In vitro Effect of Pomegranate Peel Extract on *Trichomonas tenax*

Gehad T. El-Sherbini[1] and Nahla M. Shoukry[2]

[1] Department of Parasitology, Faculty of Pharmacy, October 6 University Cairo, Egypt.
[2] Department of Zoology, Faculty of Science, Suez Canal University, Suez, Egypt.
nahla_shoukery@yahoo.com and gody_55@yahoo.com

Abstract: The incidence of *Trichomonas tenax* (*T. tenax*) in patients with acute ulcerative gingivitis has been demonstrated in several published reports**.** Metronidazole was known as the most effective drug for human trichomoniasis**,** however, drug resistance and toxicity appeared. This study was designed in vitro to investigate the inhibitory activity of *Punica granatum (P. granatun*) ethanol extract on the growth and motility of *T. tenax* in comparison to metronidazole. Pomegranate ethanol extract group was treated with concentrations of 12.5, 25, 50, and 100 µg/ml. Metronidazole group and blank control were included. At 12 h, 24 h 48 h and 72 hr after drug treatment, the anti-*T. tenax* effect of pomegranate ethanol extract was tested by microscope counting method. The results showed 60% motility of *T.tenax* trophozoite after treatment with 12.5µg/100 ml of pomegranate ethanol extract group and 25 µg/ml showed higher anti-*T. tenax* ($P<0.01$). The ethanol extract of pomegranate peel has a remarkable effect on *T. tenax*, and among the groups, 60% ethanol extract shows the best anti-*T. tenax* activity.

[Gehad T. El-Sherbini and Nahla M. Shoukry **In Vitro effect of pomegranate peel extract on Trichomonas tenax]** Life Science Journal 2012; 9(3):791-797]. (ISSN: 1097-8135). http://www.lifesciencesite.com. 113

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

1. Introduction

Serum 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 humans' oral cavity. It is frequently associated with pyogenic organisms in pus pockets or at the base of teeth. There are studies 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 poor oral hygiene, 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 (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). 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 (11). *P. granatum* has been used extensively as a traditional medicine in many countries (12) for the treatment of dysentery, diarrhea, helminthiasis, acidosis, hemorrhage and respiratory pathologies (13,14) In addition, *P. granatum* is reported to have antioxidant (15,16) anti-atherosclerotic (17,18), antibacterial (19,20), and antiviral (21) properties. The constituents of *P. granatum* include gallocatechins, delphinidin, cyanidin, gallic acid, ellagic acid, pelargonidin and sitosterol, which are very well known for their therapeutic properties (22). *P. 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* (23).

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*a ctivity of the extracts of *P. granatum* peel *in vitro* .

2. 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) l , 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 samples were collected as recommended by Navazesh (24). All samples were placed in sterile Petri dishes and diluted with saline at room temperature (25 to 28ºC). Immediately after dilution, the samples were examined under a light microscope.

The removal of dental calculus, as well as debris and plaque was performed according to (24). 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 of Ringer's solution containing the inoculum was inoculating in the broth selective Kupferberg, used for the growth of T. tenax ("Kupferberg Trichomonas Broth. "Difco Laboratories, Detroit, Michigan, USA), to which 0.1 g of chloramphenicol was added to prevent the growth of bacteria and other microorganisms. Two plantings were made for each patient, an aerobically and anaerobically, 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:

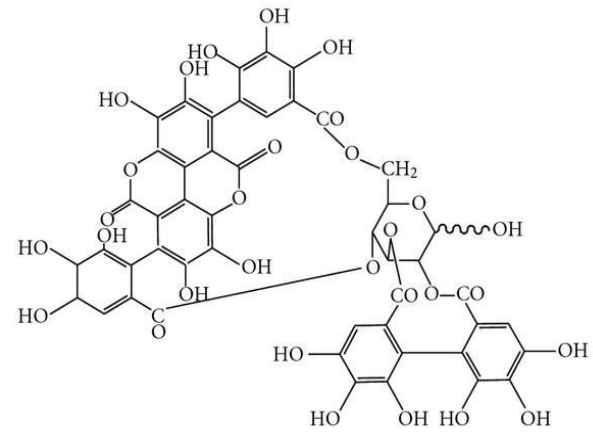
For the identification of T. tenax microscopic observations were made three times for each patient in order to determine what opportunities exist in the ability to view the scourge. These observations were made in the first instance on the same day of sampling, taking a drop of inoculum containing transport 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 above described cases, samples were examined microscopically using the light microscope (Leitz), focusing first with a low magnification lens (10 x) and then with higher magnification lenses (20 x 40 x).

