ORIGINAL ARTICLE

Assessment of Synergistic Antimicrobial Effectiveness of Glycyrrhiza glabra and Syzygium aromaticum Extracts against oral bacteria (*Streptococcus mutans*)

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ABSTRACT

Dental caries continues to be a major global health issue affecting both adults and children. is driven by dysbiosis of the dental biofilm adherent to the enamel surface. One of the microorganisms frequently associated with dental caries is S. mutans. Given that natural compounds might have fewer adverse effects than synthetic antimicrobials. ***Objectives:*** *The aim of study was to improve the antimicrobial activity of natural extracts against oral bacteria (S. mutans) by synergistic combination with a mouthwash (chlorhexidine).* ***Methodology****: A total of 100 specimens were obtained from patients clinically diagnosed by with dental caries. Methanolic extracts of these plants were prepared. Antimicrobial activity was assessed using two methods: determining the minimum inhibitory concentration (MIC), sub-minimum inhibitory concentration (sub-MIC), and minimum bacterial concentration (MBC) using the broth dilution method and determining the zone of inhibition using the well diffusion method on S. mutans sanguis agar bacitracin.* ***Results:*** *Results show that the 400 mg/ml concentration inhibited the microbial growth of S. mutans with an inhibition zone of 19.23 mm. MBC values ranged from 6.25 to 200 µg/ml, whereas the MIC and sub-MIC concentrations for all investigated extracts and chlorhexidine were 3.125–100µg/ml and 1.5625–50µg/ml, respectively. Clove and licorice are thought to possess antibacterial efficacy owing to their secondary metabolites.* ***Conclusion:*** *Natural antibacterial agents were provided in this study, which can aid in the management of dental caries. If incorporated into mouthwashes and toothpaste, these extracts may have a positive effect on lowering plaque and dental cavities.*

INTRODUCTION

Dental caries is a localized chemical degradation of the tooth surface resulting from the metabolic activity of microorganisms within a dental biofilm (plaque). It can develop at any time and affects people of all ages, ethnicities, and genders [1]. Dental caries, on the other hand, is not thought to be a classic infectious disease; rather, it is seen as a multifactorial disease involving a variety of risk factors. It occurs when there is an environmental disturbance inside the oral cavity. More than 600 bacterial species are found in the human oral cavity; however, only a few plays a role in oral health. Although the oral cavity contains a variety of bacteria, only a few of these species can cause periodontal disease and dental caries. The three bacteria most commonly linked to dental caries are *S. mutans*, *Actinomycetes*, and *Lactobacillus*[2]. Many factors accounted for the cariogenicity of streptococcus mutanssuch as short generation time, carbohydrates fermentation, and capability to resist lower pH.and their ability to production of extracellular glucan-homopolymers from sucrose, which have an important role in attachment, colonization and formation of biofilms on teeth surfaces [3]. A patient's medical history, microbiological virulence factors, and maintenance or suppression of etiological variables are all elements that should be considered during treatment[4]. Herbal medicines have gained significant recognition in dentistry for their therapeutic properties, leading to widespread acceptance and use. Interest in alternative and traditional remedies has noticeably increased in recent years, driven by a growing preference for natural approaches to oral health care [5]. Numerous studies in dentistry have demonstrated the use of conventional plants and natural products to treat tooth illnesses. These items have been shown to reduce swelling, stop the growth of oral infections, and function as antioxidants, analgesics, and antiseptics [6].

The fragrant plant *S. aromaticum* is extensively used in the food and cosmetic manifactres as a component in lotions, fragrances, and food preservation. Moreover, it is used to treat many diseases [7,8] ,blocking the formation of biofilms and various virulence factors. It is

also used as an antiseptic in the treatment of oral infections [9,10]. *Glycyrrhiza glabra* Linn. which is a member of the Fabaceae family and its root extract is widely used in traditional medicine, food supplements, and cosmetics. In traditional medicine, the methanol extract of *G. glabra* roots has shown good antioxidant activity [11,12]. It contains a wide range of bioactive compounds, including flavonoids and triterpene saponins; therefore, they are being increasingly used for their biological benefits, including cytotoxic, antibacterial, antifungal, anti-inflammatory, and antioxidant properties [13–15]. The medicinal properties of licorice in dental health have recently attracted considerable attention [16].

