

***In vitro* Antibacterial Activity of Propolis, Alum, Miswak, Green and Black Tea, Cloves Extracts Against *Porphyromonas Gingivalis* Isolated from Periodontitis Patients in Hilla City, Iraq**

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ABSTRACT

Background

Human periodontal disease has been associated with a complex microbiota, the development of destructive periodontitis seems to be the result of a specific infection. Gram positive coccoid bacteria have been related to periodontal health, while periodontal disease was associated with Gram negative rods. The clinical use of antibiotics and other antimicrobial agents, as adjuvants for the treatment of periodontitis, has been extensively investigated in the past decade. Recently, special attention has been paid to natural medication including propolis, alum and various plant extracts.

Methods

Swabs were taken from periodontal pockets of 30 patients (periodontic Department, teaching clinics of oral and dental surgery in Hilla city), *porphyromonas gingivalis* was identified according to the cultural properties, microscopic examination and biochemical tests. Antibacterial action was evaluated on this isolate by using six different aqueous extracts of *Salvadora persica* (Miswak), aluminum potassium sulphate (alum), propolis, green and black tea, cloves (*Syzygium aromaticum*).

Results

Antimicrobial activity of propolis, alum and plant aqueous extracts at 50% concentration by well-diffusion method was characterized by inhibition zones. At this concentration, the maximum inhibition zone diameters 35mm, 40 mm were found in *Salvadora persica* and alum, respectively, for propolis the inhibition zone was 30 mm, while green tea and clove give the same inhibition zone 20 mm, the minimum inhibition zone 10 mm was found in black tea.

Conclusions

In summary, the showed a wide spectrum antibacterial activity. These results suggest that, propolis, alum, *Salvadora persica* (Miswak), clove, green and black tea have antibacterial actions against black pigmented *P.gingivalis* (from periodontal pockets) and may be used for the treatment and prophylaxis against periodontal diseases.

Keywords: *Porphyromonas gingivalis*, Plant extract, Antibacterial activity

INTRODUCTION

Human periodontal disease has been associated with a complex microbiota, the development of destructive periodontitis seems to be the result of a specific infection. Gram positive coccoid bacteria have been related to periodontal health, while periodontal disease was associated with Gram negative rods. Human periodontitis is initiated and perpetuated by a small group of bacteria that colonize the subgingival region, mainly Gram negative, anaerobic or microaerophilic bacteria ¹. The black pigmented *P. gingivalis*, in particular, possess virulence factors that are relevant to the pathogenesis of oral infections ^{2, 3}. Experimentally, *P. gingivalis* has been shown to induce progression of periodontitis in Laboratory animals ⁴. Oral infections including the various clinical stages of periodontal diseases have been reported among patients of all age groups ⁵. However, there is no study so far which has established the prevalence or characteristics of black pigmented Bacteroides isolated from healthy or from oro-dental infections and diseases in Hilla city.

The clinical use of antibiotics and other antimicrobial agents, as adjuvants for the treatment of periodontitis, has been extensively investigated in the past decade ⁶. Recently, special attention has been paid to natural medication, and propolis has been reported to possess certain medicinal properties ⁷. Propolis is a natural composite balsam, produced by honey bees (*Apis mellifera*) from the gum of various plants. Bees collect vegetal exudates and form

pellets with their mandibles, mixing the exudates with wax and products of their salivary glands. The resulting material is used to strengthen the nest, provide protection from microorganisms, and as an embalming substance to cover the carcass of a hive invader ⁸. The medicinal properties of propolis have been widely investigated ⁹. Antimicrobial action of propolis has already been shown, various studies report on antibacterial, antifungic and antiparasitic actions ^{10,11}.

¹¹ demonstrated propolis antimicrobial activity against oral bacteria, as well as its action in inhibiting the production of polysaccharides, the application of propolis extract on rat molars reduced the severity of carious lesions in these animals.

One of plants was *Salvadora persica* commonly known as Msiwak, which was used as a chewing stick¹². Prophet Mohammed (peace and pray upon him) is considered by muslims the first dental educator in the oral hygiene. Since, he recommended them to use Siwak five times a day, as he said "if I had not found it hard for followers or the people, I would have ordered them to clean their teeth with Miswak prior to each pray" ¹³. Arak, a tree used for Miswak, is also known as "tooth brush tree" and "mustard plant". Although the Miswak is usually obtained from the roots of the Arak tree, some sticks are made from its branches and bark ¹⁴. The beneficial effects of Miswak in respect of oral hygiene and dental health are partly due to its mechanical action and partly due to

pharmacological actions. It was found that *Salvadora persica* root contain saponins along with tannins, silica, a small amount of resin, trimethylamine and a fairly large amount of alkaloidal constituents. Studies have indicated that *Salvadora persica* contain substances that possess plaque inhibiting and antibacterial properties against several types of cariogenic bacteria which are frequently found in the oral cavity. The growth and acid production of these bacteria is thus inhibited¹⁵.

