

Full Length Research Paper

Inhibitory effect of grape seed extract (GSE) on cariogenic bacteria

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Streptococcus mutans plays an important role in the development of dental caries in humans. Although fluoride and other preventive efforts have led to a dramatic decline in dental caries, the ability to control the actual infection has been limited. Therefore, this study was aimed at investigating in details the effect of a grape seed extract (GSE) on the growth and the biofilm formation of *S. mutans*. Cytotoxicity assay of GSE was used to determine the non-toxic concentrations to the host cell (HEp-2 cell line). Antimicrobial activity of GSE was examined *in vitro* on *S. mutans* isolate to determine the minimum inhibitory concentration (MIC) by using microtiter plate method. The potentiality of GSE on the bacterial adherence (biofilm) was also tested on growing cells on the bottom of cell culture plate. The presence of *S. mutans* increases the free radical stress, which initiates the DNA fragmentation of the host cells. In addition, the free scavenging activity of GSE was measured by using 1,1-dipicryl-2-phenyl hydrazyl (DPPH) assay; DNA fragmentation was carried out in the presence and absence of GSE treatment. The results obtained indicate that the non-toxic dose of GSE and its major constituents (gallic acid, catechin and epicatechin) was 15, 7, 5 and 15%, respectively. All the treatments have the ability to inhibit the growth of *S. mutans* and biofilm formation with priority to GSE and epicatechin (80.98 and 66.25%, respectively). Moreover, the GSE could scavenge the free radicals up to 85% and completely inhibit the DNA fragmentation. GSE, especially epicatechin therefore showed an interesting action on *S. mutans* and could be used for lowering this potentially cariogenic species in the oral cavity.

Key words: *Streptococcus mutans*, grape seed extract (GSE), cytotoxicity, biofilm, non-toxic dose.

INTRODUCTION

The most virulent of species that inhabit the human oral cavity is *Streptococcus mutans*, which has been found to be the initiator of most dental caries (Tanzer et al., 2001), and a transmissible bacterium that can be transmitted both horizontally and vertically (Loveren et al., 2000; Li et al., 2005). *S. mutans* is one of a few specialized organisms equipped with receptors that enhance adhesion to the surface of teeth. Sucrose is used by *S. mutans* to produce a sticky, extracellular, dextran-based poly saccharide that allows them to cohere, thus forming plaque (Koo et al., 2006). Biofilm formation is important for *S. mutans* to survive inside the host oral cavity and on other tissues, e.g. heart valves (Nomura et al., 2006). The ability to bind human extracellular matrix (ECM) is one of the major mechanisms for streptococcal pathogenesis (Paterson and Orihuela, 2010). Because of the strong adherence of the *S. mutans* to the ECM

components (Sklavounou and Germaine, 1980), its adherence to and invasion of host cells was tested in the current work using human epithelial cells HEp-2 as used before by Xue et al. (2011). Moreover, since many of the commensal oral streptococci produce hydrogen peroxide during aerobic growth (Carlsson et al., 1983), the free radicals stress on DNA molecule of host cells increases. The current study, therefore, investigated the potentiality of GSE to scavenge the free radicals and inhibit the DNA damage.

The most important virulence factor, however, is the acidophilicity of *S. mutans*. Unlike the majority of oral microorganisms, *S. mutans* thrives under acidic conditions and becomes the dominant bacterium in cultures with permanently reduced pH. Additionally, unlike many species present in plaque whose metabolisms slow down considerably at such a low pH, the metabolism of *S.*

mutans actually improved. Since the proton motive system is used to transport nutrients through, its cell wall in environments of low pH or high glucose concentration is modulated by hydrogen ion content, which increases with acidity (Dashper and Reynolds, 1992; Dawes, 2003). As a result, dental caries can be considered one of the most widespread and common infectious diseases on the planet. Antibiotics such as penicillin and erythromycin have been reported to effectively prevent dental caries in animals and humans, but they are never used clinically because of many adverse. Recent natural remedies with the use of medicinal plants, which are good reservoirs of chemo-therapeutants can be contributed as an alternative for antibiotic effects, such as hypersensitivity reaction, supra infections, and teeth staining (Tredwin et al., 2005). Despite the several anticaries agents available commercially, the search for an effective agent still continues.