This study suggested clove and licorice extract as an herbal medication to ascertain its efficacy against the oral pathogen *S. mutans* because few studies have been conducted on the cytotoxicity and antibacterial activities of medicinal herbs (clove and licorice) against oral infections in Iraq. Antibacterial phytochemicals found in plants and other natural sources are highly effective in preventing the growth of harmful bacteria, particularly Streptococcus. Recently, there has been a focus on identifying natural compounds that help fight *S. mutans* [17]. In order to lessen this issue, the development of novel treatment approaches for this oral condition may be helpful.

The main objective of this study was to determin the inhibitory effects of *G. glabra* and *S. aromaticum* extracts alone or in combination on *S. mutans* and compare to those of a mouthwash (chlorhexidine).

METHODOLOGY

Sample Collection and Bacterial Growth Conditions

A total of 100 specimens were obtained from patients clinically diagnosed by dental physicians with dental caries, who visited the Specialized Dental Center Hay Al Hussein Specialized Center at Mysan City-Iraq, between May 2023 and July 2023, (swabs from the mouth cavity of patients with different dental caries (pit, fissure, and dental roots)). The samples were transferred to a microbiological laboratory. The specimens were cultured in peptone water and streaked on Mutans-Sanguis agar medium. Specimens were transported to the laboratory immediately after collection and processed on the same day. The sample was vortexed (15sec,) and diluted 1:1000 in isotonic saline solution prior to inoculation. One loop (1/1000 ml of sample) was inoculated on the Mutans Sanguis Agar prepared., for 48 h under anaerobic conditions in an anaerobic environment (anaerobic container, and sterile transparent adhesive tape was used to seal the container cover) created by an anaerogen gas pack (Oxoid Ltd., England).

Preparation of Methanolic *Glycyrrhiza glabra* and *Syzygium aromaticum* Extracts

Glycyrrhiza glabra Extract

Plant roots were washed thoroughly with distilled water to remove dust. The samples were then dried in a shady place at room temperature. The dried plant material was ground to a fine powder using an electric grinder and stored at room temperature in airtight containers in the dark for further use. A methanol solution of licorice root was prepared by mixing500 g powder of root powder with 2500 ml of 95% methanol in a Soxhlet apparatus for 8h. The resulting extract was filtered through Whatman filter paper, and the solvent was evaporated under vacuum using a rotary evaporator at 60ºC for 5h. The extract was then dried at room temperature until the solvent was completely evaporated. The extract was collected in an airtight container and stored at 4^0C till further analysis [18]. One gram of the dry substrate was dispersed in 100 ml of 95% methanol to obtain a 1% stock solution.

Syzygium aromaticum Extract

The clove was washed thoroughly with distilled water to remove dust. The samples were then dried in a shady place at room temperature. The dried plant material was ground to a fine powder using an electric grinder and stored at room temperature in airtight containers in the dark for further use. The powder (200 g) was mixed with 2000 ml of 95%methanol as the solvent in a Soxhlet apparatus for 8h. The resulting extract was filtered through Whatman filter paper, and the solvent was evaporated under vacuum using a rotary evaporator at 40ºC for 5h. The extract was then dried at room temperature until the solvent was completely evaporated. The extract was collected in an airtight container and stored at 4^0C till further use [16]. One gram of the dry substrate was dispersed in 100 ml of 95% methanol to obtain a 1% stock solution. The dried concentrated crude extracts were weighed to calculate the yield using the following equation [19]:

Yield Percentage = (dry weight of the extract/dry weight of sample) x100

Phytochemical Profiling

Preliminary screening for the presence of phytoconstituents (Primary and Secondary metabolites) in all extracts was carried out as described previously[20,21].

