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EFFECT OF ROSEMARY ENDOPHYTIC FUNGAL EXTRACT ON MICROHARDNESS OF ENAMEL AND ITS INHIBITORY EFFECT ON GLUCOSYLTRANSFERASE ENZYME OF *STREPTOCOCCUS MUTANS* – AN IN VITRO STUDY

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ABSTRACT

Background: Streptococcus mutans are involved in caries by forming biofilm polysaccharides through their glucosyl transferase enzymes. Natural plant extracts can inhibit glucosyl transferase production and bacterial activity. Endophytic fungi inside plant tissues can produce bioactive compounds similar to those produced by their host plants.

Aim: Compare the inhibitory effect of rosemary endophytic fungal extract on S.mutans glucosyl transferase to rosemary and chlorhexidine and their effect on enamel microhardness.

Methods: Glucosyltransferase was purified from S.mutans. The ability of the total metabolites and endophytic fungi extracted from rosemary to inhibit the purified glucosyltransferase compared to chlorhexidine and artificial saliva.13crowns of human molars were subdivided into 4 equal parts giving 4groups for microhardness and biofilm evaluation. The microhardness was evaluated at baseline and after treating the four subgroups using the rosemary extract, chlorhexidine, artificial saliva and endophytic fungal extract for 24and72h.The biofilm inhibition was tested using SEM after 24and72h.

Results: Nine endophytic fungal strains were recovered from rosemary. Chaetomium globosum, Alternaria alternata and Aspergillus niger extracts showed positive inhibitory effect on glucosyltransferase 96.25%, 90.9%and81.74% respectively. While, those of rosemary extract, chlorhexidine and artificial saliva were 36.19%, 86.38%and0.27%. Chaetomium globosum extract showed the highest inhibitory effect on biofilm formation in comparison to chlorhexidine and rosemary extract after 24and72h. The highest microhardness value for all groups was at baseline then microhardness decreased after24 and72h except for Chaetomium globosum and rosemary extract where microhardness increased after72h to be higher than that after24h.

Conclusion : Endophytic fungal extract of rosemary can inhibit S.mutans glucosyltransferase and its biofilm formation where it can remineralize initial carious lesions.

KEYWORDS: Endophytic Fungi, Rosemarinus officinalis, Chlorhexidine, Plant Extract, Chaetomium globsum

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INTRODUCTION

The oral cavity is an intricate ecosystem of hundreds of microorganisms. These microorganisms form a biofilm which covers all the surfaces of the oral cavity, both hard and soft tissues. This biofilm is present in a state of balance, in which development of disease occurs once this balance is disturbed from the normal inhabitant of the oral cavity [1–3]. Dental caries is a widespread multifactorial disease caused by acid producing bacteria in the dental biofilm. Examples of cariogenic bacteria include *Streptococcus mutans*, *Streptococcus sorbinus*, *Streptococcus salivarius*, and *Streptococcus sanguis*. Other examples of cariogenic bacteria include *Lactobacillus acidophilis*, *Lactobacillus casei*, *Actinomyces naeslundii*, *Actinomyces viscusus* which represent a minority in the oral flora. A combination of several factors, such as carbohydrate intake, time, and diminished clearance of food debris by defective salivary flow, causes a pH shift toward acidic condition where the cariogenic microorganism dominates the imbalanced biofilm leading to caries occurrence [4–7].

Caries prevention has been studied throughout history, with more understanding of the interactions that occur in the oral biofilm, the approach to caries prevention has evolved. The goal now is no longer random elimination of oral bacteria but specific targeting of cariogenic bacteria or their virulence factors to achieve long term success [5, 7, 8]. The extracellular polysaccharide (EPS) is considered one of the main virulence factors as it plays a major role in the attachment of bacteria to tooth surface and to each other. Glucosyltransferase enzymes (GTFs) are considered one of the main enzymes that are responsible for the formation of the extracellular polysaccharides, which catalyze the formation of water-soluble glucans allowing bacterial attachment with one another. Furthermore, formation of water-insoluble glucans gives bulk to the biofilm. It was investigated that *Streptococcus mutans* are one of the main microorganisms forming the EPS through their gtfs [6, 9–11]. Many chemical agents showed antibacterial effects on oral pathogens such as chlorhexidine and fluoride. Unfortunately, many of these chemical agents showed side effects from altered taste to disturbing the oral flora in addition to the increasing antimicrobial resistance. Nowadays, scientists have started to target the natural products with antibacterial properties to reduce the chemicals side effects and overcome antimicrobial resistance [10, 12, 13]. Several researchers have reported the ability of rosemary to remineralize initial caries lesions in addition to its ability to inhibit glucosyltransferase and subsequent glucan production of *Streptococcus mutans* and *Streptococcus sorbinus* respectively [14-19].

Endophytic fungi live inside the plant tissues without causing any pathological symptoms [20–22]. It can produce different bioactive compounds similar to those produced by their host plants by using only small amount of the tested plants eliminating the need to over harvest medicinal plants which exhausts our reservoirs of these beneficial plants and endangers their existence [23–26]. Endophytic bioactive compounds were used in different industries; pharmaceutical, clinical, agriculture, and dental industries [27–29]. By screening all available data till now, very few studies were carried out to study the inhibitory effect of endophytic fungi on the bacterial glucosyltransferase enzyme. This has targeted the research to use the endophytic fungal extracts as an alternative natural source against cariogenic oral bacteria. Therefore, the objective of this study was to test the inhibitory effect of rosemary endophytic fungal extract on Streptococcus mutans glucosyltransferase in comparison to Rosemary and chlorohexidine, estimating their demineralization effect on enamel discs through microhardness testing. The null hypothesis was that there is no difference regarding the inhibition of glucosyltransferase enzyme and microhardness of enamel treated with endophytic fungal extract of rosemary compared to chlorhexidine gluconate and rosemary extract.

MATERIALS AND METHODS

Samples

Plant samples

Rosemary samples were collected from different local shops, farms over Cairo. Collection and experiments of rosemary plant followed the relevant institutional, national, and international guidelines and legislation.

