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Hypothesis

Antimicrobial effect of *Pistacia atlantica* leaf extract

Mohamad Ali Roozegar[1], Farid Azizi Jalilian[2], Mohamad Reza Havasian[3], Jafar Panahi[3] & Iraj Pakzad[4,5,*]

[1]Department of Periodentistry, Faculty of Dentistry, Ilam University of Medical Sciences, Ilam/Iran; [2]Department Microbiology, Faculty of Medicine, Hamadan University of Medical Sciences, Hamadan/ Iran; [3]Mostafa Khomeini Hospital, Ilam University of Medical Sciences, Ilam/Iran; [4]Department Microbiology, Faculty of Medicine, Ilam University of Medical Sciences, Ilam/Iran; [5]Clinical Microbiology Research Center, Ilam University of Medical Sciences, Ilam/Iran; Iraj Pakzad - Email: mpvmpv559@gmail.com; *Corresponding author

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Abstract:
The antimicrobial effect of the mastic tree (*Pistacia atlantica*) under *in vitro* conditions has been reported. Therefore, it is of interest to evaluate the effect of the plant leaf extract (aqueous) on bacterial load in mouth and saliva. The leaf of the *Pistacia atlantica* plant was collected and cleaned, dried at 40^0c and then powdered. The extraction was carried out using the maceration method in vacuum with the rotary evaporator device. Bacterial inhibition (*Streptococcus* species) by the leaf extract was studied using the disc diffusion and embedding sink diffusion methods. The values of MIC and MBC were determined. The collected data was further analyzed using t-test and repeated measure statistical tests. The disc diffusion technique showed a significant inhibitory effect for *Pistacia atlantica's* leaf extract on *S. mutans* (ATCC 35668) and *S. mitis* (ATCC 49456) with inhibition zones of 19 and 25 millimeters, respectively. This is for the highest leaf extract concentration used in this study ($p<0.01$). The values of MIC and MBC for *S. mutans* was 60, 90 µg/ml and for *S. mitis* was 75, 110 µg/ml ($p<0.01$ significance). The leaf extract has no significant effect on *S. salivarius* (ATCC 13419). Thus, the antimicrobial properties of the aqueous leaf extract from *Pistacia atlantica* is demonstrated in this study.

Keywords: *Pistacia atlantica*, leaf extract, anti-bacterial, *Streptococcus mutans*, *Streptococcus mitis*, *Streptococcus salivarius*

Background:
Streptococcus mutans is gram-positive *cocci* and is a facultative anaerobe that exists in the normal flora of the human mouth. It is the most important factor in dental caries **[1, 2].** *S. salivarius* is a type of streptococcus which exists in the normal flora of the mouth and in the upper respiratory system of humans. This bacterium is known as an opportunistic pathogen. It often causes septicemia in patients with neutropenia in the blood circulatory system **[3, 4].**

S. mitis is an alpha hemolytic and mesophilic type of *Streptococcus* (genus), which inhabits the oral cavity. These bacteria can cause endocarditis **[5, 6].** The treatment of the disease using extracts from several plant parts is known through regional yet traditional practice and possible documentation in several parts of the world. The modern drug discovery process using advanced robotic screening of traditionally known plant parts is well known **[7].** The use of plant derived herbal products as food supplements for medication is largely in practice **[8-9].** The *Pistacia* (genus) plant is known for its medicinal property. The plant species *P. atlantica* mutica (sub species) and *P. atlantica* kurdica (sub species) is in the northern mountains of the Iranian Ilam province.

The antimicrobial properties of the native species are known **[10-12].** The use of different species of *Pistachio* as antibacterial, antifungal, antiviral, anti-atherogenic, hypoglycemia, antitumor and facilitating hepatic function is known **[9].** Therefore, it is of interest to evaluate the effect of *P. atlantica* leaf extract (aqueous) on bacterial (*S. mutans, S. mitis and S. salivarius.*) load in mouth and saliva.

Methodology:
Collection and extraction of the P. atlantica leaf
The plant leaf was collected from the mountains of the Ilam province. The leaf is rinsed with water, dried at 40^0c and then

powdered **[13].** Subsequently, 10 grams of powdered plant leaf was mixed with 200 ml of boiled distilled water and the solution was mixed constantly for 20 minutes. It was then poured into a close lid container and kept at room temperature. The solution was passed through a fabric filter with fine texture. The filtered extracted solution was centrifuged for 15 minutes at 3500 rpm and then it was exposed to air until the solvent had completely evaporated to retain the powder **[14].**

Bacteria

Bacterial standard strains of *Streptococcus mutans* (ATCC 35668), *Streptococcus mitis* (ATCC 49456) and *Streptococcus salivarius* (ATCC 13419) were used. The BHI broth and agar and chocolate agar culturing medium was used in an environment with 5% CO_2 for initial culturing. The sensitivity test of the bacteria was analyzed in the Mueller Hinton agar medium (Pronadisk co. Italy) containing 5% de-fibrinated blood was used.