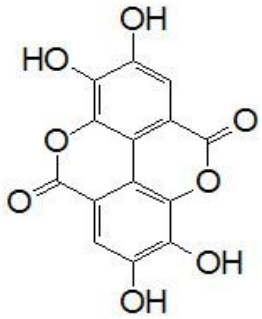
Extraction of plant material

Preparation of the Plant Extract

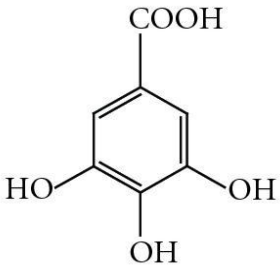
Fresh pomegranates (500 gram) were obtained (in order to prepare fresh extraction) from a public market. The peels of pomegranate were separated and oven dried at 33°C for 7 days. The dried peels were powdered in an electric grinder and stored in plastic bags for the next step. A sample of 100 gm powder was extracted using 200ml ethanol (99.9%) in an electric blender for 30 min. This suspension was filtered three times per day for 30 days. New methanol was used each time. Then methanol was removed in a rotary evaporator to produce a dry powder. The final material was dissolved in ethanol for obtaining concentrations of 12.5µg/ml, 25µg/ml, 50µg/ml and 100 µg/ml of dry plant powder (23, 24, 25).



(a)



(b)



(c)

Fig. 1: The chemical structure of *P. granatum* peel: punicalagin (a), ellagic acid (b) and gallic acid (c)

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 12.5μg/ml, 25 μg/ml, 50 μg/ml and 100 μg/ml (26).

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 different concentrations of P. granatum and metronidazole (12.5 μg/ml, 25 μg/ml, 50 μg/ml and 100 μg/ml), for12, 24, 48, and 72 h at 37°C. In addition, controls were included (cultures containing only the parasites) and submitted to the same procedures 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 [27].

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.

3.Results

The flagellate in some cases move 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 (Fig.2). Growth of *T.tenax* in culture medium is demonstrated in fig 3&4.

The present study was carried out to investigate in vitro the activity of Ethanol Extract of *P.granatum* Peel (EEPGP) 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* extract in specified times and concentrations on *T.tenax* was assessed. The inhibitory effect of extract on *Trichomonas* was assessed by counting the alive parasites 12h, 24 h, 48h and 72 hours after exposure with extracts.

Findings of this study showed that ethanol extract *P. granatum* at concentration of 12.5, and 25 μg/ml resulted in motility percentage of the parasites 60% & 40% after 12h respectively and 50% &10% after 24h of exposure respectively. Lower dose of ethanol extract *P. granatum* (12.5 μg/ml) showed complete inhibition of growth after 48 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.

Table 1: Motility percentage of *T. tenax* after exposure to various concentrations of metronidazole in comparison to normal control

Concentration (µg/ml)	% 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 %	no motility	non motile
100	No organism	No organism	No organism	No organism
NTC	98%	85 %	50%	non motile

NTC = Non Treated Culture Control

Table 2: Motility percentage of *T. tenax* after exposure to various concentrations of *P. granatum* ethanol extract in comparison to normal control

Concentration (µg/ml)	% 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 %

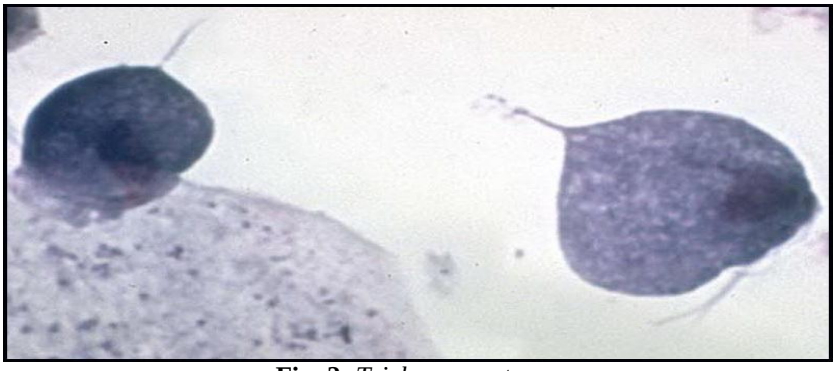


Fig. 2: *Trichomonas tenax*

Fig.3: *T. tenax* by light microscope, evidencing a flagellum (40x).

