparative analysis. *Indian J Dent Res*. 2016;27(4):433-436. doi:10.4103/0970-9290.191895

68. Utsunomiya T, Kobayashi M, Ito M, Pollard RB, Suzuki F. Glycyrrhizin Improves the Resistance of MAIDS Mice to Opportunistic Infection of *Candida albicans* through the Modulation of MAIDS-Associated Type 2 T Cell Responses. *Clin Immunol*. 2000;95(2):145-155. doi:10.1006/clim.2000.4854

69. de Oliveira JR, de Castro VC, Vilela P das GF, et al. Cytotoxicity of Brazilian

plant extracts against oral microorganisms of interest to dentistry. *BMC Complement Altern Med*. 2013;13. doi:10.1186/1472-6882-13-208

70. Yang S, Choi Y, Lee M, Kang M. Antimicrobial Effects against Oral Pathogens and Cytotoxicity of *Glycyrrhiza uralensis* Extract. *Plants*. 2020;9(838):1-12.

71. Liu C, Worthington RJ, Melander C, Wu H. A new small molecule specifically inhibits the cariogenic bacterium *Streptococcus mutans* in multispecies biofilms. *Antimicrob Agents Chemother*. 2011;55(6):2679-2687. doi:10.1128/AAC.01496-10

72. Wen ZT, Baker H V., Burne RA. Influence of BrpA on critical virulence attributes of *Streptococcus mutans. J Bacteriol*. 2006;188(8):2983-2992. doi:10.1128/JB.188.8.2983-2992.2006

73. Gillum AM, Tsay E, Kirsch D. Isolation of the *Candida albicans* gene for orotidine-5′-phosphate decarboxylase by complementation of S. cerevisiae ura3 and E. coli pyrF mutations. *Mol Gen Genet MGG*. 1984:179-183.

74. Toole GAO. Microtiter Dish Biofilm Formation Assay. *J Vis Exp*. 2011;47:10-11. doi:10.3791/2437

75. Tharakan A, Pawar M, Kale S. Effectiveness of licorice in preventing dental caries in children: A systematic review. *J Indian Soc Pedod Prev Dent*. 2020;38(4):325-331.

76. Ahn S, Park S, Ju Y, Cho E. In vitro Antimicrobial Activities of 1-Methoxyficifolinol , Licorisoflavan A , and 6 , 8-Diprenylgenistein against *Streptococcus mutans.* 2015;768:78-89. doi:10.1159/000362676

77. Ahn SJ, Cho EJ, Kim HJ, Park SN, Lim YK, Kook JK. The antimicrobial effects of deglycyrrhizinated licorice root extract on *Streptococcus mutans* UA159 in both planktonic and biofilm cultures. *Anaerobe*. 2012;18(6):590-596. doi:10.1016/j.anaerobe.2012.10.005

78. Yamashita T, Kawada-Matsuo M, Katsumata T, et al. Antibacterial activity of disodium succinoyl glycyrrhetinate, a derivative of glycyrrhetinic acid against *Streptococcus mutans. Microbiol Immunol*. 2019;63(7):251-260. doi:10.1111/1348-0421.12717

79. Fejerskov O, Kidd E. *Dental Caries; The Disease and Its Clinical Management*. 2nd ed. (Kidd OF and E, ed.).; 2008.