Microorganisms

Positive glucosyltransferase *Streptococcus mutans* ATCC 25175 was received from Ms. Hams Atef in the faculty of pharmacy, October University for Modern Sciences and Arts.

Teeth samples

13 sound extracted third molar teeth were collected from the outpatient clinic of dental school MSA University after collecting informed consent from the patients and the hospital, according to the acceptance of the ethical committee number (ETH25).

Fermentation of *S. mutans* and glucosyltransferase assay

Streptococcus mutans were cultivated in 1500 ml brain heart broth media supplemented with 5% sucrose for 15 h at 37 °C at 120 rpm**.**

The grown *Streptococcus mutans* culture was centrifuged at 4 °C for 15 minutes at 12,000 rpm. Assay mixture was prepared as follows; 1.35 ml of 0.4 gm sucrose mixed with 10 ml phosphate buffer (50 mM, pH 6) and 0.15 ml enzyme extract. The mixture was incubated at 37°C for 20 minutes. The enzymatic reaction was stopped by boiling for 20 minutes at 90 °C. To estimate the released reducing sugar, 1 ml of the mixture was mixed with 3ml of DNS reagent. After all the samples were cooled, the absorbance was estimated at 540 nm. The amount of released reducing sugar was estimated using the previously established standard curve of glucose. Glucosyltransferase was estimated by measuring the amount of released reducing sugar using DNS as reagent. Where, one unit of enzyme activity was defined as that amount of glucosyltransferase which released 1.0 umol of reducing sugar per min from sucrose [19]**.**

Purification of glucosyltransferase

Chilled acetone method

The extracellular protein precipitation was carried out by cold acetone precipitation method [30]. Acetone was kept at -80 °C prior to precipitation, 4X cold acetone was added to the culture filtrate of the selected sample and it was incubated at -20°C for 2 h, the samples were centrifuged at 13,000 rpm for 15 minutes at 4 °C to precipitate the total protein. The supernatant was discarded, and pellet obtained was dissolved in a suitable volume of 50mM Tris buffer (pH 7) and stored at -80°C till further use.

Ion Exchange chromatography

Pre-packed ion exchange column (S-Sephadex Fast Flow, 5 ml, GE) was used to fractionate the glucosyltransferase precipitate obtained from the acetone protein precipitation step. Phosphate buffer (50 mM, pH 7) was used to equilibrate the column. The sample was placed into the column with slow flow rate. Gradient concentrations of NaCl (0-1M) with a rate of (1.5 ml/min) was used to elute the enzyme. Followed with estimation of the glucosyltransferase activity and total protein estimation for the obtained fractions. Fractions that showed the highest enzymatic activity were pooled together and concentrated using chilled acetone [31].

Estimation of total protein

It was estimated for the crude enzyme and after each purification step using a Fluorometer (Qubit 3.0, LifeTech).

Characterization of proteins and molecular weight identification by SDS- PAGE

Using the protocol described by [32], the SDS working procedures were performed with slight

modifications [33], where 12% separating gel (6.8) and 5% stacking gel (pH 8.8). Through the interference of authentic protein marker (BLUEstain TM 2 protein ladder, 5-245 kDa), the molecular weight of the appeared protein bands was estimated. The marker was composed of about 0.1 - 0.4 mg/ml of each protein in the buffer (20 mM Tris-phosphate, pH 7.5 at 25°c), 2 % SDS, 0.2 mM Dithiothreitol, 3.6 M Urea, and 15% (v/v) Glycerol. Coomassie brilliant blue R-250 was used to stain the protein bands.

Isolation and Identification of endophytic fungi

Rinsing of the freshly collected aerial plant parts was done under running tap water for 10 minutes. The stem is cut into pieces of 2 cm in length each, while the leaves were cut into 4 cm^2 segments. Sodium hypochlorite and 70% ethanol were used to achieve surface sterilization then the samples were isolated on appropriate media. Malt extract agar (MEA) and Potato Dextrose Agar media (PDA) supplemented with Rose Bengal (1/1500), to suppress bacterial growth, were used for primary isolation [34]. Czapek's Yeast extract Agar (CYA), Malt Extract Agar (MEA) and Potato Dextrose Agar (PDA) and Water Agar (WA) were used for isolation and identification [35].

Recovered fungal endophytic taxa were identified morphologically to the species level on standard media based on the phenotypic means and the relevant identification keys [36–44].

Testing the inhibitory effect of endophytic fungi

The inhibitory effect was estimated by measuring the amount of released reducing sugar using DNS as reagent. Assay mixture was prepared as follows; 0.45 ml of 0.4 gm sucrose mixed with 10 ml phosphate buffer (50 mM, pH 6), 0.05 ml enzyme extract and 0.45 ml of the inhibitors tested which were artificial saliva acting as negative control, chlorhexidine gluconate acting as positive control, and endophytic fungal cultures of rosemary acting as the interventions using the previously mentioned steps [19].

Preparation of the extracts

Preparation of the endophytic fungal extract

After testing the inhibitory effect of the different fungal isolates, the positive isolates were sub-cultured on Potato dextrose broth medium (1000 mL for each isolate). The inoculated media were incubated for 15 days at 28 °C and 120 rpm. The fungal cultures were filtered and centrifuged at 8000 rpm for 20 min. The total metabolites were extracted using ethyl acetate (1:1; v/v). The mixture was filtered and the residue was re-extracted with fresh ethyl acetate overnight. The combined solution was centrifuged at 12,000 rpm for 10 min and evaporated on a rotary evaporator. The extract was reconstituted in dimethyl sulfoxide (DMSO) to a concentration of 400 mg/ml for subsequent experimentation [45].

Preparation of the rosemary extract

The dried ground leaves of rosemary were extracted with methanol (5 ml/g), using a magnetic mixer at room temperature for 3 h. After extraction, the mixture was filtered and the residue was re-extracted with fresh methanol (5 ml/g) overnight. The combined methanolic solution was centrifuged at 12,000 rpm for 10 min and evaporated on a rotary evaporator. Methanolic extract was reconstituted in dimethyl sulfoxide (DMSO) to a concentration of 400 mg/ml for subsequent experimentation [19].

