International Research Journal of Biological Sciences ISSN 2278-3202
Vol. **2(2),** 1-7, February **(2013)** *Int. Res. J. Biological Sci.*

Antibacterial Activity of *Pistacia atlantica* extracts on *Streptococcus mutans* biofilm

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Available online at: www.isca.in
Received 21st May 2012, revised 30th January 2013, accepted 3rd February 2013

Abstract

One of the important virulence properties of Streptococcus mutans *is their ability to form biofilms known as dental plaque on tooth surfaces. Recently, mastic gum has raised interest in medicine as the public is more aware of the potential hazardous side effects of conventional medications. To determine antibacterial activity of mastic gum (resin of Pistacia atlantica) against the Streptococcus mutans strains, the total numbers of viable bacteria were formed biofilm on polystyrene micro plates with THB medium and 5% sucrose. The extracte of Pistacia atlantica resin was obtained from hydrodistillation with diethylether. The concentrations of 10% to 100% of essential oil were prepared. The levels of total cultivated bacteria were measured before and after increasing the extracts of P. atlantica resin. Detrmination of MIC was showed antibacterial activity of extracts of P. atlantica. In order after increasing the extracts of mastic gum 60% and up for 60, 10 and 1 minutes, a significant decrease of total bacteria was observed . The reduction in bacteria was not significant in concentrates of 10% to 30% at 1 minute incubation. The results show that the extracts of P. atlantica resin decreased the total viable S. mutans biofilms. . In this work, the chemical composition of extracted resin was studied by GC-MS, and the majority of their components was identified. β-pinene (70 %), α- copaene (76%) and α- terpinolene (86%)were found to be the major components. Extracts of P. atlantica resin has an antibacterial activity against S mutans and may be useful for maintaining oral hygiene during dental injuries treatment.*

Keywords: *Streptococcus mutans*, biofilm, *Pistacia atlantica*, antibacterial activity.

Introduction

Essential oils as antimicrobial agents are recognized as safe natural substances to their users and for the environment and they have been considered at low risk for resistance development by pathogenic microorganisms[1].

The resiniferous pistachio tree belongs to *Pistacia*, a genus of eleven species in the Anacardiaceae family distributed in the Mediterranean and Middle Eastern areas[2]. Greece is one of the most important pistachio producing countries, along with Iran, Turkey, and India. Pistachios are commercially used as in-shell snacks, in confectionery, in ice creams, candies, bakery goods and as a flavoring[3]. The leaves are a lternate, pinnately compound, and can be either evergreen or deciduous depending on species[4]. The *Pistacia atlantica* (Betoum) is a tree which can reach 25 m in height. It is the most characteristic plant species of the pre-Saharian regions of the country[2]. This plant has also been used for the treatment of peptic ulcer and as mouth freshener[5].

The aerial part has traditionally been used as a stimulant, for its diuretic properties, and to treat hypertension, coughs, sore throats, eczema, stomach aches, kidney stones and jaundice[6]. "Gum" mastic, oleoresin exudates from the stem of this plant[7] is a source of traditional medicinal agent for the relief of upper abdominal discomfort, stomach aches, dyspepsia and peptic ulcer[8]. The chemical composition of the essential oil of this plant reveals the presence of several main compounds: myrcene (19 - 25%), a_-pinene (16%), terpinen-4-ol (22%)[9], d-3-carene (65%)[10], myrcene, limonene, terpinen-4-ol, a-pinene, b -pinene, a -phellandrene, sabinene, *para*-cymene and g-terpinene[11].

