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Antimicrobial efficacy of liquorice against *Enterococcus faecalis* biofilms in various concentrations at time-dependent variables: An *in vitro* study

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Abstract

Introduction: The purpose this study was to evaluate the inhibitory efficacy of liquorice at various concentrations against *Enterococcus faecalis* and their biofilms at time-dependent variables in 24 h, 48 h, 72 h, 120 h, and 168 h.

Materials and Methods: The antienterococcal activity of liquorice and calcium hydroxide was detected employing concentration ranging from 1–4 g and interpreted based on the zone of inhibition. The ability of liquorice to inhibit *E. faecalis* biofilms during the stages of growth kinetics on microtiter plate was assessed, and the biofilm architecture was evaluated by scanning electron microscope (SEM).

Results: Statistically significant antienterococcal was observed at 3 and 4 g of liquorice against 24 and 48 h on microtiter plates. This observation was also complimented by SEM studies of biofilm architecture cultivated in root canals.

Conclusions: *E. faecalis* biofilms at 24 h and 48 h were highly susceptible to liquorice at concentration of 3 and 4 g.

Keywords: Antimicrobial efficacy; biofilms; calcium hydroxide; *Enterococcus faecalis;* liquorice

INTRODUCTION

Enterococcus faecalis is associated with root canal treatment failures owing to their ability of growth in nutrition limited and harsh environmental conditions. Biofilms established by *E. faecalis* in association with other pathogenic bacteria complicate the problem manifold.[1] A successful endodontic treatment requires complete elimination or reduction of *E. faecalis* or their biofilms from anatomically complex root canal architecture.[2] Various traditional herbs and chemical compounds have been evaluated for their inhibitory spectrum against root canal flora. In ancient literature, liquorice has been reported to present antimicrobial flavoring preparation.[3] This study evaluates the activity of liquorice on both *E. faecalis* culture and its biofilms at various stages of growth kinetics to explore its possibility as root canal medicament.

MATERIALS AND METHODS

Enterococcus faecalis strain and chemicals used for the study

E. faecalis strain (RC115) was isolated from the root canal of a patient exhibiting repeated root canal treated failure. The

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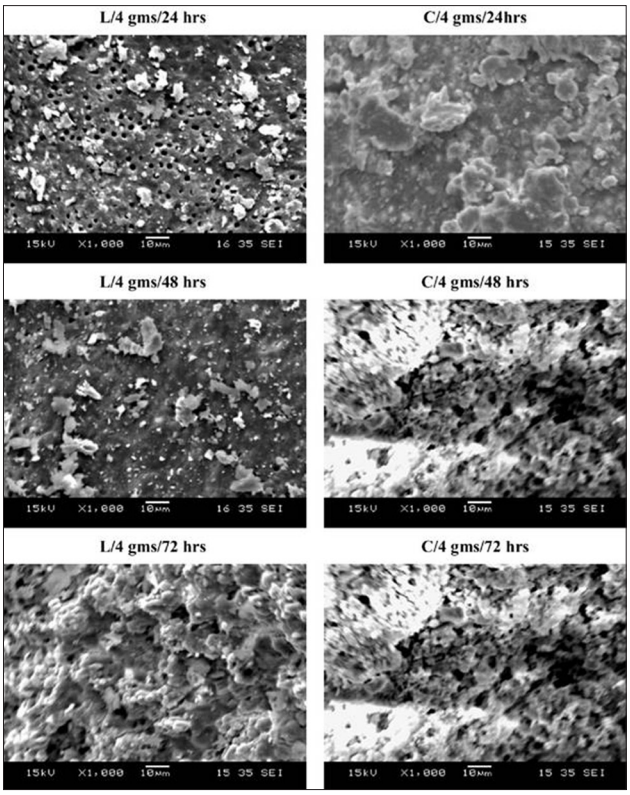


Figure 1: Inhibition of *Enterococcus faecalis* biofilms by medicament at concentration of 4 g. L: Liquorice, C: Calciumhydroxide