Meanwhile, natural products have shown to be a good alternative to synthetic chemical substances for caries prevention (Bakri and Douglas, 2005; Prabu et al., 2006; Amadi et al., 2007). Wine grapes' seed extract (*Vitis vinifera* L.) has a broad spectrum of medicinal and therapeutic properties due to its high concentration of proanthocyanidins (Ray et al., 2001; Wang et al., 2005, Chang et al., 2006; El-Adawi et al., 2011; Abd El-Wahab et al., 2008). The grape seed extract (GSE) has proven to also act as antibacterial agent (Jayaprakasha et al., 2003; Ahn et al., 2007). In addition, a previous study has screened the antibacterial effect on *S. mutans* (Smullen et al., 2007). However, more studies, particularly *in vitro* study is necessary to establish conclusive evidence for the effectiveness and the clinical applications of these compounds in the prevention of dental caries.

Due to the biofilm complexity in dental plaque and its protection against antimicrobial agents as stated by Furiga et al. (2009), the current study was undertaken to investigate the multi-dimensional influence of GSE on virulence related traits of *S. mutans*. The investigated traits are related to plaque formation potentially resulting in damage to host teeth (caries) as well as traits related to adherence in host cells and tissues (free radicals stress and DNA fragmentation). Both aspects are important for its pathogenesis. Our data were derived from *in vitro* experiments.

MATERIALS AND METHODS

Grape seeds extraction

The GSE was extracted and analyzed by high performance liquid chromatography (HPLC) as described before by El-Adawi et al. (2011). Briefly, the crushed grape seeds were subjected to preliminary treatment (defatting); crushed seeds were soaked in suitable volume of hexane overnight. Next, the defatted seeds were extracted by pressurized hot water and lyophilized using lyophilizer (Telstar, Spain). The extract was then subjected to HPLC analysis and the resultant chromatograms agreed with the standard

chromatograms produced by National Science Foundation (NSF). The major constituents of GSE, gallic acid, catechin and epicatechin were purchased from Sigma-Aldrich Co., Germany.

Dental plaque collection and growth conditions

The clinical strain of the *S. mutans* were isolated from the dental plaque of Egyptian patient, and the plaque sample was stored in a 1.5 ml Eppendorf tube containing 500 μ L of 1X phosphate buffered saline (PBS) at -20°C before analysis. The samples were diluted 100-fold in 1X PBS and plated on Mitis-Salivarius (MS) agar (Difco) supplemented with kanamycin sulfate (Km; 250 g/ml, Wako Pure Chemical Industries, Osaka, Japan), and streptomycin (Sp; 1000 g/ml, Sigma Chemical Co., St. Louis, MO, USA) using a sterilized cotton ball. Subsequently, the plates were incubated for 2 days at 37°C in an anaerobic chamber containing 10% hydrogen (H₂), 5% carbon dioxide (CO₂), and 80% nitrogen (N₂).

Bacterial genomic DNA preparation

The bacterial genomic DNAs were prepared according to the manufacturer's instructions. The DNA concentrations were determined by measuring the optical density (OD) at 260 and 280 nm, using an ultraviolet (UV) spectrophotometer (Ultraspec 2000, Pharmacia Biotech., UK).

16S rRNA gene sequencing

The bacterium was characterized and identified by 16S rRNA gene sequencing using universal primers as described by El-Helow (2001). The forward and reverse primers were of the following sequences, respectively: AGAGTTTGATCMTGGCTCAG and TACGGYACCTTGTTACGACTT. The 16S rRNA sequence was aligned with published sequences from the NCBI database using Clustal X software. The phylogenetic tree was displayed using the TREEVIEW program.

Culture of HEp-2 cells

The HEp-2 cells was purchased from Vacsera, Cairo, Egypt, and grown on tissue culture plastic ware in Dulbecco's modified Eagle's medium (DMEM; Life Technologies Inc., Rockville, MD, USA) supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) antibiotic antimycotic solution (Gibco BRL Laboratories, Grand Island, N.Y.) at 37°C in 5% CO₂ with 80% humidity.

Cytotoxicity assay

For the determination of treatments concentration that does not exert a toxic effect on HEp-2, the cytotoxic assay was performed. A cell suspension of 6×10^4 cell/ml was collected and seeded in 96-well plates (100 μ L cell suspension per well). The plates were incubated at 37°C in humidified 5% CO₂ for 24 h. After obtaining a semi confluent cell layer, the exhausted old medium were discarded and 100 μ L of different treatment concentrations (μ g/100 μ L DMEM) or DMEM medium (as a negative control) were added. The cell plates were incubated at 35°C in a humidified atmosphere of 5% CO₂ and 95% air for 3 days. After 3 days, 100 μ L of neutral red stain was added to each well and incubated for 3 h at 37°C in humidified 5% CO₂ (Borenfreund and Puerner, 1985). Only living cells are permeable to neutral red and these were incorporated into liposomes, providing a quantitative assay to the cytotoxic effects. Excessive dyes were discarded and the stained cells were fixed

with 100 μ L fixing solution (0.5% formalin with 1% CaCl₂) for 1 min, then cells were destained in 100 μ L destaining solution (50% ethanol with 1% acetic acid glacial) for 5 min by shaking. The stain intensity was assayed using automated enzyme-linked immunosorbent assay (ELISA) microplate reader adjusted at 540 nm (reference filters 620 nm).

