

In Vitro Effect of Pomegranate Extract on *Trichomonas Tenax*

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Abstract

The incidence of *T. tenax* in patients with acute ulcerative gingivitis has been demonstrated in several published reports about it. Metronidazole was known as the most effective drug for human trichomoniasis, however, drug resistance and toxicity appeared. This study was designed to investigate the *in vitro* inhibitory activity of *p. granatum* ethanol extract on the growth and motility of *Trichomonas tenax* in comparison to metronidazole. Pomegranate ethanol extract group was divided into four groups with concentration of 12.5, 25, 50, and 100 mg/ml, respectively, metronidazole group and blank control. Metronidazole group was given 10 µg/ml of the drug. Each group had 4 wells with 125 µl *T. tenax* (2×10^5 /ml). At 12 hr, 24 hr, 48 hr and 72 hr after drug treatment, the anti-*T. tenax* effect of pomegranate ethanol extract was tested by microscope counting method. The results showed that the better effect on anti-*T. tenax* 60% pomegranate ethanol extract group with concentrations of 12.5 mg/ml and 25 mg/ml showed higher anti-*T. tenax* ($P < 0.01$). The ethanol extract of pomegranate granules has a remarkable effect on *T. tenax*, and among the groups, 60% ethanol extract shows the best anti-*T. tenax* activity.

Keywords: *Trichomonas tenax*; *Punica granatum*; Metronidazole; Herbal medication

Introduction

The human oral cavity is home to numerous microorganisms. *Trichomonas tenax* (*Trichomonas buccalis*) is a regular guest of human oral cavity microorganism [1]. It is an anaerobic species that lives as a commensal in the mouth of human oral cavity. It is frequently associated with pyogenic organisms in pus pockets or at the base of teeth. There are studies that relate to its prevalence in patients with Marginal Chronic Periodontitis [2]. Transmission is through saliva, droplet spray, and kissing or use of contaminated dishes and drinking water [3]. World widely, its prevalence in the mouth ranges from 4 to 53% [4,5].

The detection of *T. tenax* in the human oral cavity is an indication of hygiene poor oral, so that its incidence increases significantly in patients with periodontal problems, this being three to four times higher than in periodontal healthy subjects [6].

Since the organism is believed to enter the respiratory tract by aspiration from the oropharynx and then cause bronchopulmonary trichomoniasis, the importance of oral infections has been increased recently [7].

The development of drug resistance in human pathogens against commonly used treatment has necessitated a search for new therapeutic agents from other sources. Recently, there has been considerable interest in the use of plant materials as an alternative method to control pathogenic microorganisms [8,9], and many compounds of plant products have been shown to be specifically targeted against resistant pathogens [10].

Punica granatum, which belongs to the family of Punicaceae, is commonly known as pomegranate, grenade, granats and punica apple [10]. *Punica granatum* has been used extensively as a traditional medicine in many countries [9] for the treatment of dysentery, diarrhea, helminthiasis, acidosis, hemorrhage and respiratory pathologies [11,12] In addition, *P. granatum* is reported to have antioxidant [9,10] anti-atherosclerotic [13,14], antibacterial [15,16] and antiviral [17] properties. The constituents of *P. granatum* include galloatechins, delphinidin, cyanidin, gallic acid, ellagic acid, pelargonidin and sitosterol, which are very well known for their therapeutic properties

[18]. *Punica granatum* peel is used to treat infections found in human sexual organs as well as mastitis, acne, folliculitis, pile, allergic dermatitis, tympanitis, scalds, diarrhea, dysentery and as an antioxidant. In addition, it is reported that the extracts of *P. granatum* have antimicrobial activity against *Salmonella* [18].

Pomegranate components have properties that could promote oral health, including reducing the risk of gingivitis. However, to date, no studies regarding the anti-*T. tenax* activity of *P. granatum* extract have been conducted. Therefore, the goal of this study is to evaluate the anti-*T. tenax* activity of the extracts of *P. granatum* peel *in vitro*.

Materials and Methods

Patients

The periodontiums of 51 patients were clinically examined, and diagnosis and classification of the periodontium was done according to the Periodontal Screening and Recording (PSR) I, in agreement with the Military Academy of Periodontology and Dental Association criteria. Twenty patients were diagnosed with gingivitis (EG1), 22 with periodontitis (EG2) and 9 presented a healthy periodontium (CG). The patients were also asked about the use of medications and systemic conditions which might predispose them to the development of periodontal disease.

Sample collection

Samples of saliva and dental biofilm/calculi were collected from all patients in the morning, before any oral hygiene. After determining the frontal mandibular area most affected by periodontal disease (by means of PSR), dental biofilm/calculi samples were collected by scraping the area with sterile periodontal curettes. Unstimulated saliva sam-

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ples were collected as recommended by Navazesh. All samples were placed in sterile Petri dishes and diluted with saline at room temperature (25 to 28°C). Immediately after dilution, the samples were examined under a light microscope.