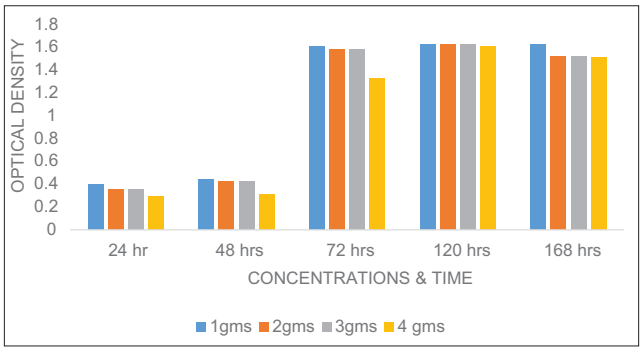


Chart 1: Detection of anti-*Enterococcus faecalis* activity of compound (liquorice) biofilm using optical density readings

isolate was identified and speciated based on conventional methods and polymerase chain reaction.

A dry liquorice extract (Sami labs Pvt. Ltd.,) employed for the study was obtained in a solid state which appears light yellow-to-brown powder. Calcium hydroxide (prime dent) was chosen as a control compound.

Efficacy of liquorice on *Enterococcus faecalis* culture

Screening for the active concentration of liquorice by disk-diffusion assay

Sterile paper discs were separately impregnated with liquorice and calcium hydroxide each at concentration

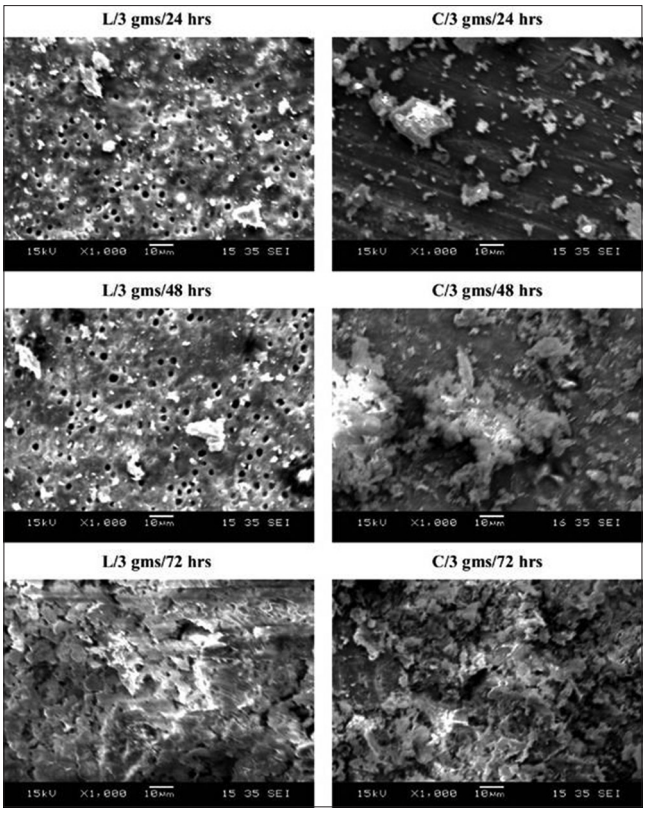


Figure 2: Inhibition of *Enterococcus faecalis* biofilms by medicament at concentration of 3 g. L: Liquorice, C: Calcium hydroxide

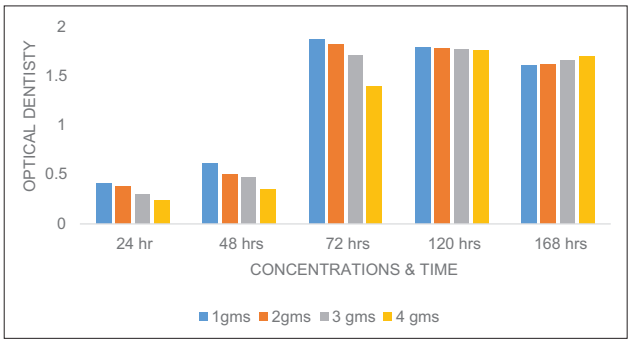


Chart 2: Detection of anti-*Enterococcus faecalis* activity of compound (calcium hydroxide) biofilm using optical density readings

of 1, 2, 3, and 4 g/ml and air dried aseptically. Overnight, broth cultures of *E. faecalis* RC115 on the brain–heart infusion broth (HiMedia) were adjusted to 0.5 McFarland standards and used as inoculum. A lawn culture of the inoculum was made on the dry surface of Mueller–Hinton agar plate (HiMedia). Liquorice and calcium hydroxide discs at various concentrations were placed on inoculated media at a distance of 2 cm. The plates were incubated overnight in a capnophilic environment, and following incubation, then the plates were observed for the zone of inhibition around the medicated paper discs.

