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In-Vitro comparative Study of Antimicrobial Activity of Two Plant Extracts and Probiotic Strain against Isolated oral Cariogenic Pathogen.

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ABSTRACT

Dental caries is a prevalent oral infectious disease. *Streptococcus mutans* is considered a crucial pathogen in the pathogenesis of dental caries. The aim of this study was to compare *in vitro* between the antibacterial properties of ethanol extract of propolis, aqueous extract of Miswak and probiotic strain as well as their mixture against *Streptococcus mutans* growth using agar disc diffusion technique. The antimicrobial activity was evaluated by measuring the diameter of zones of inhibition (in mm) and expressed as Mean ± Standard Error (SE). The results showed that the mixture of Propolis extract, Miswak extract and Probiotic strain had the maximum antibacterial activity with the mean zone of inhibition (18.3 ± 1.2) followed by Propolis extract (16.8 ± 0.8), Miswak extract (13.8 ± 0.6) and Probiotic strain (12.9 ± 0.9). These plant extracts and the Probiotic strain appear to have a potent antibacterial effect. Thus, they could be used as therapeutic agents against cariogenic bacteria as well as good alternatives to synthetic chemicals.

Keywords: Antimicrobial activity, Propolis extract, Miswak extract, Probiotic strain, inhibition zone

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INTRODUCTION

Dental caries is one of the most common infectious diseases affecting humans. *Streptococcus mutans* (*S. mutans*) has been consistently linked with the formation of human dental caries and is the most commonly implicated initiator and plaque-resident bacterium. *S. mutans* begins demineralization and the metabolism of simple carbohydrates; this produces acid as a by-product, which leads to tissue loss and further bacterial penetration [1]. Given the incidence of oral disease, increased resistance by bacteria to antibiotics, adverse affects of some antibacterial agents currently used in dentistry, there is a need for alternative prevention and treatment options that are safe, effective and economical. Natural phytochemicals isolated from plant extracts used as traditional medicines are considered as good alternatives [2]. Miswak (Salvadora persica) is one of the most commonly used medicinal plants for oral hygiene among global Muslim community [3]. Miswak is a chewing stick (natural toothbrush) prepared from the twigs of Arak tree. Extracts from Miswak showed antibacterial activity against *Streptococcus mutans* and plaque control [4-6]. Moreover, Propolis is a natural product that honeybees collect from living plants and it has been shown to exert in vitro antibacterial action against a number of oral micro-organisms and inhibits cell adhesion as well as water insoluble glucan formation [7]. In recent times, Probiotic technology represents a breakthrough approach to maintaining oral health by utilizing natural beneficial bacteria commonly found in healthy mouths to provide a natural defense against those bacteria thought to be harmful to teeth and gums [8]. Therefore, this study aimed to compare between the antibacterial properties of natural compounds involving; ethanol extract of Propolis, aqueous extract of Miswak, Probiotic strain as well as their mixture against *Streptococcus. mutans* isolated from a patient diagnosed to have dental caries.

MATERIALS AND METHODS

Collection of Plant material

Propolis powder was obtained from a honey bee Egyptian supplier. The specimens were stored at 4°C in a dry and dark place until processing. Miswak chewing sticks were utilized from the Egyptian market. The collected Miswak plant sticks were dried at room temperature for 10 days then powdered in an electric blender, packed in plastic bags and stored at 4°C till further use.

Extraction

The dried propolis powder was extracted in ethanol solvent. 30g powdered substance was extracted in 300ml ethanol (1:10 w/v). For Miswak 300ml of sterile distilled water was added to 30g Miswak powder. Each ethanol Propolis mixture and aqueous Miswak mixture was allowed to soak for 48 hours at 4°C. After extraction, each mixture was centrifuged at 2000 rpm for 15 minutes and filtered with Whatman No. 4 filter paper. The clear liquid filtrate of each plant extract was stored at 4°C until use.