Artificial Saliva preparation

The composition of artificial saliva was as follows; Methyl-p- hydroxybenzoate 2.00 g/L, sodium carboxymethyl cellulose 10.00g/L, KCl 0.625 g/L, $MgCl_2$.6H2O 0.059 g/l, $CaCl_2$.2H2O 0.166 g/L, K_2HPO_4 0.804 g/L, KH_2PO_4 0.326 g/L. pH was adjusted to 6.75 using KOH [46].

Chlorhexidine gluconate solution preparation 0.12% chlorhexidine gluconate solution was prepared with sterile-distilled water [47].

Estimation of inhibitory effect of the different extracts

The inhibitory effect of endophytic fungal extracts of rosemary (which showed positive inhibitory effect on glucosyltransferase enzyme from previously mentioned step), rosemary extract, chlorhexidine gluconate and artificial saliva was estimated by measuring the amount of released reducing sugar using DNS as reagent on the purified enzyme as previously mentioned [19].

Preparation of enamel specimens

The 13 extracted human third molars were cleaned using a low speed handpiece, polishing brush and polishing paste under water coolant. Then, the teeth were examined carefully using Stereomicroscope (X20) to ensure that the enamel is sound followed by storage in distilled water till time of usage for a period no longer than 1 month and during which period the distilled water was changed daily [48, 49]. The teeth were embedded in self cured acrylic resin blocks; approximately 1- 2 mm apical to the cementoenamel junction. The labial and lingual surfaces of each tooth were measured in a mesio-distal direction using a graph paper and a pencil to mark the middle part, then the crown was cut in 2 halves parallel to the long axis of the tooth in a bucco-lingual direction using a microtome under water coolant. Similarly, the mesial and distal surfaces were marked at the middle part and again cut along the long axis of the tooth in a mesio-distal direction using a microtome. Crown was then separated from the roots using cutting discs giving 4 parts of each tooth. Each part of the 4 parts of the same tooth were embedded from the dentin side in an acrylic resin block exposing the enamel surface [50]. Flattening of enamel surface was done using 800 and 1,200 grit silicon carbide paper in a circular motion, which was followed by polishing with 1 μm polishing suspension with a polishing cloth [51–53]. The specimens were then sterilized using an autoclave and divided among the 4 experimental groups with 13 specimens per group.

Treatment strategies

The enamel specimens were placed in glass beakers where 1 ml of different media of artificial saliva, chlorhexidine gluconate, rosemary extract and endophytic fungal extract of rosemary from the species which showed the highest inhibitory effect on gtfs solutions was applied on the enamel specimens in addition to 5 ml *Streptococcus mutans* suspension previously described enriched with 1% freshly prepared sucrose solution from 20% stock solution prepared with sterile distilled water for 24h [54] and 72h and incubated at 37°C. In which 24h period was enough to achieve in vitro enamel colonization and acid production [50] and was equivalent to 1 month in the oral environment [55]. Every 24h the specimens were removed, rinsed with sterile deionized water and placed in a new culture media. The pH of the culture was evaluated using a pH meter on 2h intervals to confirm the acidogenicity of the culture to simulate the oral environment.

Biofilm detection by SEM

After 24 and 72 h, glutaraldehyde (2.5%) was used in collection of PBS solution to fixate the specimens with adherent biofilms followed by washing with sterile water and dehydration using ethanol. An anhydrous carbon desiccator was used to perform carbon shadowing and reduced pressure dehydration. Scanning electron microscope was used to view the specimens at X3500 [56].

Microhardness evaluation

All 52 specimens were evaluated at baseline and after surface treatment using Digital display Vickers Microhardness tester Wilson Hardness with a Vickers diamond indenter and a load of 50g was applied on the enamel surface for 15 seconds. 5 indentations were done on each surface which will be applied equally over a circle not closer than 0.5mm from each other. Microhardness value was determined using the equation:

$$HV = 1.854\ F/d^2$$

Where, HV is Vickers hardness which is Kg force per square mm (KgF/mm2), F is the force applied to

the diamond in Kg- force, d is the average length of the diagonal left by the indenter in mm [57].

Statistical analysis

The data was explored for normality by checking the data distribution, and using the Shapiro-Wilk test. Data were found to be normally distributed and were analyzed using one-way ANOVA followed by Tukey's post hoc test for intergroup comparisons and repeated measures ANOVA followed by Bonferroni post hoc test for intragroup comparisons using R software for macOS Ver. 2023.03.0+386. The significance level was set at $p \leq 0.05$.

RESULTS

Production and Purification of Glucosyltransferase

Glucosyltransferase was produced with an activity of 206.72 ±5.9 IU/ml, specific activity of 0.007 IU/μg. The enzyme was partially purified on two main steps; protein precipitation using chilled acetone and ion exchange column chromatography (S-Sephadex Fast Flow).

The specific activity of the enzyme after the total protein precipitation was estimated to be 0.06 IU/μg and the purification fold was increased to 8.09. The precipitated protein was applied to S-Sephadex Fast Flow column eluting the enzyme with gradient of NaCl (0-1M, pH 7.0). The fractions eluted with 0.3 M NaCl showed the highest enzymatic activity, furthermore, it was collected together and concentrated using chilled acetone. The specific activity of the concentrated fractions was estimated to be 0.237 IU/μg and the purification fold increased to 31.77-fold (Table 1).

The molecular weight was estimated to be at the size of 70 kDa after comparing it to the standard protein ladder (Fig. 1).