The anti- bacterial activity

Minimum inhibitory concentrations (MIC) were determined in duplicate by serial dilution method; the concentrations of the treatments (GSE, gallic, catechin and epicatechin) ranged from 3 to 9%. The efficacy was evaluated by microplate reader assay method. Aliquot of 100 μ L of pre-inoculated *S. mutans* (10⁶ CFU/ml) in Luria-Bertani (LB) broth supplemented with 10% vitamin C was transferred to each well of 96-well plate. The same volume of the treatments was added to each well in replica, and the plates were incubated under anaerobic conditions at 37°C for 18 h. After incubation, the absorbance of the plates was determined using an automated ELISA microplate reader adjusted at 620 nm. The inhibition percentage of the extracts was calculated according to the following equation

$$\text{Inhibition percentage} = (A - A1 / A0) \times 100$$

Where A is the absorbance of the treatment group; A1 is the absorbance of the blank, and A0 is the absorbance of the control group.

Quantitative assay of biofilm inhibition

To assess the effect of the treatments on the biofilm formation, the quantitative assay of biofilm formation was done according to Sheikh et al. (2001), with some modifications. In brief, 200 μ L of a previously prepared overnight bacterial culture in Luria broth was inoculated in 96-well flat-bottom microtiter polystyrene plates with 100 μ L of the nontoxic dose of the treatments or 100 μ L of L.B (as control). The plates were incubated overnight (18 h) at 37°C with shaking and visualized by staining with 0.5% crystal violet for 5 min after washing with water. The biofilm was quantified in duplicate after adding 200 μ L of 95% ethanol, by an ELISA assay plate reader at 570 nm. Strain EAEC 042 was used as a positive control and *Escherichia coli* HB101 was used as a negative control.

Inhibition of *S. mutans* adhesion to HEP-2 cell (in vitro)

HEP-2 cells were maintained as confluent monolayers in complete DMEM medium in T75 flasks at 37°C in a 5% CO₂ atmosphere, 24 h before the adherence assays. Then monolayers were washed with pre-warmed PBS, and trypsinized for 2 min. Subsequently, the cells were scraped from the flask into 72 ml of DMEM/FCS/antibiotics. Three tissue culture plates (12 wells) per flask of HEP-2 cells were prepared by depositing one sterile 13 mm glass cover slip to each well, 2 ml of the HEP-2 cell suspension was added to each well incubated at 37°C in a previous conditions until the monolayers were 60 to 70% confluent (usually 24 h). The bacterial strain under test was inoculated into L.B broth for 16 - 18 h, in anaerobic conditions at 37°C. On the day of the assay, the existing medium in the wells was discarded and the monolayers were washed three times with DMEM; 2 ml fresh DMEM without supplements was added to each well, followed by 100 μ L of appropriate bacterial suspension. Plates were incubated at 37°C for 3 h. Unbound bacteria were removed by washing three times with phosphate buffered saline, then the cells were washed briefly with methanol, which was discarded and mixed with fresh methanol (1

ml per well) for 1 min. Methanol was replaced by 2 ml per well of Giemsa stain, freshly diluted 1:10 in water. This was left for 30 min at room temperature. Finally, the Giemsa stain was discarded and the monolayers were washed sequentially with 3 to 4 ml each of tap water and slides were read at 100X magnification under oil immersion.

Determination of antioxidant capacity

The antioxidant capacity with 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) (1 mM, 0.5 ml) was determined as described by Amarowicz et al. (2004). The sample (4 ml) was previously dissolved in methanol in concentrations of 2.0 to 20.0 μ g/ml, and the solutions were homogenized. The reaction occurred in ambient temperature for 30 min, protected from light. A solution of DPPH• (0.5 ml) in methanol (4 ml) was used as a control, and another solution of butylated hydroxytoluene (BHT; 2 mg) in methanol (4 ml) with DPPH• solution (0.5 ml) was used as the blank. The absorbance values were measured at 517 nm and converted to a percentage of antioxidant activity or percentage of DPPH• consumed (AA%), using the following formula:

$$AA\% = 100 - [(Abs_{sample} - Abs_{blank}) \times 100] / Abs_{control}$$

Where Abs is the absorbance.

