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Research Article

THE EFFECTS OF PROPOLIS HONEY CANDY CONSUMPTION ON MYELOPEROXIDASE ACTIVITY IN STIMULATED SALIVA

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ABSTRACT

Objective: Propolis is a natural product that contains flavonoids and has antibacterial effects that could decrease myeloperoxidase (MPO) activity in the saliva. Propolis honey candy is currently being developed and to analyze the effects of propolis honey candy on MPO activity in stimulated saliva.

Methods: Stimulated saliva samples were collected from individuals who met the inclusion criteria before and after consumption of propolis honey candy twice a day for 7 days. Salivary samples were centrifuged to separate the supernatant and pellet. A 100-µl aliquot of the supernatant was directly added to the wells of a 96-well plate and mixed with 100 µl of substrate solution containing 3,3'-diaminobenzidine, guaiacol, dapsone, and Tris-HCl buffer. After incubation for 30 min at room temperature, MPO activity was measured by subtracting the absorbance value (wavelength of 450 nm) of the saliva samples from that of the blank control (distilled water).

Results: The absorbance value of MPO activity of propolis honey candy was 0.071 before consumption and 0.076 after consumption.

Conclusion: MPO activity significantly increased after the consumption of propolis honey candy (Wilcoxon signed-rank test, $p<0.05$).

Keywords: Flavonoid, Myeloperoxidase activity, Propolis.

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INTRODUCTION

The use of herbs as traditional medicines in Indonesia has been passed from one generation to the next [1]. Many previous studies have reported the safety of traditional herbal medicines and the general lack of side effects when consumed at normal dosages. Herbs are composed of complex organic matters, as the body, suggesting that traditional medicines and herbs will be well-received [2]. Propolis is one such herb with a long history as a traditional medicine in Indonesia that is still used today [3].

Propolis is a non-toxic resin substance that is gathered by honeybees from the sap or buds of various plant sources that are digested by enzymes in the honeybee saliva and mixed with plant pollen [3]. Propolis contains 50% resin substances and balm, which consist of flavonoids, phenolic acid, caffeic acid phenethyl ester, 30% beeswax, 10% essential oils, 5% bee pollen, and 5% plant materials [4]. The substances contained in propolis have activities against microbes (bacteria, fungi, protozoa, and viruses), inflammation, pain, cancer, and oxygen radical formation [4].

The main antibacterial components of propolis are flavonoids, which include galangin, chrysin, and pinocembrin, which produce hydrogen peroxide (H_2O_2), a substance that has the capacity to induce oxidative damage to bacterial DNA. The damage to bacterial DNA causes a quantifiable decrease in the bacterial population susceptible to H_2O_2 and prevents activation of the immune response, such as the activation of enzyme myeloperoxidase (MPO) in the saliva [5,6].

Saliva is a complex solution secreted by the major and minor salivary glands that contain gingival crevicular fluid; transudate from the oral mucosa, nasal mucosa, and pharynx; non-adherent bacteria; food particles; desquamated epithelial cells; and blood cells [7]. Saliva is composed of 99% water (H_2O) and 1% other components that include electrolytes, several kinds of proteins, and by-products of glucose and nitrogen metabolism that help to maintain oral hygiene [8].

Peroxidase is an enzyme found in saliva that acts against microorganisms [9]. MPO is natural peroxidase produced by neutrophils and monocytes in the gingival crevicular fluid that acts as a catalyst for chloride ion oxidation through H_2O_2, which is metabolized by oral bacteria into hypochlorite (OCl^-) and H_2O. OCl^- acts as an antibacterial agent that prevents the growth and metabolism of oral bacteria, including cariogenic strains [9].

The mechanism of saliva MPO is dependent on the abundance of H_2O_2 produced by oral bacteria [10]. If the quantity of H_2O_2 is not optimal, the chloride ions remain unoxidized, which reduces the effectiveness of the antibacterial potential of this system [11]. The abundance of H_2O_2 is decreased along with available bacteria [11]. Therefore, the administration of antibacterial agents, such as propolis, can reduce the number of bacteria that produce H_2O_2, causing a subsequent decrease in MPO activity [6].

Propolis is available in several forms, including pastes, oils, extracts, powders, injectables, mouthwash, capsules, tablets, and sprays [4]. Previous studies have described the incorporation of propolis in hard candies. At present propolis candy is being developed and be commercially available soon [12].

Previous studies have reported that honey propolis candy can reduce the prevalence of *Streptococcus mutans* more effectively than honey candies that also have antibacterial effects [13]. Furthermore, propolis has been shown to decrease MPO activity in the saliva [14]. However, no study has yet investigated the effect of propolis in the form of hard candy on MPO activity in the saliva.

Based on these findings, the aim of the present study was to investigate the effects of honey propolis candy on MPO activity in the saliva.

METHODS

This clinical experimental research was conducted in the Oral Biology Laboratory of the Faculty of Dentistry, Universitas Indonesia, from

August to November 2014. The study cohort included 120 dental students from Universitas Indonesia who fulfilled the inclusion criteria. The samples evaluated in this study were stimulated saliva collected before and after treatment.