TABLE (1) Purification profile of glucosyltransferase

Step	Activity (IU/mL)	Protein (μg/mL)	Specific activity (IU/μg)	Purification Fold	Enzyme recovery (%)
Culture filtrate	206.72 ±5.9	27600	0.007	1	100
Acetone	122.40 ± 7.79	2020	0.06	8.09	59.21
Ion-exchange	91.39 ± 3.15	384	0.237	31.77	44.21

Estimation of glucosyltransferase molecular weight by SDS PAGE

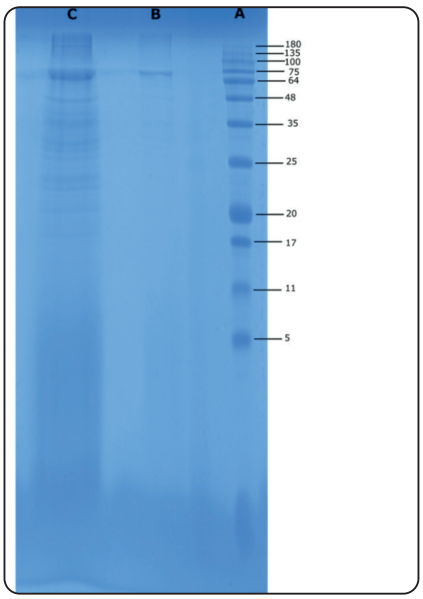

Fig. (1) Purified glucosyltransferase where A: ladder, B: purified enzyme and C: crude enzyme

Isolation and Purification of endophytic fungi

Nine different endophytic fungal strains were recovered from *Rosemarinus officinalis*. *Aspergillus niger* showed the highest count with 20 (CFU), followed by *Aspergillus flavus*, *Alternaria alternata*, *Chaetomiun globosum*, *Alternaria atra*, *Cladosporium cladosporioides*, *Alternaria solani*, *Mucor* and yeast, with a total count of 19, 15, 10, 7, 4, 3, 1 and 5 (CFU) respectively (Fig. 2).

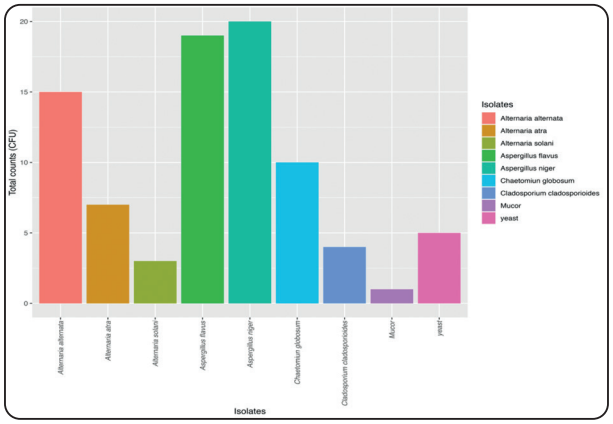

Fig. (2): Isolated endophytic fungi from Rosmarinus officinalis and their total count

Estimation of the inhibitory effect of the extracts

Three strains showed a positive inhibitory effect against glucosyltransferase, namely *Aspergillus niger* Tiegh, *Chaetomium globosum* Kunze and *Alternaria alternata* (Fr.) Keissl, with inhibition percentages of 81.74, 96.25 & 90.9 % respectively in comparison to the negative control. While, the other six strains showed negative results. Also, chlorhexidine gluconate showed an 86.38% inhibition, rosemary extract 36.19% inhibition and artificial saliva with 0.27% inhibition (Table 2, Fig. 3).

All the strains were identified morphologically and the positive strains were deposited in the culture collection of Suez Canal University fungarium under the accession numbers of SCUF0000314, SCUF0000315 & SCUF0000316 for *Aspergillus niger* Tiegh., *Chaetomium globosum* Kunze & *Alternaria alternata* (Fr.) Keissl. respectively.

TABLE (2) Glucosyltransferase activity and inhibitory effect of Chaetomium globosum Kunze, Alternaria alternata (Fr.) Keissl, Aspergillus niger Tiegh, chlorhexidine gluconate, rosemary extract and Artificial saliva

Fractions	Activity (IU/mL) ± SEM	% Inhibition
Chaetomium globosum Kunze	7.75*± 0.02	96.25
Alternaria alternata (Fr.) Keissl	18.81± 0.01	90.9
Aspergillus niger Tiegh	37.75± 0.08	81.74
Chlorhexidine Gluconate	28.15± 0.12	86.38
Rosemary	131.91±0.24	36.19
Artificial Saliva	206.16±0.16	0.27

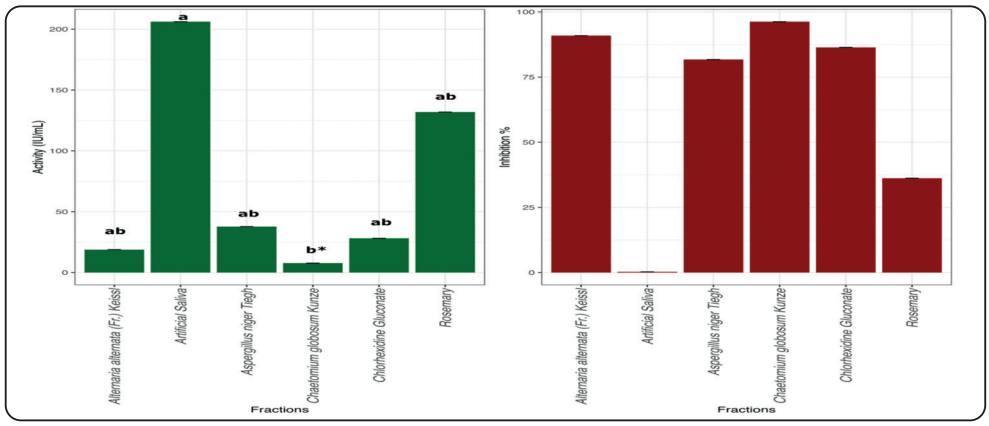

Fig. (3) Glucosyltransferase activity and inhibitory effect of endophytic fungal extracts isolated from rosemary.

Detection of Streptococcus mutans biofilm inhibition by SEM

The effect of the Rosemary & *Chaetomium globosum* Kunze extracts to inhibit the formation of *Streptococcus mutans* biofilm were tested against CHX as positive control and artificial saliva and DMSO as negative controls.

After 24 hours, *Chaetomium globosum* and rosemary extracts showed scattered cells of *Streptococcus mutans* on the surface of the prepared sample teeth with no signs of biofilm formation. *Chaetomium globosum* extract showed a high inhibitory effect on biofilm formation in comparison to chlorhexidine and rosemary extracts (Fig. 4 A, B & C), followed by the rosemary extract (Fig. 4 B). While the artificial saliva and DMSO showed mature biofilm formation (Fig. 4 D & E).