Inhibition of DNA fragmentation induced by *S. mutans*

A distinctive feature of apoptosis at the biochemical level is DNA fragmentation. This method was used as a semi-quantitative method for measuring apoptosis. Such phenomenon, described for the first time by Wyllie (1980), can be visualized by an agarose gel electrophoresis analysis. The present protocol provides a method for qualitative determination of DNA fragmentation in the presence and absence of treatments (GSE, gallic acid, catechin and epicatechin).

Effect of the extract on the production of acids

To determine the effect of the grape extract on the production of acid, the method described by Ooshima et al. (2000) was used, with certain modifications. *S. mutans* was seeded (1 ml in 100 ml red phenol broth containing 1% glucose and the extract). This was incubated at 37°C in microaerophilic atmosphere, and at regular intervals a sample (4 ml) of the culture was removed and its pH measured in a pH meter.

RESULTS

As shown in Figure 1. Distinctive colonies of *S. mutans* on Murashige and Skoog (MS) agar medium were isolated. Colonies morphologically resembling *S. mutans* in the MS medium were quantitated and characterized according to the identification criteria described by Facklam (1977) and Coykendall (1989). To investigate the phylogenetic affiliation of this strain, the complete 16S rRNA gene was amplified, sequenced and deposited at GenBank. Comparison of the obtained sequence with other sequences available at NCBI database revealed the greatest similarity to the corresponding sequence of *Streptococcus* sp. A phylogenetic tree was constructed using Clustal X program (Figure 2).

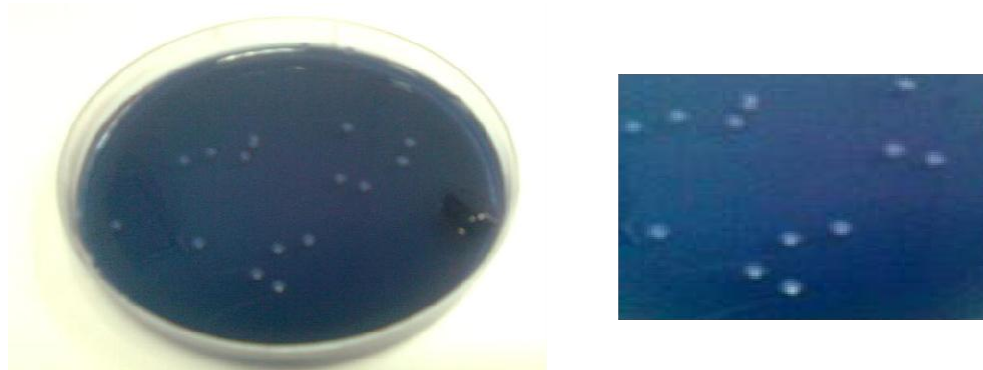


Figure 1. Isolated oral *Streptococcus* from dental plaque in Mitis-Salivarius agar culture medium after 48 h incubation at 37°C and 5% CO₂.