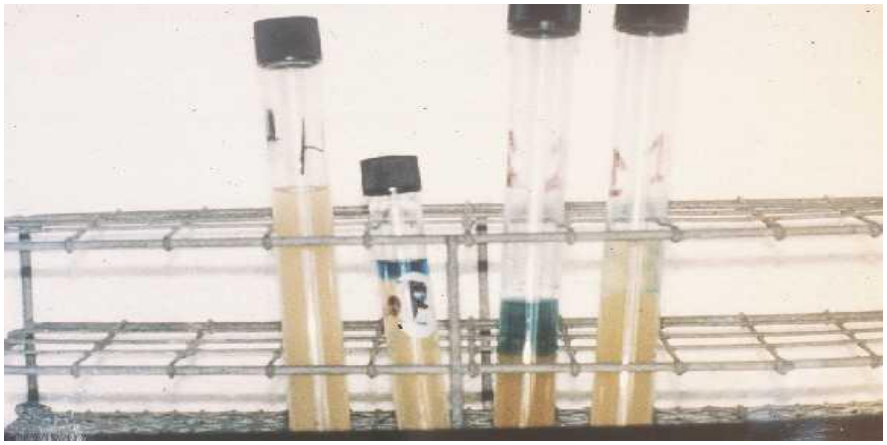


Fig.4: Growth of *T. tenax* in culture medium

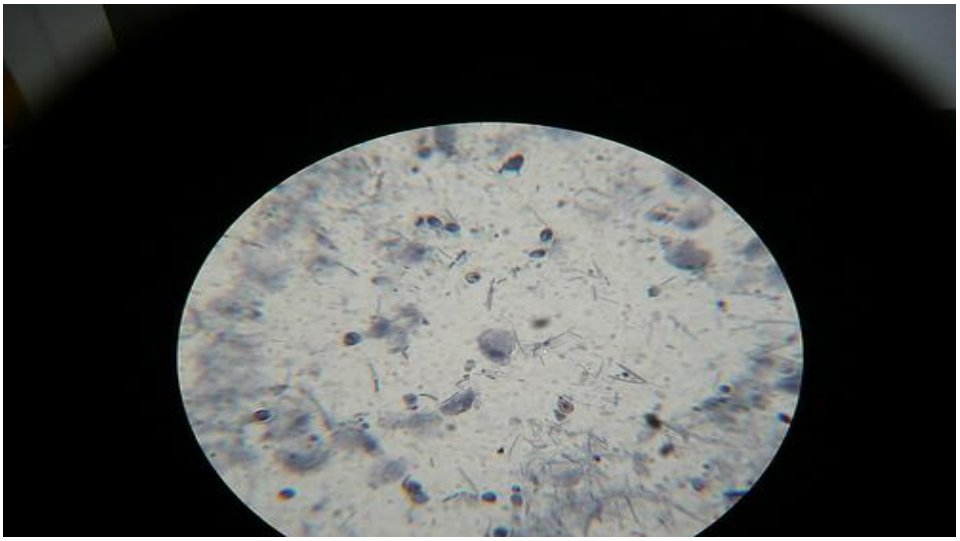


Fig 5: Selective Kupferberg (KTB).

4. Discussion

In the recent years, the use of plants with preventive and therapeutic effects contributes to health care needs (28).

There are three main reasons for interest in the treating and healing power of plant extracts. 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, *P. granatum* 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 irus, inhibition of LDL oxidation and decrease in atheromatous plaque formation and reduction of systolic blood pressure (29, 30, 31).

While the vast majority of studies of oral Microbiology relate to various aspects of bacteriology, buccal Protozoology has virtually forgotten, despite the high prevalence of infections in adults where *T. tenax* etiologic agent. (32)

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* was observed in the oral cavity (33, 34).

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 samples should be inoculated in the most appropriate culture media and allow accurate identification of this species. (35, 36)

Conclusions

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 *T. tenax* infections.

Acknowledgments

We thank The Military Academy of Periodontology and Dental Association Clinic staffs and all the participants who shared their time for working on this study.

Conflict of interest: None declared.

