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Efficacy of Non-Cytotoxic Doses of Some Medicinal Plant Extracts as Antibacterial and Anti-Biofilm Agents Against Cariogenic Bacterium *Streptococcus mutans*

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Oral diseases are major health problems with dental caries and periodontal diseases among the most important preventable global infectious diseases. Increasing of refractory periodontal diseases is due to non-sensitivity of cariogenic bacteria to antibiotics, and the lack of new alternatives will be threat to danger imminent. This serious increase in the non-sensitivity of microbes to antibiotics may be opened to search for alternative approaches for exploring of new drugs with specific therapeutic activities. In this study cariogenic bacterium *Streptococcus mutans* strain ATCC 25175 was used as a test organism for evaluation the efficacy of water extracts of eight different medicinal plants as antibacterial and anti-biofilm agents. The best percentage of inhibition of these extracts against *S. mutans* strain ATCC 25175 was recorded as; Pomegranate peelÃ CloveÃ CoffeeeÃ green tea. The cytotoxicity of the most potent extracts has been measured against fibroblast cells. Generally, the green tea extract was the safer extract followed by coffee, pomegranate peel and clove extracts. The recorded IC_{50} of the all used plant extracts on fibroblast cells ranged from 10-1.25%. with exception for green tea extract that didn't show IC_{50} even with the maximum used concentration. The potency of the three selected extracts to to inhibt the biofilm formation by the tested strain could be summarized by the following order: pomegranate peelÃ coffeeÃ clove. Oral health influences the general quality of life and poor oral health is linked to chronic conditions and systemic diseases. Finally, give a recommendation for using some of these extracts to restrict the bacterial growth and reduction of the prevalence of periodontitis and cariogenic diseases.

Keywords: Oral diseases – Medicinal plants – Cytotoxicity – Antibacterial and anti-biofilm agents.

Dental caries are considered as one of the most prevalent oral infections affecting mankind worldwide. The initiation and progression of this infection are mainly produced by endogenous oral bacterial species and their metabolites, including *Streptococcus mutans*, *Streptococcus sobrinus* and others[1,2].

The acid producing *S. mutans* inhabiting the mouth causes damage by dissolving tooth

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structures in the presence of fermentable carbohydrates such as sucrose, fructose, and glucose[3]. The food debris, acid, bacteria, and saliva combine in the mouth to form a sticky substance called "plaque" that adheres to the teeth. If plaque is not removed thoroughly and routinely, tooth decay will not only begin, but flourish[4].

In addition, *S. mutans* produces glucosyltransferases (Gtfs) and synthesizes glucans from sucrose. Glucans are critical for bacterial accumulation on the tooth surface and the formation of cariogenic biofilms[5]. Furthermore, *S. mutans* survive at low pH values and generate acids that result in the deminer-alization of tooth enamel, thereby initiating dental caries[6]. Therefore, it has been proposed that disruption of the ability of *S. mutans* to form acids and glucans is an effective therapeutic approach for the treatment of dental caries[7].

The limitation of this kind of oral infection might be achieved by using antimicrobial mouth rinses such as triclosan and chlorhexidine. The usual usage of these chemicals is limited for their undesirable side effects, including tooth staining, taste alteration and development of hypersensitivity reactions[8,9]. 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[10] in addition to the development of bacterial resistance against them[9]. That is why there is a strong need for natural antibacterial alternatives. One of the potent alternatives are medicinal plants which could be traced as far back as the beginning of human civilization.

Medicinal plants are a source of great economic value all over the world[11]. Recent natural remedies with the use of medicinal plants, which are good reservoirs of chemotherapeutants can be, contributed as an alternative for antibiotic effects such as hypersensitivity reaction, supra infections, and teeth staining[10]. In addition, using of antibiotics for prevention of systemic infections originated from the oral cavity is not recommended because of the risk that bacteria will develop resistance to them. However, the discovery of extracts or oils of medicinal plants with antimicrobial and anti-inflammatory activity will be more safe and acceptable[9].

The use of plants and plant products as medicines could be traced as far back as the beginning of human civilization. Medicinal plants are a source of great economic value all over the world[11]. Herbal medicine is still the mainstay of 75-80% of the whole population and the major part of traditional therapy involves the use of plant extract and their active constituents. Following the advent of modern medicine, herbal medicine suffered a setback, but during last two or three decades, advances in phytochemistry and in identification of plant compounds, effective against certain diseases have renewed the interest in herbal medicines[12]. In recent years, human pathogenic microorganisms have developed resistance in response to the indiscriminate use of commercial antimicrobial drugs commonly employed in the treatment of infectious diseases[13].