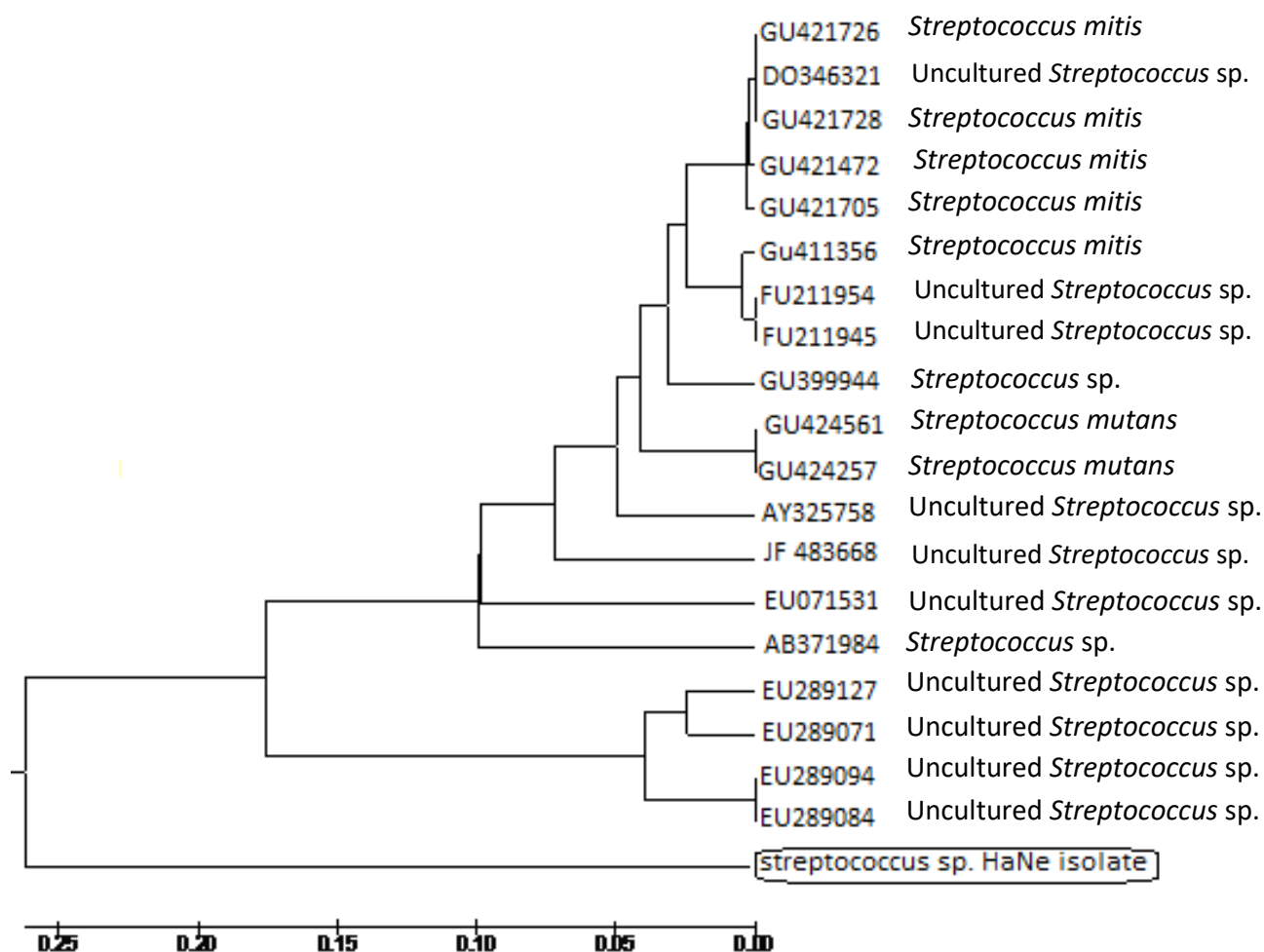


Figure 2. Phylogenetic dendrogram obtained by distance matrix analysis showing the position of the streptococcus isolate (HaNe) among *Streptococcus* sp.

Cytotoxicity

The results of cytotoxicity test were presented in Table 1. Results indicated the GSE and epicatechin shared the same nontoxic dose, followed by gallic acid and catechin.

Bacterial growth

Regarding the inhibitory effect of the treatments on *S. mutans*, a significant bacterial growth inhibition was noticed in the case of epicatechin at highly non-toxic

Table 1. The cytotoxicity test results of the evaluation of the non-toxic dose for each treatment.

Treatment	Non-toxic dose (%)
Grape seeds	15
Gallic acid	7
Catechin	5
Epicatechin	15

dose (15%), on which it inhibited the bacterial growth with more than 80% as shown in Table 2.

The antioxidant activity

The potentiality of GSE as free radical scavenger is shown in Figure 3. The activity was recorded for all treatments with comparatively high activity for both GSE and epicatechin (85 and 77.6%, respectively).

Fragmentation assay

Due to the correlation between free radicals stress and the induction of DNA fragmentation, it was necessary to check the DNA fragmentation in the case of *S. mutans* infection and the role of treatment as inhibitor. In Figure 4, there was no DNA fragmentation observed for any of the treatments, but the infected HEP2 cells have high DNA fragmentation.

Biofilm assay

Aqueous crude extract and the pure form of major constituents were shown to affect the cariogenic properties of *S. mutans*, as exhibited by the ability of the treatments to inhibit adhesion of the bacteria to flat-bottom microtiter plates and inhibit the production of biofilm (Table 3).

Adhesion

The inhibition of biofilm was confirmed in adhesion test, where the adhesion of *S. mutans* to the HEP 2 cells was inhibited in the presence of epicatechin (Figure 5).

Acid production

Figure 6 illustrated the drop of pH value from 6.5 to 3.0 (highly acidic) due to the bacterial growth and acidproduction which has been seized in the presence of epicatechin treatment to the pH value 4.8.