For the removal of dental calculus, as well as debris and plaque subgingival periodontal curettes used tartrectomos previously sterilized and, taking the material with the latter the bottom of the periodontal pockets of patients with PMC were also taken plaque samples tooth around the neck of the teeth of patients, employing previously sterile curettes. Dental calculus removed from the experimental group, was ground prior to planting and microscopic observation, using previously sterilized glass rods.

Transport of samples

The samples were placed in vials containing transport medium (sterile Ringer solution) and taken to the laboratory for further planting and microscopic observation.

Inoculation of the samples

Once the samples were taken to the laboratory, 0.1 ml was taken. Ringer's solution containing the inoculum, using sterile 1 ml pipettes, inoculating the above-mentioned amount in the broth selective Kupferberg, used for the growth of *T. tenax* ("Kupferberg *Trichomonas* Broth," Difco Laboratories, Detroit, Michigan, USA), to which were added 0.1 g of chloramphenicol to prevent the growth of bacteria and other microorganisms. Two plantings were made for each patient, an aerobically and anaerobically another, using the jug designed for this purpose (Gas Pak). Seeded culture media were taken to the oven at 37°C for 72 hours.

Microscopic observation

For the identification of *T. tenax* microscopic observations were made three times for each patient in order to determine what opportunities exist in the ability to view the scourge. These observations were made in the first instance on the same day of sampling, taking a drop of inoculum containing transport medium with the previously sterile platinum loop and placed onto a glass slide. The two remaining microscopic observations were performed at 72 hours of selective media incubated in an oven, taking a drop in the previously sterile platinum loop, both the stock that was planted under aerobic conditions as was shown in the anaerobic placing in each case on the surface of its respective blade slide.

In each of the cases described above, was placed on the drop of a slide coverslip microbial suspension, then the observation for the cool, using the light microscope (Leitz), focusing first with a low magnification lens (10 ×) and then with higher magnification lenses (20 × 40 ×).

Extraction of plant material

Preparation of the plant extract: Firstly fresh pomegranates (500 gram) were obtained (in order to prepare fresh extraction) from a public market. The peels of pomegranate were separated and oven dried at 33°C for 7 days. The dried peels were powdered in an electric grinder and stored in plastic bags for the next step. A 100 gm sample of powder was extracted using 200 ml methanol (99.9%) in an electric blender for 30 min. This suspension was filtered three times per day for 30 days. New methanol dissolvent was used each time. Then methanol was removed in a rotary evaporator to produce a dry powder. The final material was dissolved in methanol for obtaining concentrations of 4 mg/ml, 8 mg/ml and 12 mg/ml of dry plant powder [17-19].

Metronidazole

It was supplied as 500 mg tablets (Rhone Poulenc Rorer, France).

Tablets were dissolved in distilled water, and then diluted in incubation medium to yield 12.5 µg/ml, 25 µg/ml and 50 µg/ml and 100 µg/ml [20].

Growth inhibition assay

The effect of *P. granatum* on the growth of the *T. tenax* trophozoites was studied as follows: 2x10⁵ trophozoites were incubated in Selective Kupferberg (KTB) medium with drugs in different concentrations: *p.*

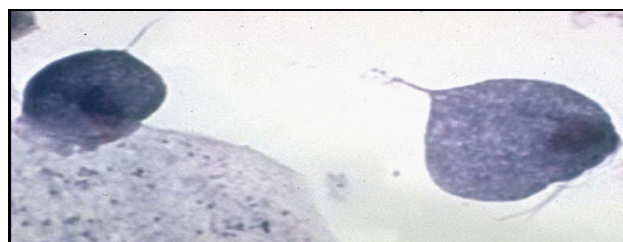


Figure 1: *Trichomonastenax*.



Figure 2: *T. tenax* by light microscope, evidencing a flagellum (40x).

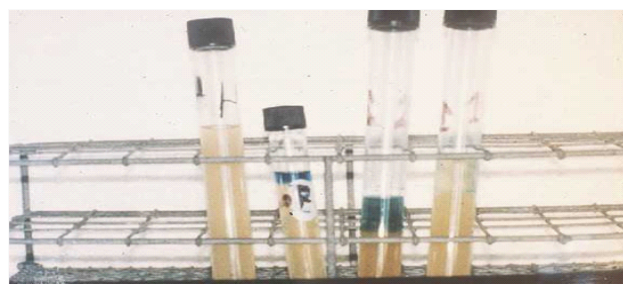


Figure 3: Growth of *T. tenax* in culture medium.

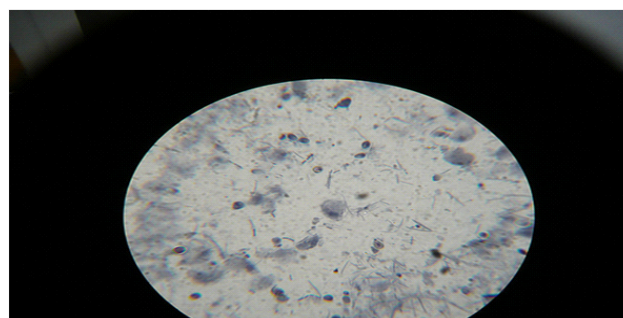


Figure 4: Selective Kupferberg (KTB).

granatum and metronidazole (12.5 µg/ml, 25 mg/ml, 50 mg/ml and 100 mg/ml), for 12, 24, 48, and 72 h at 37°C. In addition, controls were included (cultures containing only the parasites) and submitted to the same procedure used for the experimental cultures.