Natural products have been used for centuries in treating human diseases and they contain components of therapeutic value. Natural products are environmentally safer, easily available, and cheap¹⁶. Alum (Aluminum potassium sulfate), the crystallized double sulphates with the formula $KAl(SO_4)_2 \cdot 12H_2O$, are generally odourless, colourless crystalline solids that turn white in air, which is used as an astringent and antiseptic in various food preparation processes such as pickling and fermentation and as a flocculants for water purification among other things¹⁷.

The chemical material as alum has many benefits as it has antibacterial effect on *Pseudomonas aeruginosa*¹⁸, in which maximum inhibition zone reached was 35 mm when it used as a solution in 80% concentration. Also, it has antiyeast effect that inhibits the growth of *candida albicans* in which its effect on the budding process¹⁹.

Green tea (*Camellia sinensis*) is widely consumed in China, Japan, Korea, and Morocco²⁰. It has been considered as a healthy beverage since ancient times. The traditional Chinese medicine has recommended this plant for headaches, body aches, general pain, digestion, depression, as an energizer, and in general to prolong life²¹. Green tea also has many oral health benefits. It is considered a healthful beverage due to the biological activity of its polyphenols namely catechins. Among the

polyphenols, Epigallo Catechin 3 Gallate (EGCG) and Epicatechin 3 Gallate (ECG) are the most predominant catechins. The antioxidant, antimicrobial, anticollagenase, antimutagenic, and hemopreventive properties of these catechins proved to be helpful in the treatment of chronic diseases like periodontal disease. It has cognitive function and positive impact on bone density, caries, periodontal disease, and diabetes²². It is the second most consumed beverage in the world aside from water, coffee, and carbonated soft drinks²³. Approximately 76–78% of the tea produced and consumed is black tea, 20–22% is green tea. Green tea is obtained by macerating and heat drying this flush, whereas black tea is derived by fermentation of flush before heat drying. Both types of tea share many pharmacologically active components although at variable concentrations²³.

Clove (*Syzygium aromaticum*) is the second most important spice of the world, as judged from the world trade, being next only to black pepper. According to an ITC survey the total world, the USA and India are the largest importers of cloves. the dried clove bud contains free eugenol, eugenol acetate and caryophyllene. Although these substances amount to some 99% of the oil One of the main components of clove oil obtained from the dried flower-buds of *Syzygium aromaticum* is beta-caryophyllene, it has local anesthetic activity. The essential oil of clove is a colorless or light yellowish extract obtained from dried flower buds by steam distillation²⁴. The volatile oils of clove of *S. aromaticum* were assessed for antibacterial activity against 25 different genera of bacteria²⁵, which include animal and plant pathogens, food poisoning and spoilage bacteria. A crude MeOH extract of clove (*S. aromaticum*) exhibited preferential growth inhibitory activity against Gram-negative anaerobic periodontal oral pathogens, including *Porphyromonas*

gingivalis and *Prevotella intermedia* and it has been reported that the essential oil of *Syzygium aromaticum* presented the largest antimicrobial activity^{26,27}.

The aim of this study was to investigate the antimicrobial “*in vitro*” action of aqueous extract for propolis, *Salvadora persica*, alum, green and black tea, cloves against *p.gingivalis*

METHODS

Swabs were taken from periodontal pockets of 30 patients (periodontic Department, teaching clinics of oral and dental surgery), thereafter, cultured on blood agar plates immediately incubated anaerobically in the anaerobic jar at 37°C for 72 hours plus 10% CO₂. Then subjected to identification according to the cultural properties, microscopic examination and biochemical tests catalase (-), Indole (+), vancomycin sensitivity²⁸. Cultural morphology on anaerobic blood agar with long incubation period 5-7 days, a characteristic black pigmented colonies, convex, 1-2 µm in diameter. Gram negative coccobacilli were observed microscopically, in some cases appear as diplococci surrounded with a capsule or hollow. The sensitivity to vancomycin was used to differentiate this bacteria from closely related periodontal oral bacteroides e.g *prevotella intermedia*. Vancomycin disk (30µg) was used for confirmation of *p. gingivalis*.

Preparation of aqueous extracts

Propolis extract

Propolis samples were collected from hives of honey bees of Al- Museiab, Iraqi during spring and summer seasons of 2013. Propolis samples were cleaned, free of wax, paint, wood, cut into small pieces, and placed in clean container. 50 gram of propolis were mixed with 100 ml of double D.W. in dark brown container and left for 7 to 14 days at

room temperature in dark place. For 2 weeks, the container was shaken 2 or 3 times per day and returned to warm dark place. The liquid was filtered through Whatman No.1 filter paper and the water was evaporated by oven at 45°C, then the extract was weighed and stored in dark clean container for further using. Water or aqueous extract was dissolved by distilled water, sterilized by filtration (using Millipore 0.45 filter paper), and the requisite dilutions were prepared.