DISCUSSION

It is well known that different cell lines exhibit different sensitivities to cytotoxic compounds. Therefore, the use of HEP2 cell line was considered necessary in the testing of cytotoxicity of GSE and the major constituents for dental caries study. As shown in Table 1, the crude extract (GSE) and epicatechin have the same non-toxic dose (15%), which was much higher (more safer) than both gallic acid and catechin (7 and 5%). The anti-bacterial results revealed that GSE and the major constituents exhibited anti bacterial activity against *S. mutans*; the capacity of epicatechin to inhibit *S. mutans* was much higher (80%) than the crude extract (GSE) at the same non-toxic dose (Table 2). These results suggest that epicatechin may be the principle factor responsible for bacterial inhibition. The crude extract also had an antioxidant activity (85%) higher than epicatechin, gallic acid and catechin, according to measures taken with the DPPH• radical (2,2-diphenyl-1-picrylhydrazyl) method (Figure 2). This was important to the findings of the study because gum disease originates due to the bacteria's presence and its biofilm protection, but the disease progresses because of an excess release of reactive oxygen species that trigger the inflammatory process. Grape seed extract's antioxidant abilities may quench the free radicals implicated in the progression of gum disease. We continued our investigations to test the incidence of DNA fragmentation of HEP2 cells due to the infection with *S. mutans*, and then demonstrated the effect of each treatment. For all treatments, a complete protection of DNA from fragmentation was observed (Figure 4).

Also, because the development of dental caries can be attributed to events that occur at the tooth pellicle-biofilm interface, it is desirable to determine potential inhibitor of biofilm formation. Moreover, surface-adsorbed glucosyl transferase (Gtf) enzymes display an increased resistance to most of the common inhibiting agents, including commercially available anti-plaque/anti-caries agents when compared to the same enzymes in solution (Wunder and Bowen, 1999). Thus, inhibition of surface-adsorbed Gtfs may play a significant (and more relevant) role in preventing the formation of pathogenic biofilms related to dental caries. In the present study, we further expanded *in vitro* findings by showing that epicatechin could reduce the development of biofilm by 66.25% at the non-toxic dose (Table 3). Furthermore, epicatechin affected the adhesion of *S. mutans* cells to the HEP2 cell lines (Figure 5), which was consistent with previous reports that *S. mutans* was found to adhere to oral epithelial cells HEP2 (Xue et al., 2011). It is noteworthy that epicatechin affected the acid production and acid tolerance of *S. mutans* cells within biofilms as observed in the pH-drop assay, which confirms and extends a previous observation showing disruptive effects of the agent against planktonic cells of *S. mutans* (Table 3).

Table 2. The bacterial inhibition (%) presented for each treatment corresponding to different concentrations.

Concentration (%)	Bacterial inhibition (%)			
	GSE	Gallic acid	Catechin	Epicatechin
5	2	27	32.6*	55.4
7	36.6	62*	72.11	74.65
10	55.6	68.3	74.6	80
15	63.2*	70.1	75.3	80.98*

*Represents the bacterial inhibition at the non toxic dose.

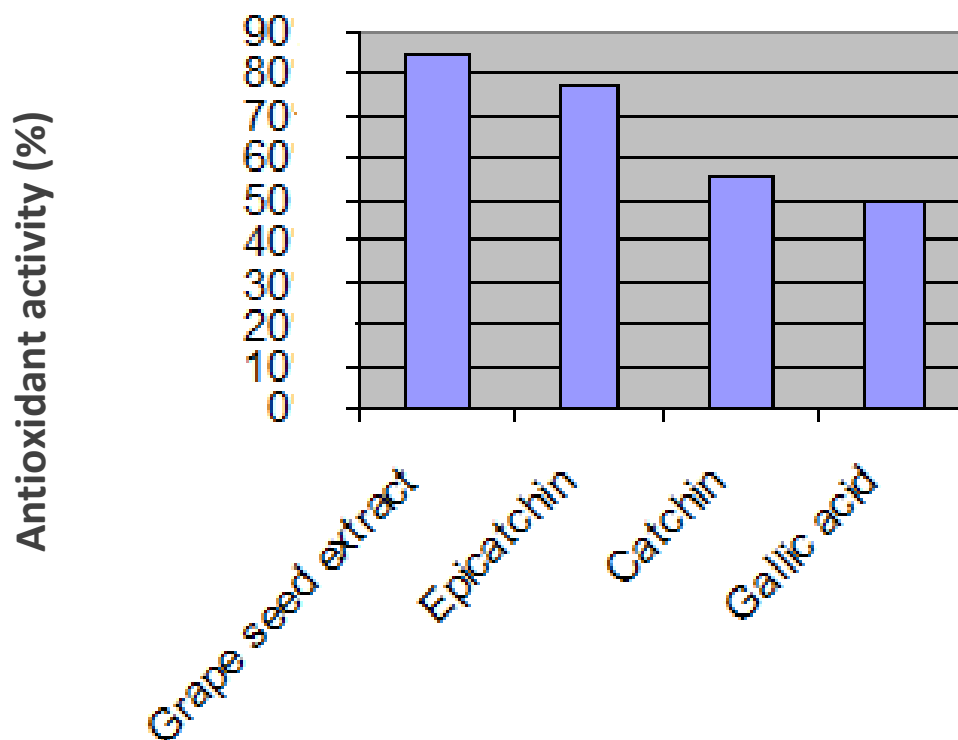


Figure 3. The antioxidant capacity with the DPPH· radical. Results are presented as percentage for each treatment.