The essential oil of the resin proved to be very active against micro-organisms and fungi, whereas the oils from the leaves and the twigs showed a moderate activity against the bacteria and was completely inactive against the fungi[12]. The antioxidant properties of the leaves phenolic compounds were reported[8,13,14]. The Chios mastic gum (CMG) is also known to contain compounds that inhibit the proliferation and induce the death of HCT116 human colon cancer cells in vitro[15]. The two most common types of dental disease and periodontal disease, are plaque-related infections[2]. *S.mutans* is generally known to be the principle causative of dental caries[16]. These bacteria metabolize carbohydrates and producing an adhesive polysaccharide such as dextran from the glucose moiety and lactic acid from the fructose moiety. The synthesis of sticky, insoluble glucan promotes the firm adherence of the organism to the

tooth surface that contributes to the formation of dental plaque. Therefore, a rinsing solution with inhibitory effect on plaque formation with anti-microbial activity will be very useful. Some of these phytopharmaceuticals have been shown to be good alternative to synthetic chemical substances for caries prevention[17]. *Streptococcus mutans* are able to synthesis extra c ellular polymers. Biofilm formation conduce chronic infection and resulting to high resistance toward antibiotics makes a serious problem to treatment these patients. In this study *Streptococcus mutans* strains was selected with prevalent capability in biofilm formation for testing antimicrobial effects of extracted *Pistacia atlantica* gum mastic. Antimicrobial, anti-inflammatory and insecticidal activities of essential oils and crude extracts of leaves and gums of *Pistacia* species (specifically, *P. lentiscus*) have been reported previously[12]. The chemical composition[12] and antimicrobial activities[18] of essential oil from the leaves of *P. vera* have been reported. In Iran, the role of *P. vera* in treatment of diarrhea has been known for many years [5]. The aim of this study is to evaluate the effects of extract obtained from mastic of *P. athlatica* on planktonic and biofilm cells of *S.mutans* and analysis of components of its.

Material and Methods

Plant Material and Extraction: The resin of *P. atlantica* (pistachio tree of the Atlas) was collected from the Bukan region (46.212 length, 36.522 width and 1373 m height from sea) west of Iran between May-June 2010, which corresponds to the period of oleoresin formation. The essential oil was extracted from the resin by hydrodistillation with diethylether. The combined hydroalcoholic ex088 tract was filtered through filter paper and evaporated to dryness under reduced pressure in a Rota-vapor and then stored in the dark at 4°C with no air contact. The extract was further used for screening purposes[19, 20].

Chromatographic Analysis: The GC-MS analysis of the samples was undertaken using a Shimadzu GC-17A, QP-5000 GC-MS system, operating in electron ionization (EI) mode with an ionization energy of 70 eV. The instrument was equipped with a capillary column (30 m, 0.25 mm i.d., 0.25 μm film thickness) with helium as carrier gas at 1 mL/min flow rate. Column temperature was initially kept for 1 min at 60 ºC, gradually increased to 180ºC at a rate of 3.5 ºC/min, and finally increased to 280 ºC at a rate of 20 ºC/min and kept there for 2 min. The injector and interface were set at 220 and 250 ºC, respectively. The gas chromatograph operated in the split mode with a split ratio of 93:1. The injection volume was 1 μL. The injected solutions were solution of mastic oil in ethanol (50% v/v), diethyl ether solutions of each standard (1% v/v), the diethyl etherl soluble part of mastic gum (30 mg/mL sample before filtration), and the collected fractions from the distillation[3].

Biofilm formation: *Streptococcus mutans* (ATCC 700611) biofilm was constituted on 96-well (flat bottom) polystyrene micro plates (Pooya Teb Co.). Microtiter plates were initiated with 18-h THB cultures transferred into fresh medium. THB contains 5% sucrose and biofilms incubated at 37°C, in a 5% aerobic atmosphere to an optical density at 600 nm (OD600) of 0.5. The cultures were diluted 1:100 in fresh THB, and then 200 µl of the cell suspension was inoculated into the wells. Wells containing uninoculated growth medium were used as negative controls. Plates were incubated at 37°C in a 5% CO_2 aerobic atmosphere for 16 to 24 h. Before biofilm quantification, growth was assessed by measuring the absorbance of cultures in the wells at 600 nm by using an enzyme-linked immunosorbent assay (ELISA) reader (Bio-Rad) and of planktonic cultures grown under the same conditions (A_{600}) was measured[21]. Media and unattached bacterial cells were decanted from the wells after 5 min of agitation at 200 rpm on a shaker and the remaining planktonic or loosely bound cells were removed by gentle rinsing with 200 µl of sterile distilled water. The plates were then blotted on paper towels and air dried, and adherent bacteria were stained with 50 µl of 0.1% crystal violet for 15 min at room temperature. After two rinses with 200 µl of water each time, the bound dye was extracted from the stained cells by using 200 µl of 99% ethanol, and the plates were set on a shaker to allow full release of the dye[22]. Scanning electron microscope was used for evaluation of biofilm formation.