The aim of this study was to evaluate the ability of calculated non-cytotoxic doses of different medicinal plants as new and natural alternatives to cease the growth of *S. mutans* strain with subsequent inhibition of its biofilm formation.

MATERIALS AND METHODS

Bacterial Strain and Preservation

Streptococcus mutans ATCC 25175, DSM No: 20523 reference strains were obtained from MERCIN, Faculty of Agriculture, Ain Shams University, Cairo, Egypt. The bacterial strain was preserved by adding 250 ìl of 60% glycerol to 750 ìl overnight LB culture and kept at -80^{o}C.

Preparation of Plants Extracts

The suggested probable eight plants (Kari, Cinnamon, Coffee, Pomegranate peel, Clove, Green tea, Garlic and Ginger) were purchased from a local herbal market in Rafha governorate, Northern Border region, Saudi Arabia and were submitted to the standard extraction procedures according to Wendakoon et al.[14] with some modifications. The obtained plants were washed three times using tap water, dried and 10 grams of each plant were submitted to extraction using 100 ml of distilled water and boiled for 10 min. The mixtures were then spun down at 3000 rpm for 30 min and the obtained supernatants were kept at 4^{o}C till use.

Determination of Antibacterial Activity and Minimum Inhibitory Concentration (MIC)

The antibacterial activity against *Streptococcus mutans* were tested and the

minimum inhibitory concentration (MIC) was determined using descending concentrations of the each extract. The MIC of the eight plant extracts were diluted using sterile saline and were tested for their antibacterial activity against *S. mutans*. Each dilution was tested against nutrient broth inoculated media in microtiter plate. The plate was incubated at 30 °C for 24 h and the optical density at 600 nm was measured using ELIZA reader.

Cytotoxicity Assay

Cytotoxicity assay was used for determination of the treatment concentration that does not have a toxic effect on normal cells. In this assay, human fibroblast cells were used as a normal cell modeling, a cell suspension of 6×104 cell/ml was collected and seeded in 96-well plates (100 ìl cell suspension per well). The plates were incubated at 37°C in humidified 5% CO_2 for 24 h. After obtaining a semi confluent cell layer, about 100 ìl of different treatment concentrations were incubated with cells at the previously described conditions for 3 days. After incubation, 100 ìl of neutral red stain was added to each well[15], only living cells are permeable to neutral red and incorporated it into liposomes providing a quantitative assay to the cytotoxic effects. The stain intensity was assayed using automated ELIZA microplate reader adjusted at 540 nm (reference filters 620 nm).

Quantitative Assay of Biofilm Inhibition

The ability of the plants extracts to inhibit biofilm formation of *S. mutans* was determined according to El-Adawi, 2012; with some modifications. In brief, triplicates of 100 ìL of a previously prepared overnight bacterial culture in Luria broth were inoculated in 96-well flat-bottom Microtiter polystyrene plate with 50 ìL of the nontoxic dose of the treatments. The plate was incubated for 48 h at 30°C without shaking. The plate was decanted once and followed by washing for three times with 200 ìL sterile PBS buffer. The plate was then dried at 60°C for 1 h. The remaining biofilm was stained with 0.1% crystal violet for 15 min, decanted and washed three times with 200 ìLof sterile distilled water. The plate was air dried for 15 min followed by the addition of 150 ìL of 95% ethanol. The absorbance was measured at 570 nm using an ELISA assay plate reader. Untreated Streptococcus mutans strain was used as

Table 1. Percentage of inhibition of different concentrations of the tested plants extract against *S. mutans*

Plants	Concentrations (%)									
	10	5	2.5	1.25	0.625	0.362	0.181	0.09	0.045	0.020
Kari	13.43075	10.66522	7.592191	5.531453	-	-	-	-	-	-
Cinnamon	93.14675	66.4859	37.70788	35.03254	30.69414	24.62039	21.87274	15.25669	6.977585	-
Coffee	67.1073	35.17715	33.47795	33.40564	33.36949	27.4765	26.3919	25.88576	20.06508	19.12509
Pomegranate peel					94.03594	87.45481	86.62328	83.58641	77.11497	69.05278
Clove	99.71077	90.13015	72.27043	69.95662	61.17137	43.56471	42.8055	39.69631	35.6833	31.48952
Green tea	88.06941	89.33478	76.35575	53.83225	48.44541	43.38395	41.57628	30.33261	25.01808	15.76283
Garlic	72.84888	48.98771	36.26175	35.71945	28.30803	27.29573	17.82357	17.60665	5.965293	3.723789
Ginger	83.11641	72.5235	64.859	56.72451	50.28923	47.46927	45.98698	43.60087	16.52205	8.387563

the positive control and un-inoculated LB broth as negative control.