References

1. Wenrich DH. Comparative morphology of the flagellates of Trichomonas man. Am. J. Trop. Med. 1944, 22: 639-42.
2. Hayawan IA,Bayoumi MM.The prevalence of *Entamoeba gingivalis* and *T. tenax* in periodontal disease. J Egypt Soc Parasitol 1992;22:101-5.
3. Memlik F. Trichomonads in pleural effusion. JAMA 1968;204:1145-6.
4. Sarowaska J, Wojnicz D, Kaczkowski H, Jankowski S. The occurrence of *Entamoeba gingivalis* and *T. tenax* in patients with periodontal disease, Immunosupression and genetic diseases. Adv Clin Exp Med 2004;13:291-7.
5. Vrablic J, Tomova S, Catar G, Randova L. Morphology and diagnosis of *Entamoeba gingivalis* and *Trichomonas tenax* and their occurrence in children and adolescent. Bratisl Lek Listy 1991;92:241-6.
6. Chiche L, Donati S, Corno G, Benoit S, Granier I, Chouraki M, *et al*. *Trichomonas tenax* in pulmonary and pleural diseases. Presse Med 2005;34:1371-2.
7. Mahmoud MS, Rahman GA. Pulmonary trichomoniasis improved diagnosis by using polymerase chain reaction targeting *Trichomonas tenax* 18S r RNA gene in sputum specimens. J Egypt Soc Parasitol 2004;34:197-211.
8. F. Aqil, M. S. Khan, M. Owais, and I. Ahmad, "Effect of certain bioactive plant extracts on clinical isolates of beta-lactamase producing methicillin resistant *Staphylococcus aureus*," *Journal of Basic Microbiology*, vol. 45, pp. 106–114, 2005.
9. H. S. Parmar and A. Kar, "Medicinal values of fruit peels from *Citrus sinensis*, *Punica granatum*, and Musa paradisiaca with respect to alterations in tissue lipid peroxidation and serum concentration of glucose, insulin, and thyroid hormones," *Journal of Medicinal Food*, vol. 11, no. 2, pp. 376–381, 2008.
10. F. Aqil, M. S. Khan, M. Owais, and I. Ahmad, "Effect of certain bioactive plant extracts on clinical isolates of beta-lactamase producing methicillin resistant *Staphylococcus aureus*," *Journal of Basic Microbiology*, vol. 45, pp. 106–114, 2005.
11. A. Related, D. LinksHeber, N. P. Seeram, et al., "Safety and antioxidant activity of a pomegranate ellagitannin-enriched polyphenol dietary supplement in overweight individuals with increased waist size," *Journal of Agricultural and Food Chemistry*, vol. 55, pp. 10050–10054, 2007.
12. A. Kar , and H. S. Parmar, "Medicinal values of fruit peels from *Citrus sinensis*, *Punica granatum*, and Musa paradisiaca with respect to alterations in tissue lipid peroxidation and serum concentration of glucose, insulin, and thyroid hormones," *Journal of Medicinal Food*, vol. 11, no. 2, pp. 376–381, 2008.
13. D. Ricci, L. Giamperi, A. Bucchini, and D. Fraternale, "Antioxidant activity of *Punica granatum*fruits," *Fitoterapia*, vol. 77, no. 4, pp. 310–312, 2006.
14. A. Sánchez-Lamar, G. Fonseca, J. L. Fuentes et al., "Assessment of the genotoxic risk of *Punica granatum* L. (Punicaceae) whole fruit extracts," *Journal of Ethnopharmacology*, vol. 115, no. 3, pp. 416–422, 2007.
15. A. Related, D. LinksHeber, N. P. Seeram, et al., "Safety and antioxidant activity of a pomegranate ellagitannin-enriched polyphenol dietary supplement in overweight individuals with increased waist size," *Journal of Agricultural and Food Chemistry*, vol. 55, pp. 10050–10054, 2007.
16. H. S. Parmar and A. Kar, "Medicinal values of fruit peels from *Citrus sinensis*, *Punica granatum*, and Musa paradisiaca with respect to alterations in tissue lipid peroxidation and serum concentration of glucose, insulin, and thyroid hormones," *Journal of Medicinal Food*, vol. 11, no. 2, pp. 376–381, 2008.
17. M. Aviram, M. Rosenblat, D. Gaitini, et al., "Pomegranate juice consumption for 3 years by patients with carotid artery stenosis reduces common carotid intima-media thickness, blood pressure and LDL oxidation," *Clinical Nutrition*, vol. 23, pp. 423–433, 2004.