After 72 hours, *Chaetomium globosum* and rosemary extracts continued to show scattered cells of *Streptococcus mutans* on the surface of the prepared sample teeth with no signs of biofilm formation. *Chaetomium globosum* extract showed the highest inhibitory effect on biofilm formation in comparison to chlorhexidine gluconate and rosemary extracts (Fig 5 A, B & C). The rosemary extract showed an increase in the number of the scattered cells (Fig 5 B) in comparison with the image taken after 24 h for the same group (Fig 4 B). While the artificial saliva and DMSO samples continued to show mature biofilm formation. (Fig 5 D & E).

Testing the microhardness for the treated teeth

When comparing the differences between the used extracts there was a significant difference between values measured at different intervals ($p<0.001$). In the artificial saliva group, the highest

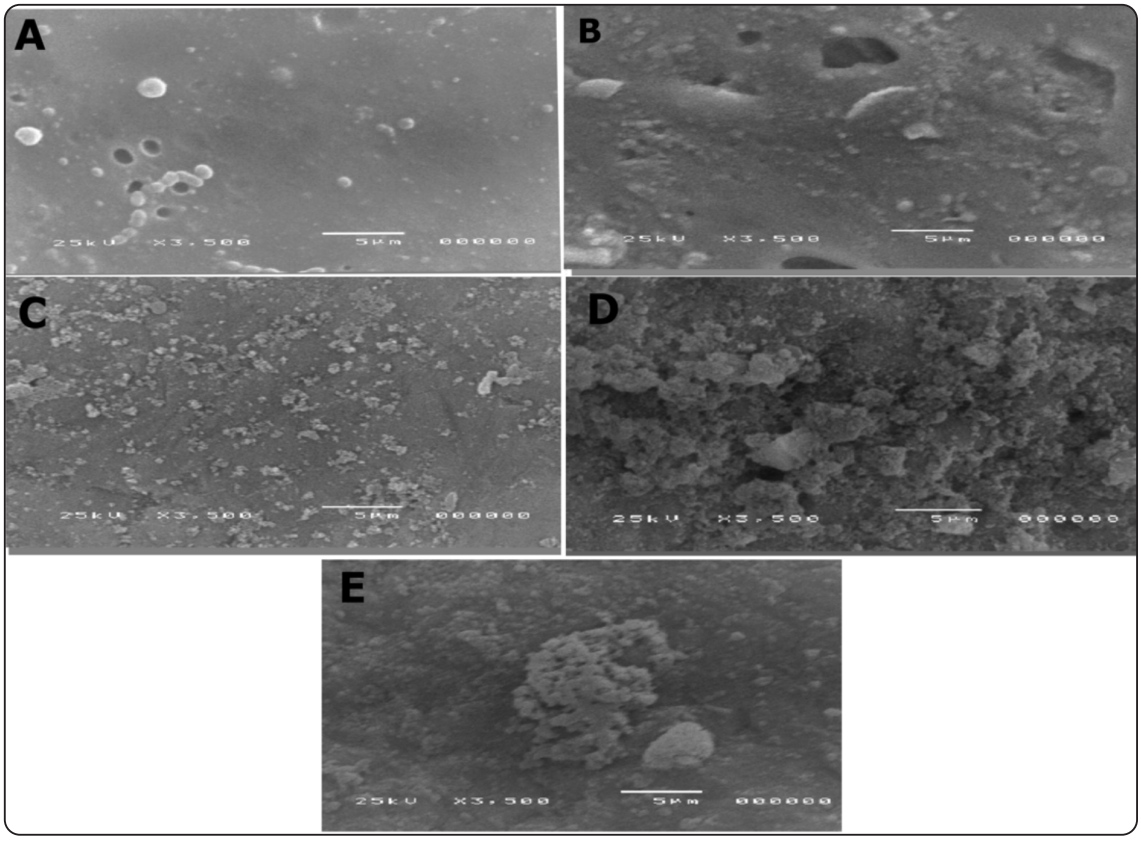

Fig. (4) SEM images of the specimens after 24 h under x3500 magnification. A: Chaetomium globosum Kunze extract, B: Rosemary extract, C: Chlorhexidine gluconate, D: Artificial saliva, E: DMSO