RESULTS

Determination of the MIC and antimicrobial activity of the tested extracts

According to the examined plants; eight water extracts of the tested medicinal plants (Kari, Cinnamon, Coffee, Pomegranate peel, Clove, Green tea, Garlic and Ginger) were prepared and their different dilutions were tested for their antimicrobial activity against *S. mutans* using ELIZA reader equipment. The percentage of inhibition of each dilution was calculated and recorded (Table 1). Some of the tested dilutions showed a high percentage of inhibition at low concentrations of the extract. The best percentage of inhibition could be summarized as follows: Pomegranate peelÃ CloveÃ CoffeeeÃ green tea. The lowest obtained concentrations that have been recorded for these four medicinal plants could be submitted for more dilutions that can achieve more dilutions for the MIC. The minimum inhibitory concentration (MIC) for most of the tested concentrations was also recorded (Table 2). The best percentage of inhibition was recorded as GingerÃ CinnamonÃ KariÃ Garlic.

Table 2. Minimum inhibitory concentration (MIC) and percentage of inhibition for Kari, Cinnamon, Garlic and Ginger against *S. mutans* strain

Plant Extracts	Recorded MIC (g/100 ml)	Percentage of inhibition (%)
Kari	1.25	5.531453
Cinnamon	0.045	6.977585
Garlic	0.02	3.723789
Ginger	0.02	8.387563

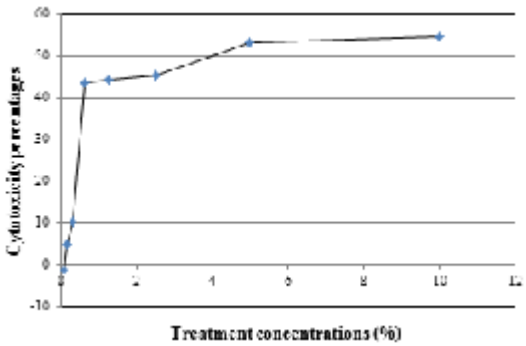

Fig. 2. Cytotoxicity of pomegranate peel extract against fibroplast cells with IC_{50} determination

Cytotoxicity Determination of Some Selected Medicinal Plants

The safety pattern of the most potent and selected plant extract was checked on fibroblast cells using neutral red assay protocol. The viability of the cells was quantitatively measured after 48 h of incubation. Generally, beginning with 10%, the green tea extract was the safer plant extract (Figure 4) followed by coffee extract then both plant extracts (pomegranate peel and clove) as shown in figures 1, 2 and 3. The recorded IC_{50} of the all used plant extracts on fibroblast cells ranged from 10-1.25% with exception for green tea extract that didn't show IC_{50} even with the maximum used concentration. By referring to the antibacterial results, the IC_{50} of plant extract pomegranate peel (2.5%) exhibited antibacterial activities percentage over than 94.03.

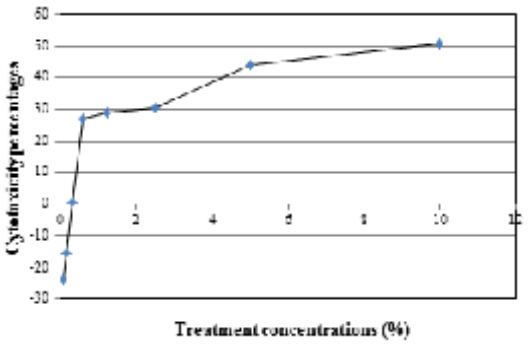

Fig. 1. Cytotoxicity of coffe extract against fibroplast cells with IC_{50} determination

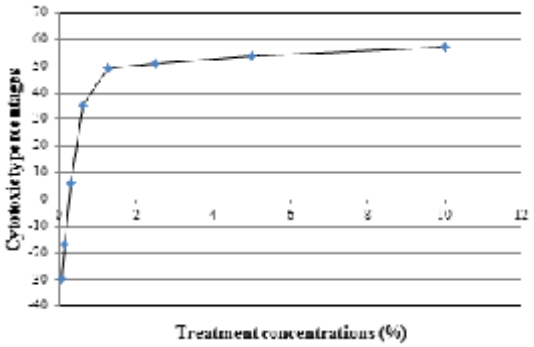

Fig. 3. Cytotoxicity of clove extract against fibroplast cells with IC_{50} determination