Alum extract

Fifty gram of alum material was purchased from the local market at Hilla City, Iraq, 2013, and was identified in the College of Medicine, Department of Chemistry, Babylon University. Crystals of alum KAl(SO₄)₂·12(H₂O). Dissolved 50 gm by 100ml distilled water completely in hot (distilled) water at 92 °C, to obtain a final concentration of 50 % at pH 3.6.

Plants extract

Dried plants green and black tea, cloves and *Salvadora persica* (Miswak) used in this study were obtained from the local market at Hilla City, Iraq, 2013. Distilled water was boiled, dried plants were added to the water and left to cool. Later on, these contents were mixed by the blender and filtered to remove the large, un homogenized particles to get clear aqueous extracts. The extracts were kept at 4°C until to be use.

In vitro Antimicrobial activity testing using Agar well diffusion assay NCCLS²⁹

The agar well diffusion method was used for the determination of antibacterial activity of propolis, *Salvadora persica* (Miswak), aluminum potassium sulphate (alum), green and black tea, cloves aqueous extracts by using bacterial isolates taken from periodontal pockets to evaluate its effects on the isolated bacteria. Loopfull growth from bacterial isolate was inoculated into nutrient

broth incubated at 37 °C for 18 hours. The bacterial suspensions were diluted with normal saline. Adjust the turbidity and compare with standard tube (McFarland number 0.5) to yield a uniform suspension containing 1.5×10^8 CFU / ml. Muller- Hinton agar was inoculated with 0.1ml of bacterial inoculum .Using cork borer, wells were made on the cultured media. The aqueous extracts were considered as the 50% concentration. Then, 0.1ml of aqueous extracts were added to wells, then the plates left for 30 min in refrigerator at 4°C, thereafter, they were incubated at 37°C for 24 hrs. The activity of aqueous extracts was determined by measuring the diameter of inhibition zone in millimeter³⁰.

RESULTS & DISCUSSION

Determination of antimicrobial activity

The agar well diffusion assay is most commonly used to determine antimicrobial susceptibility. In this study used aqueous extracts by agar well diffusion assay. In the present study at 50% concentration of the aqueous extracts was tested for their inhibitory activity on gram-negative black pigmented *p.gingivalis* isolates. All test isolates were inhibited by aqueous extracts at 50% concentration. The maximum inhibition zone was observed in alum and Miswak aqueous extracts respectively (40 mm, 35 mm), and the minimum was in black tea (10mm) Figure (1)³¹ found that 60% alum concentration gives inhibition zone of 29 mm diameter on *Pseudomonas aeruginosa*, while 50 and 40% alum concentrations were 25 and 22 mm diameter, respectively. Therefore, alum is used in wound and burns disinfection and in treatment of ulcers in the oral cavity.

The antibacterial actions of 50% concentration of *Salvadora persica* (Miswak) aqueous extract on *p. gingivalis* isolated from periodontitis pockets revealed that Miswak is effective in oral therapy and give high

inhibition zone (35mm).³² tested the antibacterial activity of *Salvadora persica* against some oral aerobic and anaerobic bacteria and reported that the extract of these sticks had a drastic effect on the growth of *Staphylococcus aureus*, and a variable effect on other bacterial species. They commented that the chewing sticks they used were harvested one month earlier, and suggested that using more fresh sticks will give better result.³³ tested fresh vs. one- month-old Miswak extracts for antibacterial activity and found no difference. A comparison of alcohol and aqueous extract of Miswak was also made. It was found that alcoholic extract is more effective than aqueous extract for antibacterial activity³⁴.

In the present study has shown propolis antimicrobial activity against periodontal pathogen, *P.gingivalis* with inhibition zone 30 mm. Antimicrobial activity demonstrated in this study, confirming previous results^{35,36,37}.The verification of the antimicrobial action of the propolis extract is not surprising. The primary function of propolis in the hive is to act as a biocide, being active against invasive bacteria, fungi and even invading larvae. There are a number of studies documenting the biocidal functions of propolis,³⁷ reported that Kaempferol had antimicrobial activity against *Streptococcus mutans* and *Actinomyces viscosus* and demonstrated that the same constituent inhibited the growth of *P. intermedia* and *P. gingivalis*.