Table 1: Comparison of the mean values of liquorice and calcium hydroxide against biofilms of *Enterococcus faecalis* at varying time intervals between groups (paired samples statistics)

Hours	Groups	Mean	SD	*t*	*P*
24 h	A - Liquorice	0.38275	0.0049	1.992	0.05 (S)
	B - Calcium hydroxide	0.32650	0.0423		
48 h	A - Liquorice	0.38950	0.0581	2.369	0.01 (S)
	B - Calcium hydroxide	0.30475	0.1279		
72 h	A - Liquorice	0.37700	0.046	0.326	0.754 (NS)
	B - Calcium hydroxide	0.41425	0.351		
120 h	A - Liquorice	0.3211	0.064	1.367	0.214 (NS)
	B - Calcium hydroxide	0.43150	0.237		
168 h	A - Liquorice	0.30887	0.075	0.806	0.447 (NS)
	B - Calcium hydroxide	0.33975	0.0535		

S: Statistically significant, NS: Nonsignificant, SD: Standard deviation

Testing the antibiofilm activity of liquorice and calcium hydroxide

Preparation of biofilms

The ability of liquorice and calcium hydroxide to inhibit the biofilms *E. faecalis* was assayed on microtiter plate as per the protocol of Toledo Arana *et al*. (2001).

Testing the antibiofilm activity of liquorice and calcium hydroxide by viable count method

Biofilms of *E. faecalis* RC115 formed on microtiter plate on the days 1, 2, 3, 5, and 7 were treated individually with 3 and 4 g/ml of liquorice and calcium hydroxide. Wells without medicament were used as control. The biofilm containing microtiter plate was inoculated in a capnophilic environment for overnight. Following incubation, the microtiter plates were taken, and the viable count of *E. faecalis* from each of the liquorice and calcium hydroxide treated test wells and control wells was done.

Viable count method

The test and control wells were washed with 20 µl of phosphate-buffered saline twice. The cells in biofilms were obtained with 20 µl of phosphate-buffered saline and transferred to sterile 1.5 ml microcentrifuge tube. Ten-fold serial dilutions of the bacterial suspension from each well made and 10 µl of each dilution was plated as a lawn culture on nutrient agar using sterile spreader. The plates were incubated in capnophilic environment overnight. The cfu/ml of each dilution was calculated to determine the viable number of *E. faecalis*.

Testing the antibiofilm activity of liquorice and calcium hydroxide by optical density measurements (A_{495})

Biofilms of *E. faecalis* RC115 formed on microtiter plate on the days 1, 2, 3, 5, and 7 were observed for the inhibitory activity of liquorice and calcium hydroxide at concentration of 3 and 4 g/ml. Following incubation of biofilms with the medicament, the wells were stained and destained, and the optical density of the destaining solution was measured at A_{495} using microtiter plate reader (Toledo Arana *et al*., 2001). The test was done in duplicate. Uninoculated medium was used as control. The mean A_{495} value from the control wells was subtracted from the mean A_{495} value of test wells.

Cultivation of Biofilms on root canals

The ability of liquorice to inhibit *E. faecalis* biofilms formed on anatomically complex site in the root canal was determined by culturing biofilms on extracted premolars.

Testing of medicament (liquorice and calcium hydroxide) on Enterococcus faecalis biofilms in root canal by scanning electron microscope

Freshly extracted 15 single-rooted premolars with mature apices were selected and stored in hydrogen peroxide. The crowns were removed using standardized endodontic protocol. The tooth length was standardized to 18 mm from the root apex to the coronal border. Cleaning and shaping of samples were performed using Flexer Heat Activation rotary nickel–titanium system (Mothers Dental Products Pvt. Ltd., Ireland) as per the protocol of Shehab El-Din (2012). Then, tooth was wrapped with aluminium foil. The teeth were then air dried and autoclaved. All samples were mounted individually in wax block and sealed. Pure culture suspension of *E. faecalis* RC115 grown overnight in brain–heart infusion broth and adjusted McFarland turbidity standard was used as inoculum, and fill the root canals of the teeth were sealed and incubated as earlier. Suitable positive and negative control were included as stated earlier.