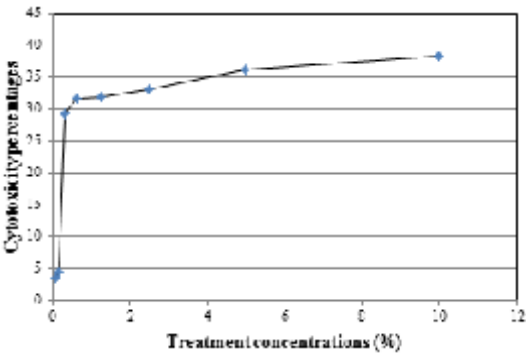

Fig. 4. Cytotoxicity of green tea extract against fibroplast cells with IC_{50} determination

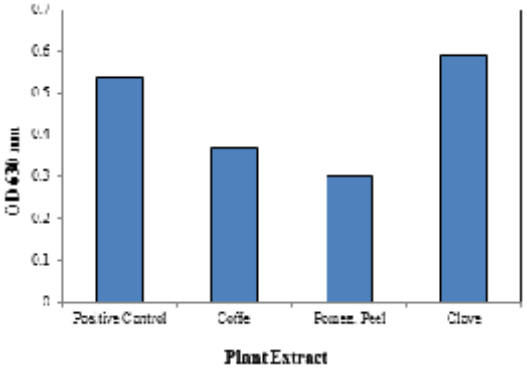

Fig. 5. Biofilm inhibition of *S. mutans* by the reported MIC of the three tested extracts, coffee, pomegranate peel and clove

Inhibtion of Biofilm Formation Using Plant Extracts

The MIC of three extracts that showed significant antibacterial activities have been selected for evaluation of their ability to inhibit the formation of biofilm by *S. mutans*. As shown in figure 5, two extracts were able to inhibit the biofilm formation partially in a good way. Both of coffee and pomegranate peel succeeded to inhibt the biofilm formation by *S. mutans* by 30 and 43% respectively. On the other hand, clove has been failed to inhibit the biofilm formation by the tested strain. The potency of the three selected extracts to inhibit the formation of biofilm could be summarized by the following order: pomegranate peelÃ coffeeÃ clove.

DISCUSSION

Periodontal disease and dental caries are among the most common diseases in affecting mankind since the early history of ancient civilizations[16,17]. At these time periods, people used medicinal plants as potent drugs for the treatment of these diseases. Since the discovery of penicillin, they have used antibiotics as alternatives for medicinal plants. The recent problems were arisen as a result of antibiotic resistance by many pathogenic microbes. Most of the recent drugs are depending on elimination of the bacterial pathogen by its destruction or prevention of biofilm formation[18].

The results of the present study revealed that, the eight tested medicinal plants can cease the *S. mutans* growth efficiently. These individual extracts showed potent antibacterial activity against the cariogenic properties of *S. mutans*. This observation confirmed that these plant extracts possess bactericidal compounds, which inhibit a bacterial strain that is responsible for the growth of dental caries [19]. Sometimes, the combination between two or many extracts can exert a synergistic effect against the pathogen, which indicating more strong effect over single extract against the pathogen of interest[20].

As time is passing, the microbes have modern potentials to resist antibiotics and antimicrobial agents. Modern research in microbiological field is recently concerned with the development of the natural sources for management of diseases and in particular, oral diseases. The elimination of the bacteria that cause oral diseases is mainley depending on its destruction or prevent the formation of biofilm, which is crucial for its existence[18].

The concept of biofilm destruction by the chosen medicinal plant lead us to examine the best recorded MIC of the extracts to inhibit the formation of biofilm by *S.mutans*. As shown in figure 5, two of the three examined extracts showed almost 30 and 43% of inhibition for coffee and pomegranate peel respectively, compared with the positive control (*S. mutans* with no additions). The cytotoxicity results revealed that the recorded MIC for the three selected extracts is safe on human fibroblast cells. The most safer one was coffee followed by pomegranate peel and clove. These results emphasis that the extracts could be used

as antimicrobial agents against *S. mutans* with safe effect to human cells.

CONCLUSION

The obtained results revealed that medicinal plants are considered as potent alternatives for antibiotics for the treatment of bacterial infections with studied and calculated MIC that can harm the bacterial units without cytotoxic effect against the human cells. We could recommend the preparation of a triple mixture of clove, pomegranate peel and coffee as a mouth wash for the treatment of caries causing agent with safety issue.

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