Antibacterial Activity of *Glycyrrhiza glabra* and *Syzygium aromaticum* Extracts

Well Diffusion Methods

The antimicrobial activity of the methanolic extracts of clove buds, licorice roots, and their combination was determined by the agar well diffusion method to determine the zone of inhibition against *S. mutans* bacteria. The bacterial culture was inoculated onto Muller Hinton agar plates, and a sterile cotton swab was inserted into the suspension to remove excess fluid. The swab was then streaked on the plate to ensure uniform

growth, and the plates were dried for 15 minutes. Five wells (6 mm diameter) were punched in inoculated agar, and a stock solution of plant extract was prepared by dissolving dried extracts in methanol to a final concentration of 400 mg/ml. 20 µl of each concentration (50, 100, 200, 300, and 400) was transferred to the wells using a micropipette. The extract was diffused onto plates for 10 minutes, followed by anaerobic growth and incubation at 37°C for 24 hours. Results were recorded by measuring the inhibition zone diameter, and the experiments were conducted in triplicates [22].

Determination of Minimum Inhibitory Concentration (MIC), (Sub-MIC) and (MBC)

To determine the efficacy of *G. glabra*, *S. aromaticum* and their combined extract, as well as mouthwash (chlorhexidine), on *S. mutans* bacteria, a broth microdilution assay was used to estimate the MIC, Sub-Minimum Inhibitory Concentration (sub-MIC), and Minimum Bactericidal Concentration (MBC) according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [23]. A study was conducted on S. mutans in sterile, polystyrene 96-well-flat bottom plates. A stock solution was added and diluted two-fold with brain heart infusion broth (BHIB), and each well was inoculated with bacterial suspension equivalent to McFarland standard no 0.5. The final concentrations reached were 200-1.56µg/ml. Free controls were kept for each test plate, and all plates were incubated at 370C for 24 hours in an anaerobic environment created by an anaerogen gas pack. Resazurin solution (30 µL) was added to each well of a microtiter plate and incubated at 37^0 C for 30 min. No color changes were observed; blue/purple indicated no bacterial growth, whereas pink/colorless indicated bacterial growth [17]. Rows with no color change were scored above the MIC, while the last blue well was recorded as MIC, and the next pink well was recorded as sub-MIC.

Statistical Analysis

Analysis of variance (ANOVA) was used to analyze the differences between the studied treatments according to a Completely Randomized Design (CRD), and the Least Significant Difference (LSD) test was used to compare significance between means. Statistical significance was considered when the P-value was ≤ 0.01[24].

RESULTS

Glycyrrhiza glabra and *Syzygium aromaticum*

Extraction yields

The physical characteristics of The dried extracts of *S. aromaticum* and *G. glabra* were dark brown and light brown, respectively. The extraction yield serves as a gauge of the efficiency of the solvent for extracting particular constituents from the raw material. It will provide insight into how plant's extractability is under different conditions. The extraction yields from 500 g of clove buds or Licorice roots in methanol solvent were determined (Table 1).

Table 1: The percentage of crude *S. aromaticum* and *G. glabra* extract yield.

Plant	Part of plant	Sample weight(g)	Extract Colour	Extract dry weight (g)	Percentage of dry extract weight (%)
S. aromaticum	Flower buds	500	Dark brown	140.91	28.18
G. glabra	Roots	500	Light brown	98.69	19.74

Qualitative Screening of Phytochemicals in *Syzygium aromaticum* and *Glycyrrhiza glabra* Extracts

The presence or absence of certain phytoconstituents can be ascertained by applying phytochemical screening techniques using different chemical reagents. The methanolic extracts of *S. aromaticum* and *G. glabra* are listed in Table 2.

Table 2: Phytochemical Screening of *S. aromaticum* and *G. glabra* extracts.

Phytochemical	Phytochemical Screening		
	Clove	Licorice	Clove + Licorice
Alkaloids	+	+	+
Flavonoids	+ +	++	++
Polyphenolic Compounds	+	+	+
Tannins	++	++	++
Terpenes	+	+	+
Saponins	+	+	+
Glycosides	+	+	+
Steroid	+	+	+
Proteins	-	+	+
Coumarins	+	+	+
Carbohydrate	+	+	+
Volatile	+	+	+
Amino Acid	-	+	+
Fat and Oil	+	+	+

(+)=Minimal reaction (++) = Moderate reaction (+++) = Strong reaction

Antibacterial Activity of *Glycyrrhiza glabra* and *Syzygium aromaticum* Extract

Well Diffusion Method:

All *S. aromaticum*, *G. glabra* and combined extracts were investigated for their antimicrobial activity against three isolates *S. mutans* gram-positive bacteria using the simple agar well diffusion method (Table 3; Figures 1-3). The effect of methanolic extracts of *S. aromaticum*, *G. glabra* and the combined extract on the three strongest *S.mutans* isolates from dental caries was studied because of their high resistance to antibiotics.