Addition of Medicaments on biofilms in root canal

Biofilms on the days 1, 2, and 3 were taken and treated with liquorice and calcium hydroxide each at concentrations of 3 and 4 g/ml. Following addition of medications, the tooth was incubated as earlier and prepared for scanning electron microscopic analysis.

Scanning electron microscope analysis of medicated biofilm in root canals

All teeth were washed with PBS after 24 h, 48 h, and 72 h, respectively, and the samples were cut into longitudinal sections with safe-sided diamond disc mounted on a high-speed contra-angle with water coolant. Each sample was mounted and gold sputter was coated (JFC 1600 auto fine coater) with a 200 Å layer of gold-palladium. Observation was performed using a JEOL JSM-5610LV scanning electron microscope (SEM) at 15 kV.

RESULTS

The results were tabulated and statistically analyzed using paired samples statistics [Table 1].

1. Screening for the antienterococcal activity of liquorice and calcium hydroxide

Results of disk-diffusion employing concentration range of medicaments from 1 to 4 g show zone of inhibition of 13 and 14 mm for liquorice and for calcium hydroxide at 3 and 4 g, respectively

2. Antibiofilm activity of medicaments on microtiter plates
   The OD values of A_{595} for biofilms tested with liquorice showed <0.4 at 24 h, and at 168 h ranged from 1.4 to 1.6, the OD values at A_{495} on calcium hydroxide treated biofilms showed higher values >0.4 at 24 h and >1.5 at 168 h. The highest OD values were found at 72 h with a range of 1.5–1.6 for liquorice treated biofilm of 1.7–1.9 for calcium hydroxide treated biofilm [Charts 1 and 2]
3. In an effort to mimic the activity of liquorice in an anatomical complex site, enterococcal biofilms were cultivated in extracted premolar root canals. The ability of liquorice in inhibiting the 24 and 48-h-old biofilms was well evident in observing the reduction in biofilm architecture through SEM studies [Figures 1 and 2].

DISCUSSION

Enterococcus species live as commensals in the human intestinal lumen, human female genital tract, and the oral cavity.[4] The prevalence of *E. faecalis* is increased in oral rinse samples from patients receiving initial endodontic treatment, those midway through treatment, and patients receiving endodontic retreatment when compared to those with no endodontic history. Therefore, it is important to consider treatment regimens aimed at eliminating or preventing the infection of *E. faecalis* during each of these phases.

Preparing the apical portion of the root canal to a larger instrument size will help eliminate intracanal microorganisms by reaching areas which are inaccessible by smaller master apical files.[5] In addition, larger apical preparation sizes facilitate removal of the innermost (pulpal) dentin. This provides the potential to remove intratubular bacteria and open the dentinal tubules to allow antimicrobials to penetrate more effectively.[6]

Biomechanical cleaning and shaping of the root canal with the assistance of antimicrobial irrigants may fail to eliminate bacteria from the root canal.[7] Residual bacteria in the dentinal tubules may multiply which negatively affects the repair of periradicular apparatus.[8] The use of a biocompatible intracanal medication possessing antimicrobial properties between appointments may reduce or eliminate bacteria in the root canal system and also significantly increase the success of root canal therapy.[9]

According to Gurib–fakim,[10] there are four basic ways in which plants that are used by tribal peoples are valuable in modern medicine.

1. Plants used as sources of direct therapeutic agents
2. Plants are also used as sources of starting points for the elaboration of semisynthetic compounds
3. Plants can serve as sources of substances that can be used as models for new synthetic compounds
4. Plants can also be used as taxonomic markers for the discovery of new compounds.