Bacterial viability: An aliquot (0.1 mL) of the homogenized suspension was serially diluted and plated on tryptic soy agar or blood agar by means of a spiral plater. The plates were incubated in 5% CO_2 at 37°C for 48 h, and then the number of cfu was determined. The sonication procedure provided the maximum recoverable counts as determined experimentally[23,24].

Evaluation of the antibacterial activity: The disc diffusion method was used for the determination of the antibacterial activity (Gulluce *et al*., 2003). Sterile Discs, 6 mm in diameter (Wattman paper No.1), impregnated with 5 and 10 µL of extracted (0.316 g/mL) were placed in Petri dishes on Mueller-Hinton agar, which had been surface spread with 1 mL of logarithmic phase bacteria adjusted to a 108 UFC/mL fixed by the optical density (OD = 0.08 and 0.1). The Petri dishes were then incubated for 18 h at 37°C. The diameter of the inhibition zone was measured to compare the in vitro antibacterial activity.

Determination of MIC values: The MIC of the resin of *P. atlantica* was measured by the liquid serial dilution culture method using 10 mL of sterile 5% sucrose-Trypticase Soy Broth (TSB). The diethylether and aqueous extracts were diluted with water. Bacteria ($1x10^6$ cfu mL^{-1}) were added to each culture tube containing serially diluted test extract or control and incubated for 24 h at 37°C[25, 26].

Determination of bactericidal activity: To estimate whether inhibition of growth was bactericidal, 10 times MIC of the pistachio extracts were used in the experiment. Samples were collected over an extended period. The stocks concentration used were 1 mg mL^{-1} of diethyl ether extract and 100 mg mL^{-1} of aqueous extract for *Streptococcus mutans*. These samples were diluted and inoculated onto a plate at each appropriate time. After incubation, the number of colonies was counted. Further, to investigate the effect of the extracts on non-multiplying bacterial cells, resting bacterial cells were prepared. The growing cells were harvested, washed three times with 50 mM tris-HCl buffer (pH 7.3, TB) and used for determination under aerobic condition. The lowest concentration of the extracts that inhibited growth was noted (Kamrani, *et. al*., 2007). MIC for planktonic cells and biofilm forms of bacteria was determined. Then the ability of biofilm formation in presence of 5% sucrose was indicated.

Results and Discussion

Antibacterial activity was determined by measuring the diameter zone inhibition. Diethylether extract showed more inhibitory effect than aqueous and chloroformic extract (table 1) on all test bacteria and used for rest of this study. At concentration of 100% always have highest inhibitory zone.

Table-1
Antibacterial activity of mastic gum of *Pistacia atlantica* on *S.mutans* strains

Concentration of extracts	Disc diffusion assay (inhibition zone mm)	MIC* (mg/mL)
Control	0	O
10%	14.5	8.54
20%	15.2	7.12
30%	16.3	6.34
40%	17.5	5.67
50%	18.6	4.75
60%	20.2	2.78
70%	21.1	2.13
80%	21.6	1.56
90%	22.8	0.08
100%	31.6	0.05

*:Disc diameter 6 mm average of three consecutive trials MIC: Minimal Inhibitory Concentration, concentration range 0.05-8.54 mg/mL. The antimicrobial test was done using the agar-well diffusion method on BHI agar and incubated for 24 h at 37°C. Values are given in mm and expressed as mean ± SEM (n = 5).