Disk diffusion Method

This method was completed using a 1x 10^8 CFU/ml suspension of bacteria and blank disks with a diameter of 6.4mm (MAST Co. UK) treated with different concentrations of the extract (5, 10, 20, 40, 80 and 100 mg/ml) were used. Mueller Hinton agar culture medium containing 5% de-fibrinated blood under completely sterile conditions was used as described elsewhere **[15, 16].** The results were checked at 24, 48 and 72 hours after culturing. Amoxicillin with a concentration of 25 µg/ml (MAST Co. UK) was used as positive control and blank disks were used as negative control in this experiment.

Embedding sink diffusion method

The diffusion in agar method was used with slight modifications. Wells with a diameter of 5mm were created in the Mueller Hinton agar medium after adding 1 x 10^8 CFU/ml of the species to the intended medium. The bottom of each well was obstructed with Mueller Hinton agar under sterile conditions in order to prevent the extract from diffusing under the medium. 25 microns of the different concentrations (5, 10, 20, 40, 80 and 100 mg/ml) was added in each well and after 24-48 hours the results were recorded **[17, 18].**

MIC determination

The micro-broth dilution method was used to determine MIC. Solutions with concentrations (100, 200 and 400 µg/ml) were added in 2 ml Mueller Hinton Broth medium. 20 µl of bacteria suspension with turbidity equal to 0.5 McFarland was added to each tube. Then the tubes were incubated at 35°C for 24 hours **[19].**

MBC determination

The MBC determination method is similar to the method used for MIC with the difference that bacteria counting were carried out on test tubes with concentrations of MIC and or higher **[19].**

Statistical analysis

The results were analyzed using the SPSS (version 18) software. The t-test and repeated measure statistical tests were used for the analysis **[14].**

Table 1: *Streptococcus* inhibition by *P. atlantica* leaf extract using disk and embedding sink diffusion methods.

Species	**Extract** Concentration (mg/ml)	**Inhibition zone** (mm) Diffusion methods	
		Disk	Embedding sink
S. mutans	5	5	5
	10	8.5	5
	20	11	8
	40	20	15
	80	25	21
	100	25	22
S. mitis	5	0	0
	10	3.5	0
	20	7	5
	40	15.5	5
	80	18	9
	100	19	13
S. salivarius	5	0	0
	10	0	0
	20	4	4
	40	5	4
	80	5	6
	100	5	6

Results:

Disk diffusion and creating wells

The results from disk diffusion and creating wells showed a significant inhibitory effect of the aqueous leaf extract on the standard strains of *S. mutans* with a halo of 25 and 22 mm for the highest concentration of the extract **Table 1** ($P<0.01$). The results also showed the suitable effect of the extract on the standard strain of *S. mitis* under *in vitro* conditions **Table 1** ($P<0.01$). However, the data showed a weak effect on *S. salivarius* for the highest extract concentration **(Table 1)** ($P<0.01$).

MIC and MBC results

The results from the micro dilution method showed that the MIC of the extract for *S. mutans* and *S. mitis* is 60 and 75µg/ml, respectively. The MBC of the extract on *S. mutans* and *S. mitis* is 90 and 115 µg/ml, respectively ($P<0.01$).

Discussion:

The use of medicinal plants derived compounds for treatment of illness is traditional and often believed to be simple yet naturally safe **[20].** The application of herbal medicine as anti-microbial agent is an alternative solution where anti-biotic resistance is ascertained **[21-24].** The undesired effects of several synthetic compounds as drugs are known **[25].** Therefore, it is of interest to evaluate the effect of *P. atlantica* leaf extract (aqueous) on bacterial (*S. mutans, S. mitis* and *S. salivarius*) load in mouth and saliva. The *Pistacia* plant is known for its medicinal properties and its antibacterial effects are known **[26, 27].** The two sub-species of *P. atlantica* mutica and *P. atlantica* kurdica are available in plenty in the northern mountains of Ilam province in Iran. Their use for medicinal purpose is of relevance.

We studied the anti-bacterial effect of its leaf extract (aqueous) on selected *Streptococcus* species (*S. mutans, S. mitis* and *S. salivarius*). Results showed the strong effect of the *P. atlantica* plant leaf extract on *S. mutans* with a MIC of 60 and MBC of 90

µg/ml. The aqueous extract of this plant also had desirable effects on *S. mitis* with a MIC of 90 and MBC of 115 µg/ml. However, the leaf extract has no significant effect on *S. salivarius* (ATCC 13419). Azimi Laysar *et al.* (2013) showed the effect of different concentrations of Nigella on the growth of *Streptococcus mutans* **[28].** The results showed that the effects were not as strong as the leaf extract of *P. atlantica.* Azizianet et al (2013) showed the antimicrobial effect of the plant *P. atlantica* aqueous extract on *Streptococcus mutans* **[29].** Results showed that the inhibitory effect of the extract was stronger than amoxicillin disks but this difference is not statistically significant (P>0.01). Thus, results show that the antimicrobial effects of the aqueous extract from the leaf of the *Pistacia atlantica* plant are stronger and more desirable compared to other plant extracts **[30, 31, 32, 33].**

Conclusion:

Data presented in this report show the effect of the plant *Pistacia atlantica* leaf extract on *S. mutans* and its desirable effects on *S. mitis*. The leaf extract is rich in phenol compounds and it is implied to be associated with anti-bacterial properties **[34].** Further studies are necessary to determine the anti-cellular effects of the plant extract.

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