Table 3: Zone of inhibition (mm) of *S. aromaticum* and *G. glabra* crude extracts against *S. mutans*.

Isolates	Treatment	Extract Concentration(mg/ml)					Mean	LSD 0.01
		50	100	200	300	400		
74	Clove	11.33	12.33	13.67	14.67	16.0	11.33	0.87
	Licorice	13.67	15.67	18.33	17.67	18.0	13.89	0.92
	Clove + Licorice	12.67	14.67	17.0	22.67	23.67	15.11	0.79
	Mean	12.56	14.22	16.33	18.33	19.22	13.44	_
80	Clove	13.67	15.0	16.67	19.33	20.67	14.22	0.85
	Licorice	12.67	15.0	16.67	17.0	18.33	13.28	1.31
	Clove + Licorice	13.67	15.67	16.67	18.33	17.67	13.67	0.99
	Mean	13.33	15.22	16.66	18.22	18.89	13.72	_
46	Clove	12.0	12.67	15.67	17.0	19.33	12.78	0.79
	Licorice	12.67	15.0	16.67	17.0	18.33	13.28	0.71
	Clove + Licorice	12.67	14.67	17.0	20.0	21.33	14.28	1.13
	Mean	12.44	14.11	16.44	18.0	19.67	13.44	_
Mean		12.78	14.52	16.48	18.18	19.23	13.54	_
LSD 0.01	Concentrations	0.27					1.40	
LSD 0.01	Concentrations x Treatments x Isolates	0.82						
P≤ 0.01: Highly Significant N.S. Not Significant								

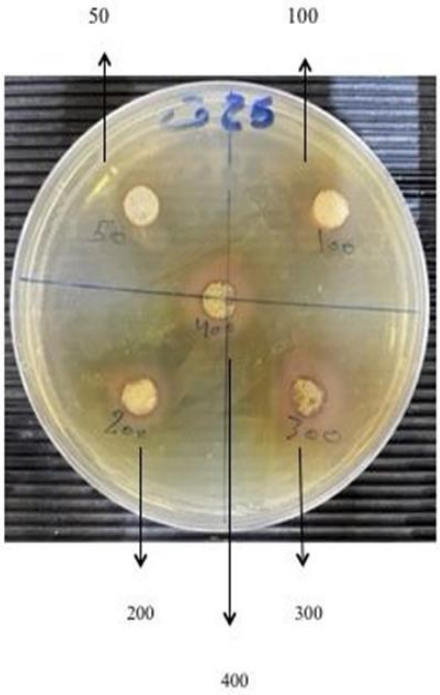

Fig. 1: Antibacterial activity of *S. aromaticum* extract against *S. mutans*.