The test agent sensitized the biofilms' cells to acidification to the point that the final pH values (pH 4.8) were significantly higher than those in the presence of the vehicle control (pH 3.0), and this elevation in the pH value might protect the tooth enamel from demineralization (Bowden, 1990). Considering that biofilms display increased resistance, the presence of an exopolysaccharide matrix acts as a barrier to diffusion of antimicrobial agents (Stewart, 1996).

Substances that act on *S. mutans* virulence could be used to control dental caries or even to enhance the anti-cariogenic effect of other recognized agents such as fluoride. Such agents could be used to enhance the

effectiveness of the protective effects of fluoride, which (despite some antibacterial effects) does not effectively address the infectious character of the disease (Koo et al., 2005, 2008). In this context, it would be valuable to investigate the possible additive or synergistic anti-caries effects of epicatechin and fluoride, since these agents have distinct (but potentially complementary) mechanisms of action. In addition to the improvement of fluoride's anti-caries effects, the association could also effectively diminish the concentration of fluoride required for therapy (without affecting its cariostatic effectiveness). In conclusion, GSE and specifically epicatechin, may be considered a novel and promising alternative agent to

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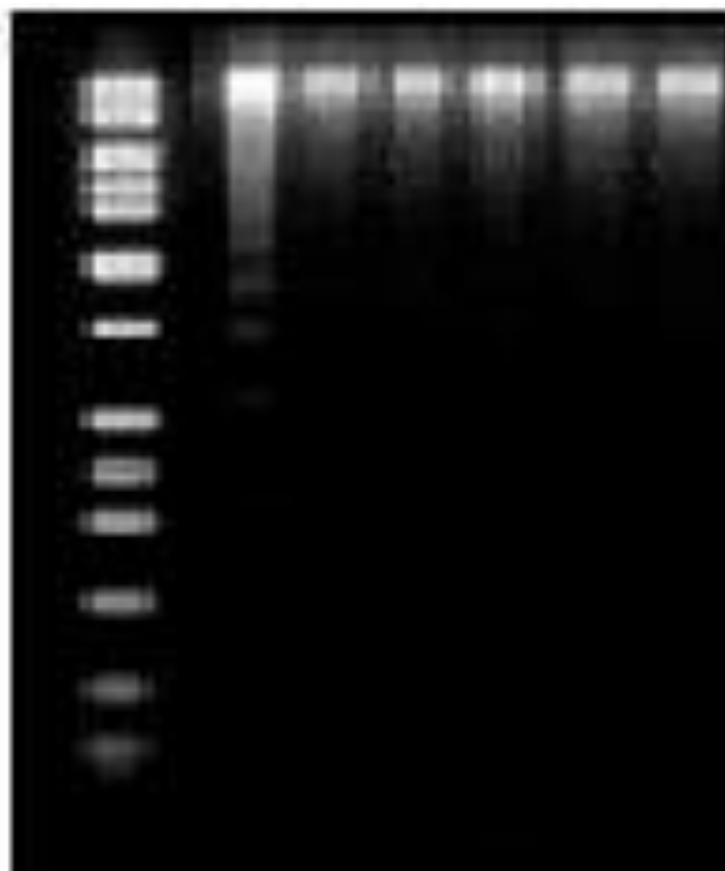


Figure 4. Agarose gel analysis of genomic DNA isolated from Hep2 cells. Lane M: DNA marker; 1: DNA of Hep-2 cells infected with *S. mutans* bacteria; 2: DNA of control cells (Hep-2 cells); 3: DNA of Hep-2 cells with *S. mutans* co-incubated with GSE; 4: DNA of Hep-2 cells with *S. mutans* co-incubated with gallic acid; 5: DNA of infected Hep-2 cells with *S. mutans* co-incubated with catechin; 6: DNA of infected Hep-2 cells with *S. mutans* co-incubated with epicatechin.

Table 3. The bacterial biofilm inhibition (%) presented for each treatment corresponding to different concentration.

Concentration (%)	Biofilm inhibition (%)			
	GSE	Gallic acid	Catechin	Epicatechin
2	34	44.75	50	55.25
2.5	37.25	49	50.01	55.5
3	39.5	49	52.5	58
3.5	41.25	51.75	53.75	58.25
4	42.75	52.25	55	58.5
4.5	43.25	53	55.75	59.5
5	44	54.2	56.25	60.75
7	44.25	54.5	57.25	61.25
10	50.5	54.75	57.75	62
15	51.75	57.5	62.75	66.25*

*Represents the maximum inhibition for bacterial biofilm growth.