18. H. S. Parmar and A. Kar, "Protective role of Citrus sinensis, Musa paradisiaca, and *Punica granatum* peels against diet-induced atherosclerosis and thyroid dysfunctions in rats," *Nutrition Research*, vol. 27, no. 11, pp. 710–718, 2007.
19. L. C. Braga, J. W. Shupp, C. Cummings et al., "Pomegranate extract inhibits *Staphylococcus aureus* growth and subsequent enterotoxin production," *Journal of Ethnopharmacology*, vol. 96, no. 1-2, pp. 335–339, 2005.
20. S. Naz, R. Siddiqi, S. Ahmad, S. A. Rasool, and S. A. Sayeed, "Antibacterial activity directed isolation of compounds from *Punica granatum*," *Journal of Food Science*, vol. 72, no. 9, pp. M341–M345, 2007.
21- J. Zhang, B. Zhan, X. Yao, Y. Gao, and J. Shong, "Antiviral activity of tannin from the pericarp of*Punica granatum* L. against genital Herpes virus in vitro," *Zhongguo Zhong yao za zhi*, vol. 20, no. 9, pp. 556–576, 1995.
22- E. P. Lansky and R. A. Newman, "*Punica granatum* (pomegranate) and its potential for prevention and treatment of inflammation and cancer," *Journal of Ethnopharmacology*, vol. 109, no. 2, pp. 177–206, 2007.
23- S. Naz, R. Siddiqi, S. Ahmad, S. A. Rasool, and S. A. Sayeed, "Antibacterial activity directed isolation of compounds from *Punica granatum*," *Journal of Food Science*, vol. 72, no. 9, pp. M341–M345, 2007.
24- Navazesh M. Methods of collecting saliva. Ann N Y Acad Sci. 1993 Sep 20;694:72-7 PMID: 8215087.
25- Vasconcelos LC, Sampaio FC, Sampaio MC, Pereira Mdo S, Higino JS, Peixoto MH. Minimum inhibitory concentration of adherence of Punica granatum Linn (pomegranate) gel against S. mutans, S. mitis and C. albicans. Braz Dent J 2006;17(3):223-7.
26- Ali NM. In vitro activity of commercially available Egyptian propolis on *T. vaginalis*. N Egypt J Med 2007; 36 (1): 7-15.
27- Palmas C, Wakelin D, Gabriele F. Transfer of immunity against Hymenolepis nana in mice with lymphoid cells or serum from infected donors. Parasitol 1984; 89: 287-293.
28- Holetz FB, Pessini GL, Sanches NR, Cortez DA, Nakamura CV, Filho BP. Screening of some plants used in the Brazilian folk medicine for the treatment of infectious diseases. Mem Inst Oswaldo Cruz 2002 Oct;97(7):1027-31.
29- Reddy MK, Gupta SK, Jacob MR, Khan SI, Ferreira D.Antioxidant, antimalarial and antimicrobial activities of tannin-rich fractions, ellagitannins and phenolic acids from *P. granatum* L. Planta Med 2007 May;73(5):461-7.
30- Kim ND, Mehta R, Yu W, Neeman I, Livney T, Amichay A, et al. Chemopreventive and adjuvant therapeutic potential of pomegranate (Punica granatum) for human breast cancer. Breast Cancer Res Treat 2002 Feb;71(3):203-17.
31- Naz S, Siddiqi R, Ahmad S, Rasool SA, Sayeed SA. Antibacterial activity directed isolation of compounds from Punica granatum. J Food Sci 2007 Nov;72(9):M341-5.
32- FERRARA A, Conca R, GRASSI L, DE CARNERI I. Its a possibile ruolo Rilievi di pathogen *Trichomonas tenax* Parodontite nella Cronica. Ann Ist Super Sanità 1986, 22 (1): 253-6.
33- Feki A, MOLET B. Importance des protozoaires *Trichomonas tenax* et *Ent gingivalis* dans Cavite buccale humaine. D'Rev Odontostomatol 1990; 19 (1): 37-45.
34- MAZZALI of Ilja R. Incidence and quantification of mycoplasma in saliva of patients with periodontal disease and in subjects with healthy gums.Acta Odontológica Venezolana 1990, 28 (2-3): 81-7.
35- Honigberg BM. Trichomonads of Importance in human medicine. In: Kreier JP editor. Parasitic Protozoa. 2 ed. New York: Acad Press, 1978. p. 392-405.
36- NOLTE W. Dental Microbiology. 4 ed. Mexico: Editorial Interamericana, 1986.