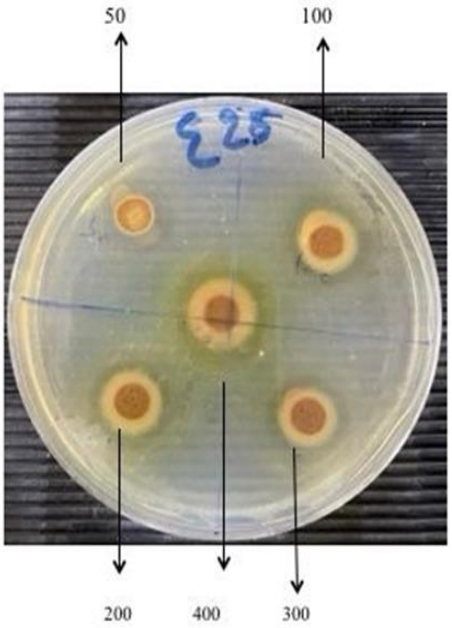

Fig. 2: Antibacterial activity of *G. glabra* extract against *S. mutans*

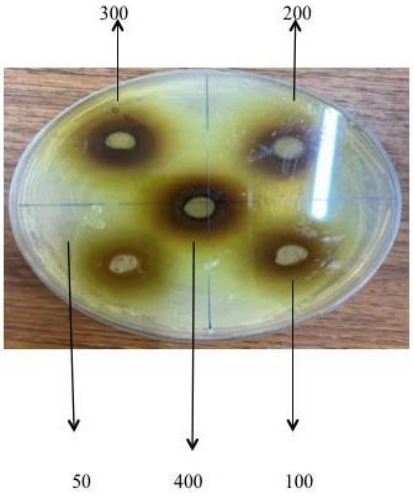

Fig. 3: Antibacterial activity of combined extract off *S. aromaticum* and G. *glabra* against *S. mutans*

Minimum Inhibitory Concentration (MIC) and Minimum Bacterial Concentration (MBC)

Based on the sensitivity to antibiotics test (unpublished data), the five strongest resistant *S. mutans* isolates were chosen to evaluate the range of intrinsic resistance of caries-related streptococci. The total MIC, Su-MIC, and MBC values of methanolic extracts of clove buds, licorice roots, and their mixture were measured against the isolates of *S. mutans*.The MIC, MBC, and sub-MIC values against *S. mutans* isolates were used to evaluate chlorhexidine and the antibacterial activity of the plant extracts. They were effective against *S. mutans* that was isolated from dental caries and showed a wide range of inhibitory capabilities.

Table 4: MBC, MIC and Sub-MIC of leaf *R. communis* extract on *E. coli* and *S. aureus* isolates

Extract		Isolates				
		74	**80**	**46**	**54**	**57**
Clove	**MBC**	25	12.5	12.5	12.5	25
	MIC	12.5	6.25	6.25	6.25	12.5
	Sub-MIC	6.25	3.125	3.125	3.125	6.25
Licorice	**MBC**	200	200	200	50	6.25
	MIC	100	100	100	25	3.125
	Sub-MIC	50	50	50	12.5	1.5625
Clove + Licorice	**MBC**	50	50	50	25	12.5
	MIC	25	25	25	12.5	6.25
	Sub-MIC	12.5	12.5	12.5	6.25	3.125
Mouthwash (Chlorhexidine)	**MBC**	25	25	25	12.5	6.25
	MIC	12.5	12.5	12.5	6.25	3.125
	Sub-MIC	6.25	6.25	6.25	3.125	1.5625

The MICs of licorice against the isolates74, 80,46and54 were less effective than those of clove or their mixture and chlorhexidine, as evidenced by the need for a higher concentration (100, 100, 100 and25µg/ml), and higher concentrations (200,200,200and 50 µg/ml) for MBC and (50, 50,50and12.5 µg/ml) for sub-MIC, respectively, to inhibit *S. mutans* bacteria, indicating that these isolates possessed intrinsic resistance to licorice extract. MIC/MBC values were similar in tested isolates 74,80 and 46; however, isolate 57 was sensitive and required only 6.25, 3.125 and1.5625 µg/ml for MBC, MIC, and sub-MIC, respectively (Figure 4). In the present study, the clove extract exhibited inhibitory effects on MBC, MIC, and Su-MBC against all isolates of *S. mutans* at values less than those of licorice, a mixture of clove and licorice, and chlorhexidine, which indicated that clove extract was more effective (Table4).

The methanolic extracts of cloves inhibited the growth of isolates 80, 46, and 54 *S. mutans* with MICs of 6.25µg/ml. The methanolic extract of clove also had bactericidal activity with an MBC of 12.5 µg/ml, and a Sub-MIC of3.125 µg/ml on the same isolates and started showing resistance after these concentrations. While showed less inhibitory activity on the isolates 74 and 57 as compared to other isolates with MIC, MBC and Sub-MIC values of 12.5, 25 and 6.25µg/ml, respectively (Figure 5).