Culture and Enumeration of selected Probiotic strain

The probiotic strain; *Lactobacillus rhamnosus* was isolated and serologically identified by dairy microbiological Lab., National Research Center., Egypt. The selected Probiotic strain was cultivated in De Man, Rogosa and Sharpe broth (Fluka and catalogue no.69966 MRS broth, Sigma-Aldrich) for 48 hours at 37°C in Co2. The cultured bacteria were centrifuged at 5000 rpm for 20 minutes to obtain pure cells (pellet). According to Reynolds et al, 2005, the total live cell numbers per one gram of pellet were calculated using the following formula: Live cells (CFUs/g) = number of colonies in the agar plate x dilution factor [9]. In the present study one gram of the previously prepared pellet was found to be contained 10 x 10^8 cells.

Preparation of the mixture

2gm of the prepared pellet was dissolved in 4ml saline. 0.5ml of each clear liquid filtrate of ethanol Propolis extract and aqueous Miswak extract were mixed together then immediately 1ml from the previously dissolved pellet was added. The mixture was stored for 24hours at 4°C before using in the experiment.

Isolation and Culture of *Streptococcus mutans*

S. mutans was isolated directly from the tooth of a patient diagnosed with dental caries by a physician. The infected area of the tooth was swabbed three times with sterile cotton wool to remove debris and saliva. The tooth was then swabbed with another sterile cotton wool then immediately streaked on the selective media Mitis Salivarius agar (Fluka and catalogue no.01337, Sigma-Aldrich) and incubated for 24hours at 37°C. *S. mutans* were easily identified on the selective media by its characteristic colony morphology (dark blue, small, and irregular margin). A single colony of *S. mutans* was isolated and placed in a test tube containing 10ml of Treptone Soya broth (TSB, Difco, Detroit, MI USA) in incubator for 24hours to allow the bacteria growth before immediately use

Antimicrobial Activity

A disc diffusion method was used to evaluate the antimicrobial activity of the two plant extract and the selected probiotic strain as well as their mixture. Treptone Soya agar (TSA, Difco, Detroit, MI USA) was poured into sterile petri dishes (15ml each) and 50µl of TSB containing *S. mutans* were dispersed on the surface of each agar plate. Sterile filter paper discs 6mm in diameter were impregnated with 20µl of each the following natural compounds; ethanol Propolis extract, aqueous Miswak extract and previously dissolved pellet in saline as well as their prepared mixture, then two sterile filter paper discs for each compound and mixture were placed on surface agar plate which inoculated by *S. mutans* and incubated at 37°c for 24 hours. The antimicrobial activity was evaluated by determining the diameter of zones of inhibition around each disc of natural compounds and their mixture.