The study cohort was limited to dental students at Universitas Indonesia, aged 19–23 years, with good general health or oral hygiene who were willing to participate and provided signed informed consent. Subjects with poor oral hygiene, periodontal disease, systemic diseases involving the oral cavity, orthodontic appliances and dentures, smoking and drinking habits, administration of antibiotics, and allergies to propolis were excluded from the study.

The independent variables in this research were honey propolis candy, honey candy, and X brand candy. The dependent variable was the MPO activity of stimulated saliva. The instruments and materials used in this research included a mouth mirror, probe, flashlight, 50-ml graduated cylinder, funnel, Eppendorf pipette, 1.5-ml microtubes, blue and yellow pipette tips, refrigerator/freezer (4°C and −80°C), centrifuge, microplate reader, microtiter plates, computer and printer, stimulated saliva samples, and reagents (guaiacol, 3,3'- diaminobenzidine [DAB], Tris-HCl buffer, H_2O_2, dapsone, honey propolis candy, honey candy, X brand candy, paraffin wax, purified water, and alcohol).

Initially, the oral hygiene of the subjects was screened for compliance with the inclusion criteria using a mouth mirror, a probe to determine pocket depths, and a flashlight to detect bleeding and other signs of oral disease. Then, saliva samples were collected from the study participants before candy consumption. Before sample collection, the subjects were instructed to not brush or use mouthwash, and not eat or drink (except mineral water) for a minimum of 1.5 h before sample collection. For sample collection, the subjects were instructed to sit up straight and relax, while chewing paraffin to stimulate the saliva, which was collected in a 50-ml graduated cylinder every 30 s for a period of 10 min. Then, the graduated cylinder was sealed, labeled with a code, and refrigerated.

The 120 research subjects were randomly allocated to one of the following three groups of 40 subjects each: A honey propolis candy group, a honey candy group, and an X brand candy group. The candies were consumed for 7 continuous days, twice per day, at morning and night, until the candy was finished.

After 7 days, the second batch of stimulated saliva samples was collected with the same method at the first sample collection before candy consumption. Then, aliquots of the stimulated saliva samples collected before and after candy consumption were transferred to 1.5-ml microtubes, which were centrifuged at 10,000 rpm for 10 min at 4°C to separate the pellet from the supernatant. The remaining sample was stored at −80°C for further use to avoid repeated freeze-thaw cycles. Then, 100-µl aliquots of the supernatant were transferred by pipette to the wells of a microtiter plate and mixed with 100 µl of the following reagents: 3.48 mM DAB, 176 mM guaiacol, 4 mM H_2O_2, and 1 mM dapsone in 0.3 M Tris-HCl buffer (pH 7.5) [15].

The saliva samples and reagents were added to triplicate wells and mixed. Purified water and reagent mix were used as blank and negative controls, respectively. Then, the plate was incubated for 30 min at room temperature in the dark. The reaction was monitored with a microplate reader at a wavelength of 450 nm that was connected to a computer. After the allotted time, the absorbance value appeared on the computer screen. Then, the optical density (OD) value of MPO of the saliva sample and that of the blank control (purified water) were compared.

All data analyses were performed using IBM SPSS Statistics for Windows, version 20.0 (IBM Corporation, Armonk, NY, USA). Data normality was assessed using the Shapiro–Wilk test (n<50 for each group). Data that were abnormally distributed were subjected to non-parametric analysis with the Wilcoxon signed-rank test, Kruskal–Wallis, and Mann–Whitney U-test. A probability $p<0.05$ was considered statistically significant.

RESULTS

Stimulated saliva samples were collected from 40 research subjects before and after the consumption of honey propolis candy, honey candy, and X brand candy each. The MPO activity was determined according to the OD value obtained from differences in the OD value of the stimulated saliva sample and that of the blank control. The results are presented in Table 1.

The difference in MPO activity in stimulated saliva before and after the consumption of honey propolis candy, honey candy, and X brand candy is shown in Table 1 and Fig. 1. The OD value of MPO activity after consumption of honey propolis candy had increased by 0.006, whereas that of the honey candy had increased by 0.006 and that of the X brand candy had decreased by 0.001. These data were processed with the Wilcoxon signed-rank test due to the abnormal distribution of data.

There were significant differences in MPO activity before and after consumption of the honey propolis candy (p=0.041 vs. 0.127), honey candy (p=0.016 vs. 0.439), and X brand candy (p=0.001 vs. 0.007). According to the results of the Shapiro–Wilk normality test, two sets of data were normally distributed ($p>0.05$), and four sets of data were abnormally distributed ($p<0.05$). Since there were fewer than 50 subjects, it was concluded that all data were abnormally distributed (Table 2).