In the present study both cloves and green tea aqueous extracts exhibit antibacterial activity with inhibition zone 20 mm for each. Extract of clove (*S. aromaticum*) exhibited preferential growth inhibitory activity against Gram-negative anaerobic periodontal oral pathogens, including *Porphyromonas gingivalis* and *Prevotella intermedia*³⁸. The clove oil is active against oral bacteria associated with dental caries and periodontal disease and

effective against a large number of other bacteria^{39, 40} has been reported antimicrobial, antifungal properties, the essential oil of *S. aromaticum* shows anti-inflammatory, cytotoxic and anesthetic activities and observed the anti-inflammatory and antinociceptive activities of eugenol essential oil in experimental animal models.³⁸ have reported the inhibitory effect of clove extract against *Staphylococcus aureus* bacteria. Antibacterial compound of clove oil is effective against food borne Gram positive bacteria (*Staphylococcus aureus*, *Bacillus cereus*, *Enterococcus faecalis* and *Listeria monocytogenes*) and Gram-negative bacteria (*Escherichia coli*, *Yersinia enterocolitica*, *Salmonella choleraesuis* and *Pseudomonas aeruginosa*^{39, 21} found that a four-week regimen of mouth washing with a dilute catechins solution reduced the mouth odor associated with periodontal disease. Tea catechins deodorizes methyl mercaptain, the main cause of mouth odor. inhibited growth and adhesion of *P. gingivalis* to buccal epithelial cells. Green tea catechins were reported to be effective in preventing gingival and periodontal inflammation. Catechins inhibit IL-17 induced production of human gingival fibroblasts^{41, 42}. Inhibition of extracellular signal regulated Kinase (ERK) decreased IL-17 induced production of human gingival fibroblasts.^{43, 44, 45} demonstrated bactericidal activity of green tea catechins against *Prevotella* and *P. gingivalis* at concentration of 1 mg/ml. They found significant reduction in markers of gingivitis after the use of slow release buccal delivery system applied over a period of 8 weeks. More recent studies have shown that some virulence factors (toxic metabolites, protein tyrosine phosphatase, and gingipains) and aetiological agents of periodontal disease are neutralised by EGCG^{46, 47}.

CONCLUSION

The alum, *Salvadora persica* (Miswak), propolis, clove and green tea have antibacterial actions against black pigmented *P. gingivalis* (from periodontal pockets) and may be used for the treatment and prophylaxis against periodontal diseases.

LIST OF ABBREVIATIONS

ERK: extracellular signal regulated Kinase
IL-17: Interleukin-17

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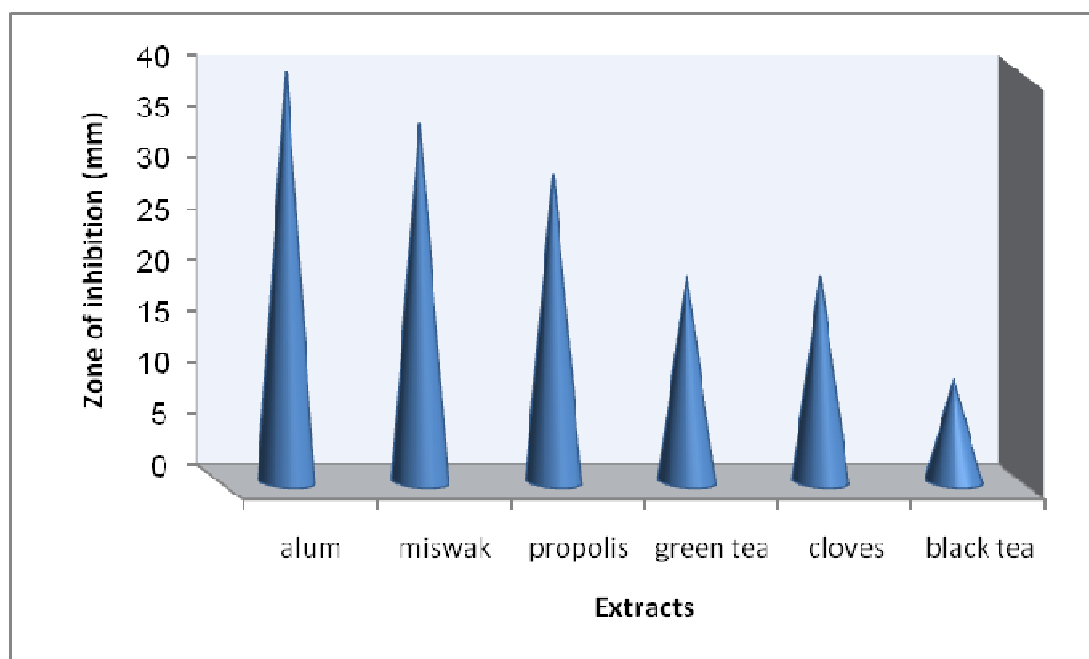


Figure (1) Antimicrobial activity at 50% concentrations of different aqueous extracts by agar well method.