RESULTS AND DISCUSSION

The results of the current study clearly demonstrated that ethanol extract of Propolis, aqueous extract of Miswak, Probiotic strain as well as their mixture inhibit the growth of *Streptococcus. mutans*, however, their effectiveness varied as presented in (Fig. -1). The maximum zone of inhibition was noticed with the mixture of all the previous natural compounds on *S. mutans* (18.3 ± 1.2), followed by each compound alone; Propolis extract (16.8 ± 0.8), Miswak extract (13.8 ± 0.6) respectively and the least zone of inhibition was observed by Probiotic strain (12.9 ± 0.9) (Table - 1). It has been widely documented that *S. mutans* is the major organism involved in dental caries due to its ability to create an acidic environment by the breakdown of carbohydrate [10, 11]. The antimicrobial activity of propolis against *S. mutans* and other oral pathogens have been reported [10, 12, 13]. The bacteriostatic and bactericidal effects of ethanol extract of Propolis (EEP) against *S. mutans* was observed by Scanning electron microscope and was showed the appearance of materials with irregular shapes around the cell surface of *S. mutans* after exposure to EEP, it demonstrated that EEP caused the injury on the cell membrane of *S. mutans* and resulted in cell leakage [13]. The result in (Table -1) confirms the antimicrobial activity of propolis to *S. mutans*. Accordingly to several studies, the antimicrobial activity of propolis might be due to the synergistic effect of main components of propolis extracts like flavonoids and caffeic acid and/or cinnamic acid, probably influence the microbial membrane or cell wall sites, resulting in functional and structural effects [14, 15]. In the present study, the result of the antimicrobial effect of Miswak extract against *S. mutans* was in agreement with previous studies that have reported that Miswak extracts were effective against the growth of the various cariogenic microorganisms including *S.mutans* [6, 16, 17]. The antimicrobial effect of Miswak extract might be attributed to various chemicals contained in its extracts such as salvadorine, chlorides, high amounts of fluoride and silica, sulphur, tannins, saponins [18, 19]. The absence of flavonoids in aqueous extract of Miswak and the high content of flavonoids present in EEP could be demonstrated the higher mean inhibition zone of Propolis extract than Miswak extract in (Fig. -2). Moreover, in the present study the selected Probiotic strain (*Lactobacillus rhamnosus*) is also known to inhibit the growth of cariogenic streptococci by producing antistreptococcal substance and the inability of *Lactobacillus rhamnosus* to ferment sucrose or lactose greatly increases its potential as a good probiotic against cariogenic *streptococci* [19]. While, The lowest mean inhibition zone presented by probiotic strain in (Fig. -2), could be due to the antimicrobials such as organic acids or bacteriocins released by *Lactobacillus rhamnosus* were degraded or unstable [20, 21]. Miswak extract displays in several study moderate bactericidal properties against a wide range of oral microorganisms and did not affect in any way on a probiotic bacterium [22]. Moreover, the various sugars that extracted from Propolis could be utilized by Probiotic strain [23, 24].Thus, the synergistic effect between probiotic strain in its mixture with propolis and Miswak extracts did not suppress the antimicrobial activity of each compound in the mixture

and this could be explained the maximum inhibition zone of the mixture than the inhibition zone was exhibited by each compound alone [25, 26]. However, tests *in vitro* do not reproduce the real conditions of the oral cavity. In addition, determination of inhibition zones values depends on the technical details that are different in various laboratories. The size of the inhibition zones depends on the diffusibility of the test substance in the agar, which is under the influence of molecular weight, negative charge, composition of samples, and the thickness and pH of the agar culture medium [27].

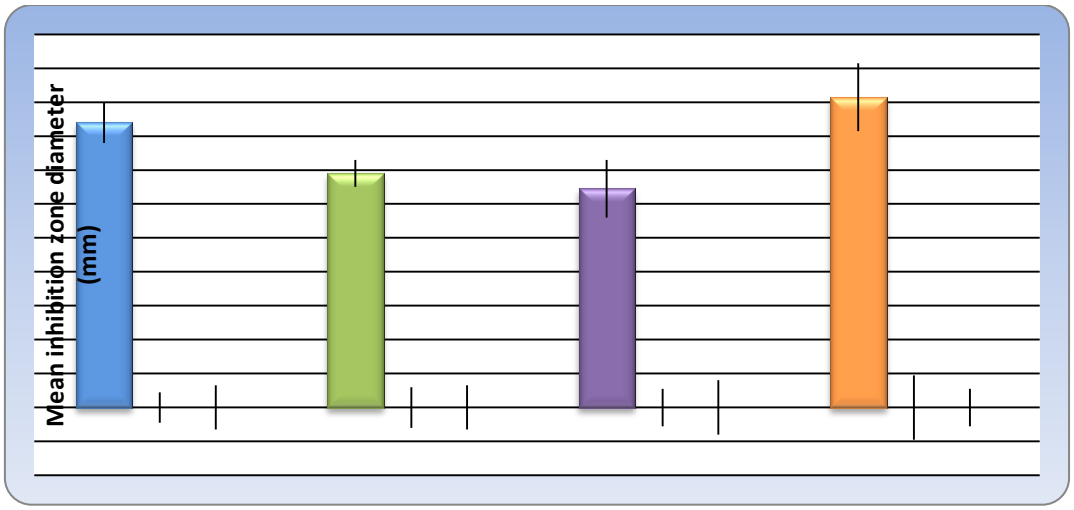

Figure 1: Antimicrobial activity of Propolis extract, Miswak extract, probiotic strain and their mixture by Agar Disc Diffusion method