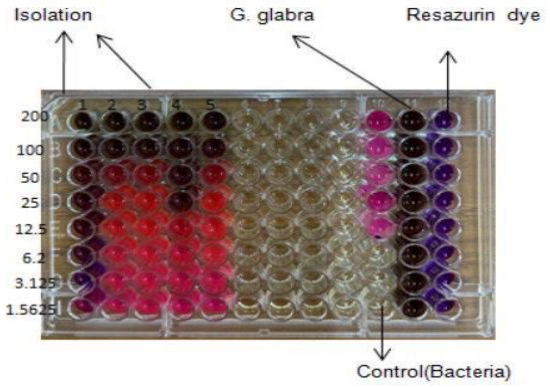

Fig. 4: MIC of root *G. glabra* extract on *S. mutans* isolates Isolate No., 5=74, 2=80, 3=46, 4=54, 57=1

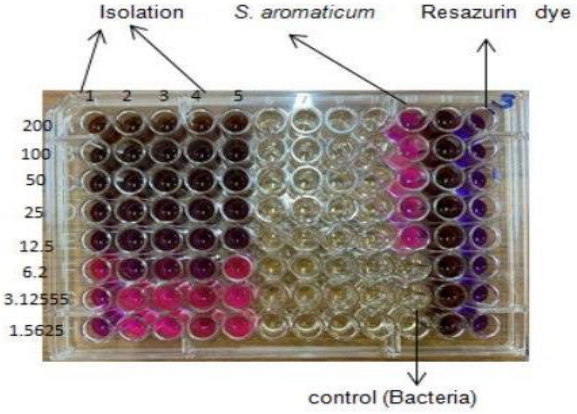

Fig. 5: MIC of S. aromaticum extract on S. mutans Isolate No., 5=74, 2=80, 3=46, 4=54, 57=1

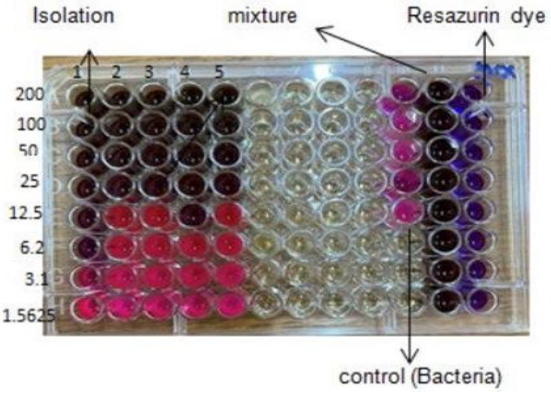

Fig. 6: MIC of the extract mixture of *G. glabra* and *S. aromaticum* against *S. mutans* isolates

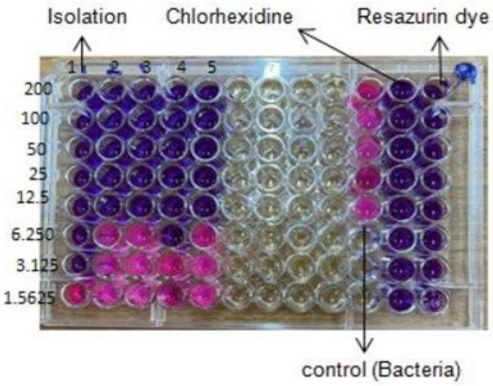

Fig. 7: MIC of Chlorhexidine on *S. mutans* isolates Isolate No., 5=74, 2=80, 3=46, 4=54, 57=1

Five S. *mutans* isolates were tested for their range of chlorhexidine resistance. Isolates 74, 80, and 46 all had MIC values of 12.5 µg/ml, but isolate 54 (6.25 µg/ml) and isolate 57 (3.125 µg/ml) MIC values declined. The MBC and sub-MIC for the isolates followed the same pattern (Table 4). Isolates 74, 80, and 46 had MBC and sub-MIC values of 25 and 6.25 µg/ml for chlorhexidine, respectively. The isolate 54 had values that decreased to 12.5 and 3.125 µg/ml, while the isolate 57 had even further declines to 6.25 and 1.5625 µg/ml (Figure 7). Based on the obtained data, the isolates were ranked 74, 80, 46, 54, and 57, in order of resistance.

DISCUSSION

This study shows that the dry weight yields of clove buds and Licorice roots methanolic extracts was found to be 114.91 g (22.98%) and 98.69 g (19.74%), respectively. This result was very close to another results of [25] who reported that the extraction of 50 g of clove powder using a methanolic solven yielded13.972g of extract. %. A previous study [26]. developed a *G. glabra* extract with the maximum yield (21.31 ± 0.64 weight percent) using the Soxhlet extraction method and methanol, which is comparable to the results of the current investigation.