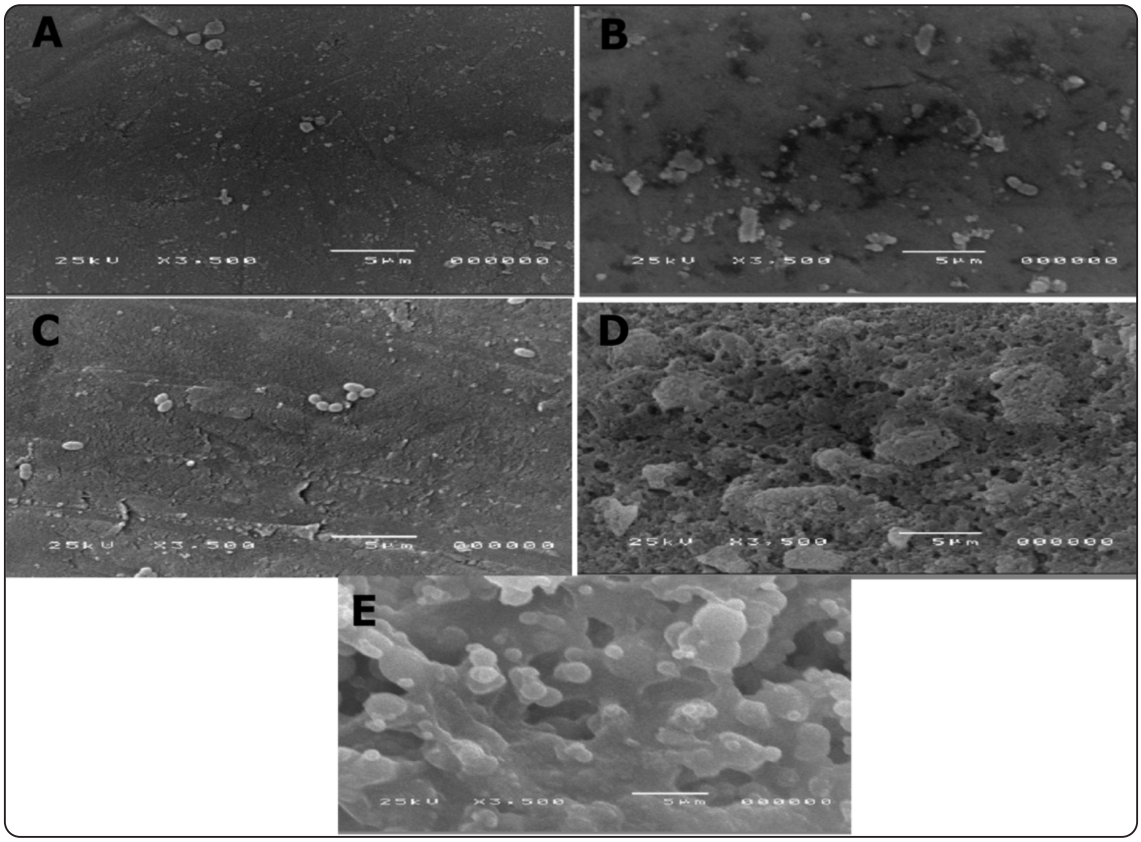

Fig. (5) SEM images of specimens after 72 h under x3500 magnification. A: Chaetomium globosum Kunze extract, B: Rosemary extract, C: Chlorhexidine gluconate, D: Artificial saliva E: DMSO

value was measured at baseline (261.93±30.16), followed by the value recorded after 24h (201.06±28.65), while the lowest value was found after 72h (152.15±19.64). In the chlorhexidine group, the highest value was measured at baseline (268.66±31.55), followed by the value recorded after 24h (193.60±35.03), while the lowest value was found after 72h (187.29±48.89). While in the rosemary extract group, the highest value was measured at baseline (265.52±36.05), followed by the value recorded after 72h (240.60±13.96), while the lowest value was found after 24h (188.10±48.26). Post hoc pairwise comparisons showed values measured after 24h to be significantly lower than values measured at other intervals ($p<0.001$).

As for the *Chaetomium globosum* extract group, there was a significant difference between values measured at different intervals ($p<0.001$). The highest value was measured at baseline (262.56±41.83), followed by the value recorded after 72h (214.16±44.33), while the lowest value was found after 24h (197.17±7.18). Post hoc pairwise comparisons showed values measured at baseline to be significantly higher than values measured at other intervals ($p<0.001$). (Fig. 6 & 7)