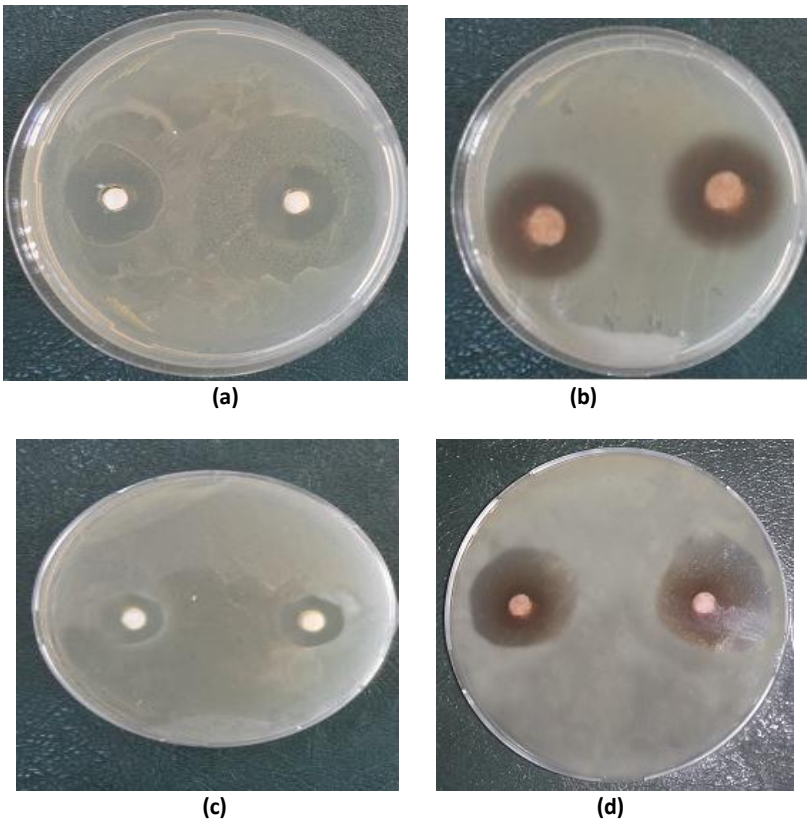

Figure 2: Zone of inhibition with (a) Miswak extract, (b) Propolis extract, (c) Probiotic strain and (d) the mixture against *Streptococcus. mutans*

Table 1: Mean, standard deviation (SD) values, results of one-way ANOVA and Tukey's tests for comparison between inhibition zones exhibited by Propolis extract, Miswak extract, probiotic strain and their mixture against *Streptococcus. Mutans*

Propolis extract	*Miswak extract*	*Probiotic strain*	*Mixture*	*P-value*
16.8 (0.8) [a]	**13.8 (0.6) [b]**	**12.9 (0.9) [c]**	**18.3 (1.2) [d]**	**<0.001***

*: Significant at P ≤ 0.05, Different superscripts are statistically significantly different

CONCLUSION

This preliminary screening study revealed that ethanol extract of Propolis, aqueous extract of Miswak and Probiotic strain posses antimicrobial activity and gave better results with the mixture of the natural previous compounds against *S.mutans* growth. Apart from the recommended use of natural antimicrobial agents, the use of propolis, miswak, probiotic alone or their mixture will help to improve the treatment and control of dental caries. Also, these natural compounds are available and inexpensive, thus they can be a great help in developing countries with financial constraints and limited oral health care facilities.

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