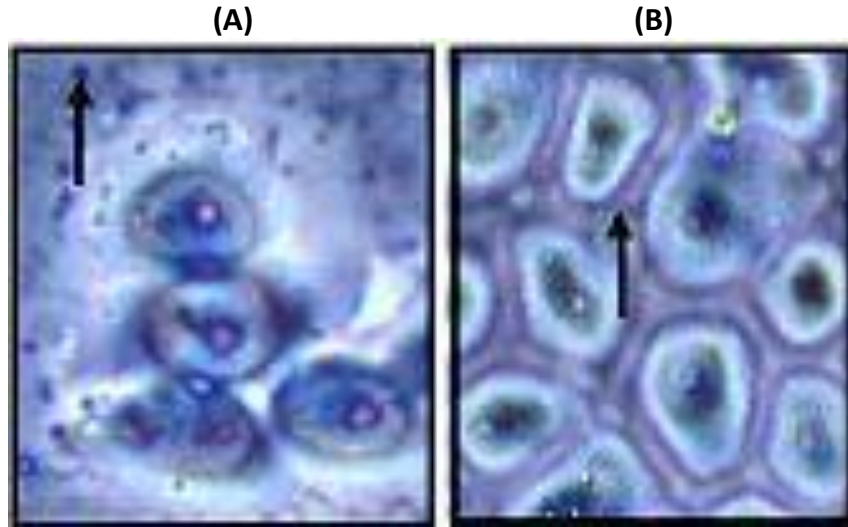


Figure 5. (A) The adhesion of *S. mutans* to the Hep-2 cells; (B) The adhesion of *S. mutans* to the Hep-2 cells in the presence of GSE.

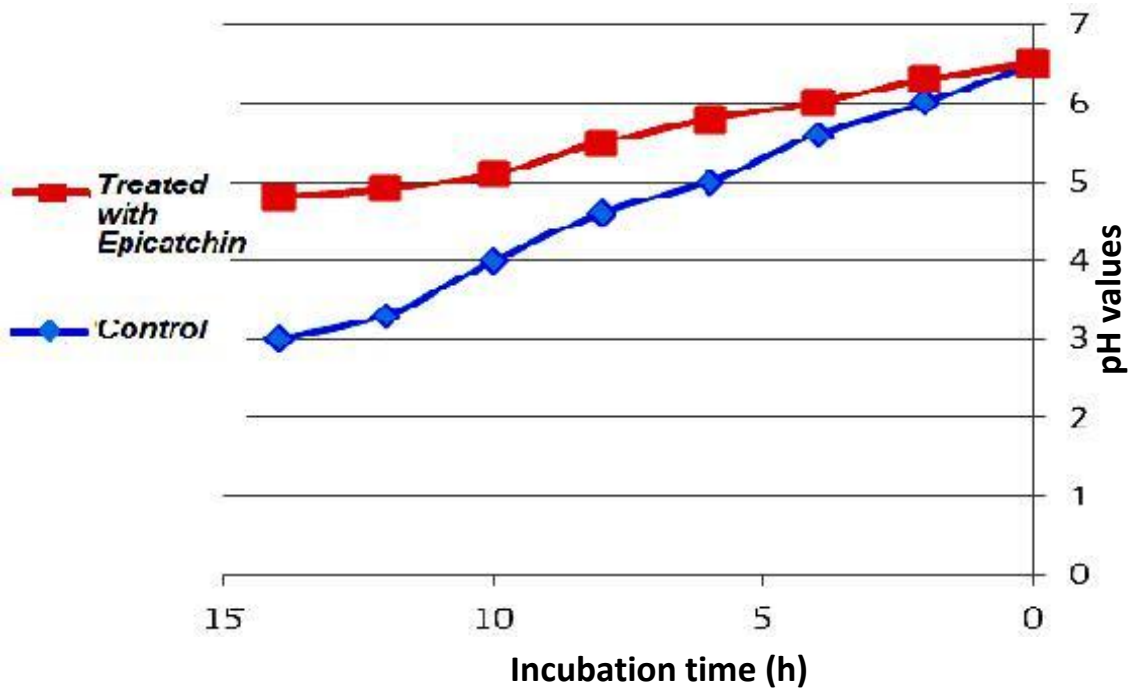


Figure 6. The changes in pH values with time due to the bacterial growth and acid production in the presence and absence of epicatechin.

prevent and control dental caries.

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