The chemical GC-MS analysis of the extracted of the whole resin showed that it was very rich in β -pinene (70 %), -α Copaene (76%) and α- Terpinolene (86%). In total, twenty five constituents were identified (98.3%). The enantiomeric analysis showed that the (+) /(-)-β -pinene ratio was 99.5:0.5, (+)/(-)-α- Copaene 85:20, (+)/(-)-Trans-verbenol 95:8 and (+)/(-)-α- Terpinolene 0:100. Table 2 contains components of extracted of the whole resin, along with their percentages.

Table-2
Chemical composition of the extracted of the whole resin as determined by GC -MS analysis

Purity (%	Percentage (%)	Composotions
65%	0.0278%	Diethyl ether
86%	0.0139%	α-Pinene
9.5%	0.0125%	Camphene
70%	0.0421%	β-Pinene
23%	0.00219%	Limonene
86%	0.0345%	α-Terpinolene
67%	0.0342%	α-Copaene
57%	0.0216%	Trans-carveol
18%	0.0150%	Verbenone
79%	0.0278%	α-Terpineol
84%	0.00986%	Trans-verbenol
64%	0.00121%	3-Carene
28%	0.00449%	Tolune
25%	0.000544%	Phenol,3-(1-methylethyl)
44%	0.000175%	β-Caryophyllene
57%	0.00665%	Hexanol,2-ethyl

In this study result haven't been seen any significant decrease in viable cells of *S.mutans* in concentration of 10 to 40% of extracted of *Pistacia atlantica*. Whereas in 60% and up was distinguished particularly decrease in viable cells of biofilm (figures 1 and 2).

The antimicrobial activity of *P. lentiscus* essential oils and its resin against different micro-organisms has been reported by several researchers[2,15,27] but little is known on the bactericidal effect of *P. atlantica.* Several members of the genus Pistacia have been chemically investigated. They are characterized mainly by the occurrence of flavonoids and flavonoid glycosides[28]. These plants have also been reported to contain phenolic compounds and triterpenoids[29, 30]. Previous works on *Pistacia vera* concern mainly the resin of the plant[31,32], the hull[33] or the nutritional value of the nut[34]. Concerning the leaves of the plant there is one chemical study of the essential oil along with its antifungal activities from leaves of *Pistacia vera* grown in Turkey[35]. Interestingly, in spite of the commercial value of fruits, the contained mastic gum has never been studied[3].

In this study, we assessed the antibacterial activity of *P. athlatica* mastic gum extracts on one of important oral bacteria *S.mutans* biofilm with the aim of preventing dental caries**.** In this microbial analyses, the diethylether extracts of *P. athlatica* showed stronger inhibitory activity compared to aqueous extracts. Diethylether extract of *P. athlatica* has been demonstrated to manifest good antimicrobial activity, as evidenced by the MICs of obtained against a *S.mutans*. The weak effect of aqueous extrac was predictable. The terpenoids have hydrophobic properties causing low solubility in aqueous media[5]. In previous studies were existed reasons for the effectiveness of mastic oil against the bacterium *H. pylori* [36].