The extract of dried liquorice root powder was used in the present study. The roots main component is glycyrrhizin, a triterpene molecule which accounts for the sweet taste of liquorice root. This compound represents a mixture of potassium-calcium-magnesium salts of glycyrrhizinic acid that varies within a range of 2%–25%. Chromatographic analysis of the root reveals a wide variety of components such as flavonoids, isoflavonoids, coumarins, saponins, glycosides, and phenols. Among the natural saponins, glycyrrhizinic acid is a molecule composed of a hydrophilic part, two molecules of glucuronic acid and a hydrophobic component, glycyrrhetic acid.[11]

In this study, the zone of inhibition was calculated in various concentrations such as 4 g, 3 g, 2 g, and 1 g of liquorice and calcium hydroxide against *E. faecalis*. The results of the study showed that after overnight incubation, the concentration of 4 g and 3 g of liquorice showed the highest zone of inhibition in plates than calcium hydroxide. This finding is in agreement with a study carried out by Badr *et al*.[12] where the antimicrobial efficacy of liquorice and calcium hydroxide was compared against *E. faecalis,* and it was concluded that liquorice extract had significant antimicrobial activity against these organisms.

Shalhav *et al*.[13] proposed that the process of evaluating antienterococcal properties of dental materials more than one assay or method should be used. For this reason, the broth dilution test on microtiter plates was also performed to evaluate the extent of antimicrobial activity and to measure the minimum inhibitory concentration of liquorice extract and calcium hydroxide. The agar diffusion test gave a qualitative value of bacterial sensitivity, and the broth dilution test gave quantitative results. The intracanal medication used ideally should reach the microorganisms located in the distant areas of the root canal system and should remain for a longer duration of time.

The biofilm results of this study revealed that liquorice extract and $Ca(OH)_2$ mixture were killed effective against *E. faecalis*, while liquorice alone had only a marginal antimicrobial effect. The weak antibacterial effect of $Ca(OH)_2$ against *E. faecalis* in this study is in agreement with studies done by Safavi *et al*. 1990[14] and Peters *et al*. 2002.[15] According to Badria *et al*., the antimicrobial effect of liquorice extract against

E. faecalis may be related to the glycyrrhizin. The mode of action of antibacterial effects of saponin seems to involve membranolytic properties, rather than simply altering the surface tension of the extracellular medium, thus being influenced by microbial population density.[16] The flavonoid content of liquorice extract is also a strong inhibitor of oxygen consumption in bacterial cells; the site of inhibition is thought to be between coenzyme Q and cytochrome C in the bacterial respiratory electron transport chain.[17]

Biofilms at growth kinetics of 24 and 48 h, when treated with liquorice, demonstrated a reduction in CFU/ml of enterococci as tested by viable count assay and the paucity of biofilm architecture by SEM studies compared to the negative group.

Saber Sel-D, El-Hady *et al*.[18] in their study assessed the mature biofilm of *E. faecalis* against various antimicrobial agents such as calcium hydroxide, amoxicillin, and clavulanate potassium, ciprofloxacin, clindamycin, and doxycline. He concluded that these agents were significantly better than calcium hydroxide, whereas in the present study, there is a reduction in biofilm forming ability of *E. faecalis* in the presence of liquorice at a concentration of 4 and 3 g and 24 and 48-h-old biofilm than calcium hydroxide, thus supporting the observations of the previous study.

A systematic review by Sathorn *et al*.[19] on the antibacterial effect of $Ca(OH)_2$ as an intracanal medicament showed limited disinfection activity within the root canal system. In a recent systematic review published by Saatchi *et al*.,[20] they report no improvement in antibacterial activity by mixing chlorhexidine and $Ca(OH)_2$.

CONCLUSIONS

Based on the results of the present study, it can be concluded that as follows:

1. Liquorice extract revealed higher activity than calcium hydroxide against enterococci by disk-diffusion method at a concentration of 3 and 4 g
2. Liquorice exhibited on better activity ($P < 0.05$) on biofilms formed on microtiter plates with statistically significant reduction in cfu/ml (0.298) and A_{495} (0.298)
3. In an effect to mimic the activity of liquorice in an anatomical complex site, enterococcal biofilms were cultivated in extracted premolar root canals. The ability of liquorice in inhibiting the 24 and 48-h-old biofilms was well evident in observing the reduction in biofilm architecture through SEM studies.

Based on the single species model used, this study shows that liquorice extract can be used as an intracanal irrigant and root canal medicament. Liquorice has been used for the treatment of many general body ailments such as eye diseases, throat infections, peptic ulcers, and liver diseases in the Indian Ayurvedic system. It has been proved to be a more biocompatible material. Further clinical trials are essential to prove liquorice as an alternative intracanal medicament.

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Conflicts of interest
There are no conflicts of interest.

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