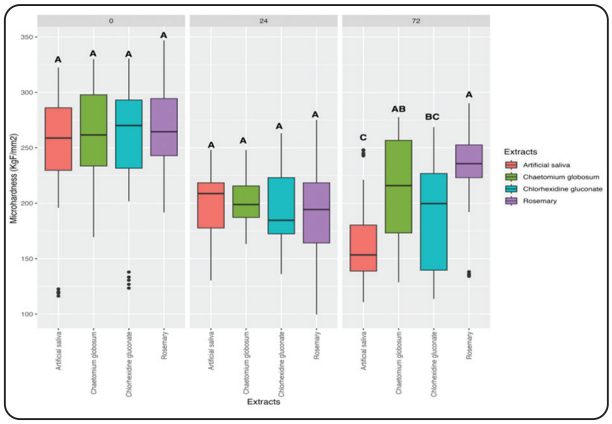

Fig. (6) The average micro-hardness values over the three-time intervals

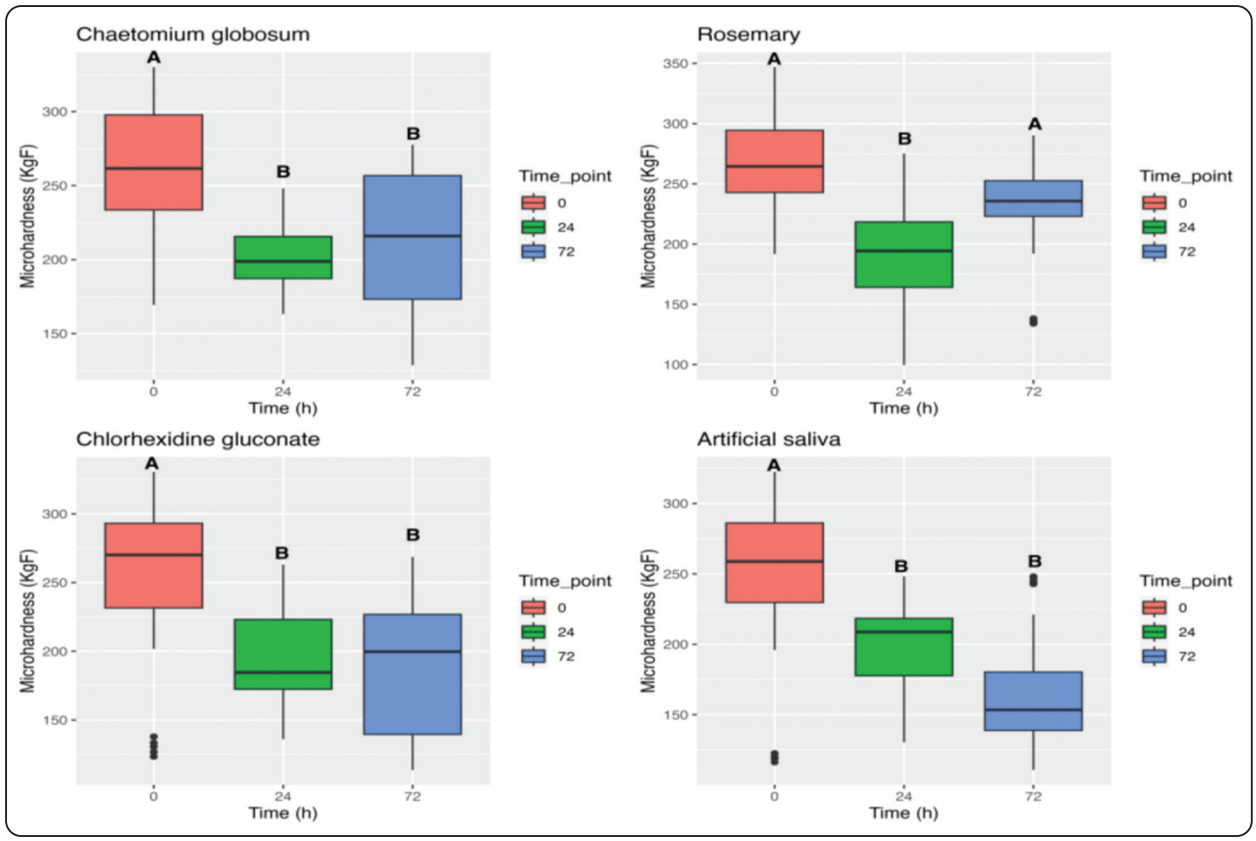

Fig. (7) The average micro-hardness values for different tested groups

Testing the pH values for the test media

When comparing the recorded pH values, there was a significant difference between different tested groups ($p<0.001$). The highest value was found in rosemary extract (6.47±0.04), followed by chlorhexidine (5.98±0.08) and *Chaetomium globosum* extract (5.98±0.02), while the lowest value was found in artificial saliva (5.62±0.03). Post hoc pairwise comparisons showed that the rosemary extract to have significantly a higher value than other groups ($p<0.001$). In addition, chlorhexidine and *Chaetomium globosum* extract showed significantly higher values than the artificial saliva ($p<0.001$) (Fig. 8).

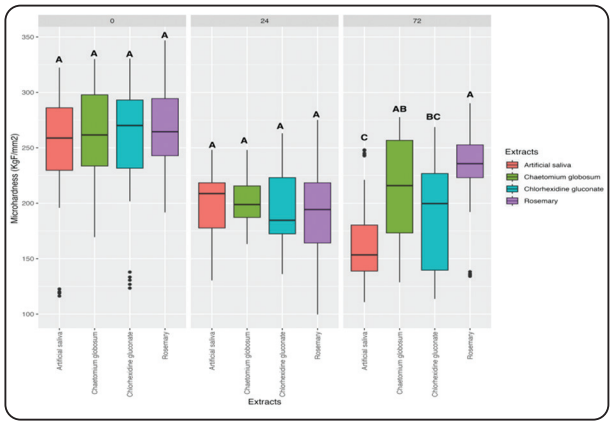

Fig. (8) The average pH values recorded after testing the four used extracts

DISCUSSION

Researchers now selectively target glucosyltransferase of cariogenic bacteria, as *Streptococcus mutans*, to inhibit the biofilm formation without adversely affecting the normal oral flora using either synthetic or natural compounds [58]. With the increasing bacterial resistance to drugs, researchers have directed their attention to finding a natural compound that can have similar activity without the undesirable side effects [59, 60]. Several studies have reported that endophytic fungi isolated from natural plants can produce metabolites, similar to their host plants, with antimicrobial and biofilm inhibition abilities [61].

The present study identified *Chaetomium globosum, Alternaria alternata* and *Aspergillus niger* as endophytic fungal strains isolated from *Rosmarinus officinalis* that managed to achieve inhibitory effect on glucosyltransferase enzyme, glucan production and subsequent biofilm formation in addition to increase microhardness with *Chaetomium globosum* giving the highest results.

Regarding the inhibition of glucosyltransferase enzyme, in the present study, rosemary and endophytic fungal extract of rosemary, obtained from the species *Chaetomium globosum*, *Alternaria alternata* and *Aspergillus niger*, managed to inhibit glucosyltransferase which was in agreement with [62–67] who studied the effect of various plant extracts to inhibit glucosyltransferase enzyme and glucan production. They stated that many researchers attributed the ability of several natural extracts including rosemary to inhibit the production of glucosyltransferase to the extract's polyphenol content which can denature the enzyme protein. However, the inhibitory effect of rosemary extract was significantly less than chlorhexidine gluconate which is in contradiction with [64] who reported similar effects between its tested natural extracts and chlorhexidine gluconate. Furthermore, endophytic fungal extract of rosemary obtained from the species *Chaetomium globosum* managed to inhibit glucosyltransferase enzyme, production of glucan and subsequent biofilm production of *Streptococcus mutans* which was in agreement with [61, 68, 69] who studied the ability of endophytic fungal extracts of several plants and reported the ability of these extracts to inhibit the targeted enzyme. This can be attributed to the ability of endophytic fungi to produce the same phytochemicals and active compounds produced by their host plants as explained by [70–76]. They explained that production of bioactive components from endophytic fungi of medicinal plants is considered an easier, cost-effective alternative method for mass production of bioactive compounds that avoids over harvesting and endangering of medicinal plants.

Regarding the inhibition of *Streptococcus mutans* biofilm formation, in the present study rosemary and endophytic fungal extracts of rosemary were observed to inhibit biofilm formation by SEM imaging. Concerning the SEM images taken after 24 h, endophytic fungal extract of rosemary from the species *Chaetomium globosum* and rosemary extracts showed scattered cells of *Streptococcus mutans* on the surface of the prepared sample teeth with no signs of biofilm formation with *Chaetomium globosum* extract showing a higher inhibitory effect on biofilm formation in comparison to chlorhexidine gluconate (positive control) and rosemary extracts Followed by the rosemary extract. While the artificial saliva and DMSO, which was used as an additional negative control to rule out the effect of the solvent used for the rosemary extract, showed mature biofilm formation. Although biofilm inhibition was established for rosemary extract, endophytic fungal extract of rosemary and chlorhexidine gluconate, microhardness readings after 24h showed a significant decrease where [77] explained that glucosyltransferase enzyme is responsible for glucan production with subsequent biofilm formation and not the acidogenic property of *Streptococcus mutans* while [78] explained that some bioactive compounds inhibit glucosyltransferase enzyme without affecting the acid production ability of *Streptococcus mutans*. Meanwhile, the SEM images taken after 72 hours showed that endophytic fungal extract of rosemary from the species *Chaetomium globosum* and rosemary extracts continued to show scattered