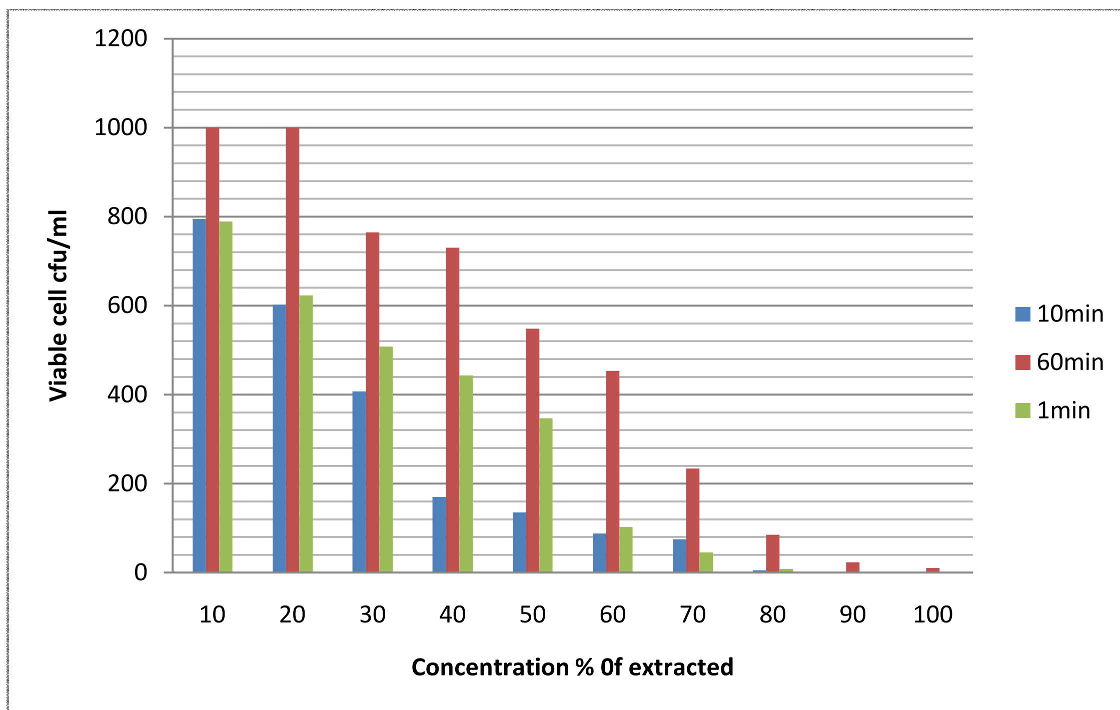

Figure-1
Relation between concentration of *Pistacia atlantica* mastic gum extracts and viable cells in biofilm of *S.mutans*

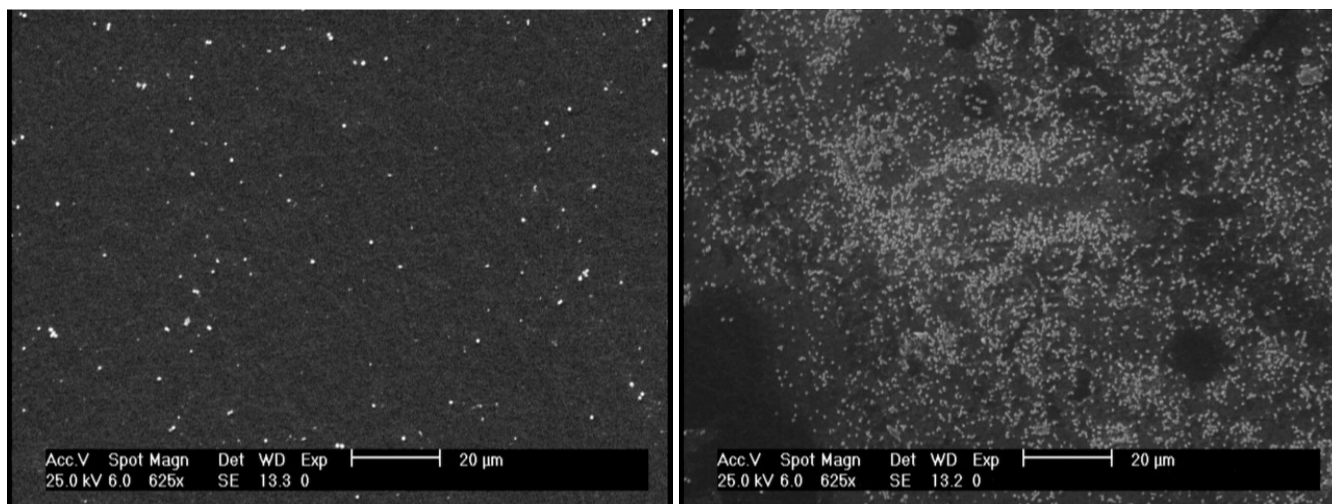

Figure-2
Scanning electron micrographs comparing biofilm formation of *S. mutans* accumulated on polystyrene tips after 48 h of inoculation treatmented with concentration of 60% of extracted of *Pistacia atlantica* (left) and without treatment (right). Images were obtained at ×625 magnification