Evaluation of the drug efficacy was done by:

1. Counting the number of trophozoites using the haemocytometer (Neubauer cell-counter chamber).
2. Calculation of the percent of inhibition of multiplication according to the equation:

$$\text{Percent inhibition of growth} = \frac{a - b}{a} \times 100$$

Where;

a=Mean number of trophozoites in control tubes and

b= Mean number of trophozoites in test tubes [21].

3. Calculation of the percent of motility of trophozoites which is the ratio of motile to total number of parasites counted per 10 High Power Field (HPF).
4. The Minimal Lethal Concentration (MLC) of *p. granatum* extract and metronidazole was determined.

Results

The observation in the cool of the sheets prepared allowed us to visualize where they were considered positive, the flagellate in some cases moving in a circular motion and other times not moving and isolated. Usually, the flagellar morphotypes of *T. tenax* can be seen with four flagella, but sometimes can be seen only with one or two flagella (Figures 1-4).

The present study was carried out to investigate the *in vitro* activity of *Punica granatum* peel (EEP GP) on the growth and motility of *T. tenax*, compared to the standard drug metronidazole. The results showed that the degree of growth inhibition was dependent upon the concentration of *P. granatum* and metronidazole

Effect of *P. granatum* extracts in specified times and concentrations on *Trichomonas tenax* were assessed. The inhibitory effect of extract on *Trichomonas* was assessed by counting the live parasites 1, 2 and 24 hours after exposure with extracts.

Findings of this study showed that ethanol extract *P. granatum* at concentration of 12.5, and 25 mg/ml killed 80% and 70% after 24 hrs and 60% and 20% of the parasites after 24 hour of exposure Moreover killed 98% of parasites at concentration of 12.5 mg/ml after 12 hours of exposure (Table 1).

Lower dose of metronidazole (12.5 µg/ml) showed complete inhibition of growth after 72 hours (Table 2).

Both of *P. granatum* and metronidazole were able to inhibit the motility of the parasite with increasing percent of immotile trophozoites after 72 hr.

Discussion

In the recent years, the use of plants with preventive and therapeutic effects contributes to health care needs [22].

There are three main reasons to be interested in the treating and healing power of plant extract. First, pharmacological studies have demonstrated that many of plants are known to possess antimicrobial agents; second, people are becoming aware of the side effects associated with the over prescription of traditional antibiotics; third, time to time resistant microorganisms against antibiotics are increasing Among these plants, *punica granate* has an important role in folk medicine. *Omegranate* is known as a rich source of pharmacological properties which have been evaluated due to antiparasitic, antibacterial, antifungal, antiproliferative, apoptotic and anti-cancer effects as well as protection against herpes virus, inhibition of LDL oxidation and decrease in atheromatous plaque formation and reduction of systolic blood pressure [18,23,24].

While the vast majority of studies of Microbiology oral relate to various aspects of bacteriology, buccal Protozoology has virtually forgotten, despite the high prevalence of infections in adults where *T. tenax* etiologic agent [25].

In this regard there are many publications that reported the complexity of the microbiota residing in dental plaque, not only in quantitative terms but also qualitative including bacteria, fungi, mycoplasma and protozoa, among them *T. tenax* as part of that system as varied ecological as it is the oral cavity [26,27].

concentration	% of motility			
	12 hr	24 hr	48 hr	72 hr
12.5	98%	80 %	60 %	non motile
25	80%	70 %	20 %	non motile
50	30%	10 %	non motile	non motile
100	No organism	No organism	No organism	No organism
NTC	98%	85 %	50%	non motile

NTC = Non Treated Culture control

Table 1: Percentage of motility of *T. tenax* after exposure to various concentration of ethanol extract in comparison to normal control.

concentration	% of motility			
	12 hr	24 hr	48 hr	72 hr
12.5	60 %	50 %	Non motile	No organism
25	40 %	10 %	No organism	No organism
50	20%	No organism	No organism	No organism
100	No organism	No organism	No organism	No organism
NTC	100%	98 %	85%	50 %

NTC = Non Treated Culture control

Table 2: Percentage of motility of *T. tenax* after exposure to various concentration of metronidazole in comparison to normal control.

Microscopic observation in the cool of the samples from dental calculus and subgingival plaque by using light microscopy is essential if you want to achieve faster discovery *T. tenax*, being able to visualize usually with one or two flagella. However, provided the samples should be inoculated in the culture media most appropriate and allow for finding more sure of this species [28,29].

Conclusion

The incidence of *T. tenax* was higher in patients and this study suggests that EEPGP might be used as an antiparasitic agent in controlling oral *Trichomonas tenax* infections.

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