The presence or absence of certain phytoconstituents can be ascertained by applying phytochemical screening techniques using different chemical reagents. For *S. aromaticum* extract, the methanol extract of *S. aromaticum* tested positive for the following secondary metabolites: alkaloids, flavonoids, polyphenolic compounds, tannins, terpenes, saponins, glycosides, steroids, coumarins, carbohydrate volatiles, and fat and oil. However, the methanol extract was negative for proteins and amino acids. These findings are consistent with previous findings[25], which indicated that the extract of clove was devoid of proteins and amino acids. The antioxidant, antibacterial, anti-inflammatory, antiviral, and anticancer activities of clove are attributed to a number of phytochemical compounds found in *S.*

aromaticum. Approximately 13 phytochemical components, including tannins, ascorbic acid, and phenolic compounds, have been qualitatively identified in several clove extracts[25]. *G. glabra* consists of numerous components that can be extracted from its roots, those compounds tested positive in this study include: alkaloids, flavonoids, polyphenolic compounds, tannins, terpenes, saponins, glycosides, steroid, proteins, coumarins, carbohydrate, volatile, amino acids, as well as fat and oil. Licorice roots contain many chemicals that have been proven to have pharmacological benefits, including anticancer and anti-a number of other disorders [27]. All of the phytochemicals presented in Table2 are tested positive in the combination of clove and licorice (Table 2).

current study confirmed the effectiveness of plant extracts on bacterial isolates, as all extracts demonstrated clear inhibitory activity against the studied strains, with no recorded resistance from any of the isolates. It was observed that increasing the concentration of clove and licorice extracts, whether individually or in combination, led to a greater diameter of inhibition zones. The synergistic effect of the combined extract was particularly notable, enhancing the antibacterial activity more effectively compared to each extract used separately

Pei *et al* reported that the combined action of natural antimicrobials is more successful in inhibiting the growth of harmful bacteria than the use of individual antimicrobials. This phenomenon is believed to occur when multiple natural antimicrobials are administered together, creating a synergistic effect [28]. The findings of the present study indicate that the zone of inhibition of clove against *S. mutans* is greater than that of [29], who reported clove extract activity against *S. mutans* with a 13 mm zone of inhibition. Additionally, the zone of inhibition was found to be larger than that of [30], who reported a 3.8 mm zone of inhibition. A. indica has been studied in dentistry because of its ability to protect against oral pathogens. Furthermore, by displaying anti-adherence activity, it alters bacterial adhesion and the organism's ability to colonize [31]. Licorice is an inexpensive substitute for synthetic antimicrobial agents, making it a safe and natural herbal root extract of plant-derived phytochemicals. It has been shown to have specific antibacterial activity, lowers oral *S. mutans* levels, and is increasingly being used to treat oral diseases [32].

The susceptibility of various *Streptococcus mutans* clinical isolates to a clove and licorice extract mixture was evaluated, indicating potential differences in drug resistance. MBC, MIC, and sub-MIC values were monitored across five clinical isolates from passages 1 through 5 to assess the resistance spectrum of these isolates to the extract mixture. Figure 6 (passages 1–5) provides an overview of the observed variations in susceptibility among the tested isolates.

The current study results showed a lower MIC/MBC than other report [33], who discovered that the MBC values observed varied from 3.9 µg/ml to 500 µg/ml, while the MIC concentrations for all 16 extracts examined ranged from 0.97µg/ml to 125µg/ml. The results of this study imply that cloves may be a significant source of antimicrobial agents. The MIC of clove extract against S. mutans was 5.5 mg/ml (30). The results of the present study are consistent with those of earlier investigations [34–36] that examined the effects of a range of herbal and medicinal plant extracts and found that they had exceptional antibacterial effectiveness against both cariogenic and non-cariogenic bacterial species.

CONCLUSIONS

Based on the findings of this study, methanolic extracts of licorice, clove, and their combination have antibacterial action against *S. mutans*, and are, therefore, promising natural antidental caries. Combining extracts from different plants can work well because one plant can compensate for the shortage in the secondary components of other plants. This was determined by combining extracts from licorice and cloves.

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