To determine whether the observed inhibition of bacterial growth by diethylether pistachio extract is bactericidal or bacteriostatic, viable cell method against *S.mutans* planktonic and biofilm forms were used. The pure diethylether extracts killed more than 98% of both planktonic and biofilm cells of *S.mutans* within 1 h. While aqueous extract showed weak bacteriostatic activity, the action of diethyether extracts was bactericidal. In contrast, the bacterial cells in biofilm were not affected by the aqueous extract and the diethylether extracts with 50% concentration and less. The antibacterial effect of diethylether extracts can be attributed to the presence of phenolic compounds in the extract and similar activity has been reported previously. Present data in agreement with

other reports proposing that *P. athlitica* have anti-bacterial activity[37].

Kordali S. and et. al. shows that the ethyl alcohol extracts obtained from the leaves of *Pistacia vera*, *Pistacia terebinthus* and *Pistacia lentiscus* were tested for antifungal activities against three pathogenic agricultural fungi[36]. Ghalem and Mohamed have been used the hydrodistilled essential oils from *Pistacia vera.* L stem exudates against three bacteria (*Escherichia coli*, *Staphylococcus aureus* and *Proteus spp*) and found with increasing essential oil resin of *P. vera* concentration, an obvious inhibitory effect on the growth of *E. coli*, *Proteus spp.* and *S. aureus*, was significantly increased[3].

The relative incubation period of diethylether extract against *S mutans* biofilms revealed that the composition of extract probably less heat sensitive or volatile and with increasing incubation time rise killed cells number in both forms. In this study we were used polystyrene surface to represent the sucrose-dependent adherence hard surface of the tooth[37].

S. mutans adherence to surfaces is mediated by glucan as well as the in vivo situation and the polystyrene adherence assay is still used in some recent studies[23]. In this study inhibition of adherence of *S. mutans* by sub-MIC concentrations of the extract would be possible that the bioactive compound(s) such as flavonoids, tannins in the *P.athlatica.* Flavonoids are known to have anti-GTase activity. This enzyme is responsible for the conversion of sucrose to sticky insoluble glucan, which promotes the firm adherence of *S. mutan s* to the surface of the tooth.

At concentrations of 90%, the extract had an immediate effect on the biofilm bacteria and this effect was retained for 60, 10 and 1 minutes. This activity is also associated with inhibition of adherence to polystyrene surface. Thus, the extract could successfully prevent plaque formation on the surface of the tooth. Based on results obtained from the present study, it is evident that the flavonoid and other phytoconstituents present in the resin extract had bactericidal and bacteriostatic activity against *S. mutans* at different concentrations. These results are almost similar to those shown by other works on the antimicrobial activity of oil mastic gum of *Pistacia vera* as well as those of similar species[3, 2].

In this study the various extracts from diethylether were analyzed by GC-MS. Various compounds were characterized: for instance, a- pinene in the essential oil has been recently reported[6], as well as monoterpenes and oxygenated sesquiterpenes as terpinen-4-ol (21.7%) or elemol (20%)[13]. In comparison with *P. lentiscus*, there are few reports in the literature about the antioxidant properties of *P. atlantica* 9.

Conclusion

The aim of this work was to evaluate the antimicrobial activities of the diethylether mastic gum extracts of *Pistacia atlantica* with their phenollic compounds against *S.mutans* biofilm that forming dental plaque[3]. In conclusion, the antibacterial activity of mastic gum as a by-product can be attributed to the combination of oral hygiene for pharmaceutical preparations such as mouthrinse.

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