cells of *Streptococcus mutans* on the surface of the prepared sample teeth with no signs of biofilm formation. Furthermore, *Chaetomium globosum* extract continued to show the highest inhibitory effect on biofilm formation in comparison to chlorhexidine gluconate and rosemary extracts. However, the rosemary extract showed an increase in the number of scattered cells in comparison with the image taken after 24 h for the same group, with no sign of biofilm formation, while the artificial saliva samples continued to show mature biofilm formation. This was in agreement with [60,63,65,66,79–82] who observed the biofilm eradication ability of many herbs including rosemary, This was attributed to the antimicrobial ability of rosemary in addition to its ability to inhibit glucan production through the inhibition of glucosyltransferase enzyme. However, the biofilm inhibitory effect of rosemary was significantly less than that of chlorhexidine which is in agreement with [83] who reported greater biofilm eradication with chlorhexidine in comparison with rosemary extract which was attributed to the broad spectrum antibacterial properties of chlorhexidine gluconate. This was a contradiction with [60] who reported higher biofilm inhibition with Rosemary compared to chlorhexidine gluconate to which it was attributed to the antibacterial potential of rosemary.

Regarding the microhardness of enamel surface, results after 24 h showed significant decrease with chlorhexidine gluconate, rosemary extract and the extract of endophytic fungus *Chaetomium globosum* from rosemary with the lowest microhardness was with the artificial saliva when compared to the baseline readings, showing insignificant differences between the different groups, meanwhile after 72h the microhardness readings showed a significant increase with rosemary extract and the extract of endophytic fungus *Chaetomium globosum* from rosemary exceeding the 24h readings where the highest value was observed in the rosemary group, while chlorhexidine gluconate and artificial saliva showed low microhardness with the lowest value observed with the artificial saliva, showing significant differences between all groups. Although the increase in microhardness observed in the rosemary extract and the extract of endophytic fungus *Chaetomium globosum* from rosemary groups did not reach the baseline readings, this suggested some form of remineralization. The results obtained after 24h were in agreement with [84],who studied the ability of rosemary and ginger honey mixture to remineralize initial caries lesions using color change with vita easy shade, they found that rosemary was of low remineralizing effect, as well as, low acid resistance after testing the effect of rosemary application after 3 and 6 minutes, meanwhile they were in contradiction after 72 h where the 72 h results were in agreement with [14, 16, 17] who concluded the ability of rosemary to remineralize carious lesions using microhardness and fluorescence testing and attributed the effect to the ability of rosemary extracts to inhibit *Streptococcus mutans* which aided in the remineralization of enamel. This difference was attributed to difference in evaluation method where [84] evaluated the remineralization of initial caries lesions by color change using vita easy shade and not microhardness readings as in the present study, and the effect was also recorded after 3 and 6 minutes and not 72 h as in the present study. Furthermore, the conducted studies induced the initial carious lesions on enamel surface artificially using lactic acid or acetic acid or pH cycling which was different from the methodology of the current study in which artificial carious lesions were induced biologically using *Streptococcus mutans* broth supplemented with sucrose. In addition, the interventions as well as the bacterial challenge were applied concurrently during the testing periods, therefore, the methodology used was mimicking the oral environment challenge.

In the current study, rosemary extract, endophytic fungal extract of rosemary obtained from the species *Chaetomium globosum* and chlorhexidine gluconate managed to increase the pH of the test medias, with significant differences between groups, where the rosemary extract showed the highest readings and the artificial saliva showed the lowest readings. This was in agreement with [79, 85–87] who studied the ability of various herbal and natural extracts to modulate the pH of the oral cavity. They

observed the ability of these extracts to raise the pH of the oral cavity which can be directly linked to inhibition of cariogenic bacteria, like *Streptococcus mutans*, by creating an unfavorable environment for their growth and favorable environment for remineralization of enamel.

The null hypothesis was that there is no difference regarding the inhibition of glucosyltransferase enzyme and microhardness of enamel treated with endophytic fungal extract of Rosemary compared to chlorhexidine gluconate and Rosemary extract. According to the results of the present study, the endophytic fungal extract of rosemary showed higher inhibition of glucosyltransferase enzyme and microhardness of enamel, therefore the null hypothesis was rejected.

Limitations of the current study ranged from the lengthy complicated procedure, from fermentation, purification to isolation to identification to preparation of extracts, which require sophisticated laboratory equipment to prepare the endophytic fungal extract of plants which was in agreement with [76, 88]. Furthermore, in vitro studies cannot completely simulate oral conditions as temperature or pH cycling. The tests were carried out against only one of the main microorganisms involved in caries development although there are many other strains that are implicated in the caries process.

CONCLUSION

Within the limitations of the current study, we can conclude that: *Chaetomium globosum* Kunze showed the highest inhibitory effect on glucosyltransferase enzymes within the endophytic fungal extracts of rosemary. Endophytic fungal extracts of rosemary showed the highest inhibitory effect on glucosyltransferase enzymes superior to rosemary and chlorhexidine gluconate. Rosemary increased the enamel microhardness superior to endophytic fungal extract and chlorhexidine gluconate after 72 h.

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Authors contribution

BB, MB, NH designed the study, MB & BB performed the experiments, MB, BB, NH and HT wrote and revised the manuscript, BB & MB analyzed the results. All authors approved the final manuscript.

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Availability of data and materials

Any data used or analyzed during the study is available to be submitted by the corresponding author upon request.

Declarations

Ethics approval and consent to participate

This study was approved by the Research Ethics committee, Faculty of Dentistry, October University for Modern Sciences and Arts under the approval number of ETH25. Written informed consents were obtained from the patients. All methods were performed in accordance with the relevant guidelines and regulations.

Consent for publication:

Not applicable.

Competing interests:

The authors declare that they have no competing interests

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