The Wilcoxon signed-rank test was used to determine the significance of differences in MPO activity before and after candy consumption. The results showed that there were indeed significant differences in MPO activity before and after the consumption of honey propolis candy (p=0.01), but not before and after consumption of honey candy (p=0.072) and X brand candy (p=0.398) (Table 2).

The Kruskal–Wallis was used to identify significant differences in MPO activity before and after the consumption of honey propolis candy, honey candy, and X brand candy. The Kruskal–Wallis test was used because the results of the Shapiro–Wilk normality test showed that the data were abnormally distributed. The results showed significant differences in MPO activity before and after consumption of honey propolis candy (p=0.002), honey candy (p=0.002), and X brand candy (p=0.048).

The Mann–Whitney U-test was performed to determine which sets of data had significant differences by the Kruskal–Wallis test by comparing the data of the honey propolis candy group versus the honey candy group, the honey candy group versus the X brand candy group, and the honey propolis candy group versus the X brand candy group. The results showed that there were no significant differences in MPO activity before and after candy consumption between the honey propolis candy and honey candy groups (p=0.747) or between the honey candy and X brand candy groups (p=0.057). However, there was

Table 1: Mean OD values of MPO activity in stimulated saliva before and after the consumption of honey propolis candy, honey candy, and X brand candy

Candy consumption	Mean OD value of MPO activity in stimulated saliva
Before consumption of honey propolis candy	0.071
After consumption of honey propolis candy	0.077
Before consumption of honey candy	0.059
After consumption of honey candy	0.065
Before consumption of X brand candy	0.072
After consumption of X brand candy	0.071

MPO: Myeloperoxidase

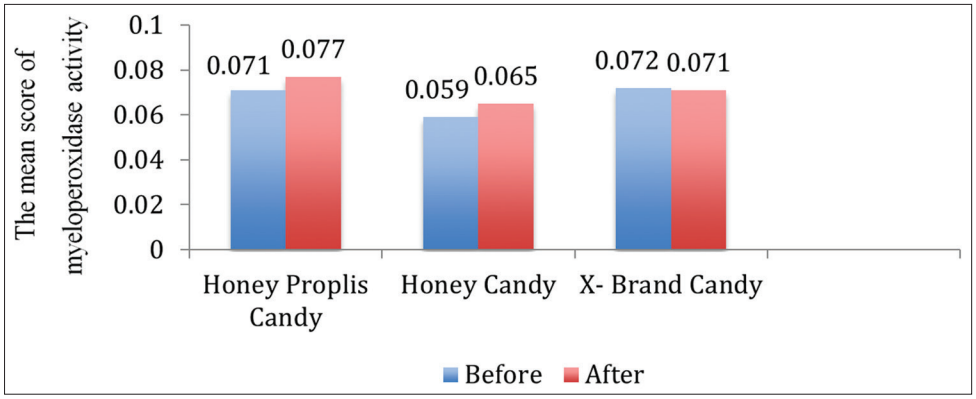

Fig. 1: The mean score of myeloperoxidase activity before and after the consumption of honey propolis candy, honey candy, and X brand candy

Table 2: Differences in MPO activity in stimulated saliva before and after consumption of honey propolis candy, honey candy, and X brand candy

Group (n=40 each)	Before consumption (mean±SD)	After consumption (mean±SD)	p (Wilcoxon test)
Honey propolis candy	0.07090±0.015447	0.07683±0.018746	0.01
Honey candy	0.05908±0.015869	0.06390±0.017457	0.072
X brand candy	0.07160±0.009904	0.07128±0.018746	0.398

MPO: Myeloperoxidase

a significant difference in MPO activity before and after consumption between the honey propolis candy and X brand candy groups according to the results of the Mann–Whitney U-test (p=0.008) (Table 3).

DISCUSSION

The aim of this study was to determine the effect of honey propolis candy on MPO activity in stimulated saliva. Previous studies reported that propolis conveyed antibacterial activities by causing damage to bacterial DNA [6]. A previous report found no significant effect on the consumption of honey propolis candy for 7 days, 2 times per day on the prevalence of *S. mutans* [13]. A decrease in MPO activity is suggestive of a decrease in the abundance of cariogenic bacteria in the oral cavity, such as *S. mutans*, which is a species of lactic acid bacteria (LAB) [16].

The MPO system uses H_2O_2 (LAB product) as a substrate and chloride ion as co-substrate within the oral cavity according to the following equation: $Cl^- + H_2O_2$ (MPO)◊ $OCl^- + H_2O$ [9]. Hence, a reduction in the abundance of cariogenic bacteria causes a reduction in the production of H_2O_2. In 2008, Sakamoto *et al.* reported a method to detect the MPO activity in saliva where MPO is separated from lipid peroxidase using a staining technique [15].

The results of the present study showed an increase in MPO activity after consumption of honey propolis candy and honey candy (Fig. 1). The mean OD value after honey propolis candy consumption had significantly increased by 0.006 (Table 2), whereas the mean OD value after consumption of honey candy increased by 0.006, but this difference was not statistically significant (Table 2). The increase in MPO activity after the consumption of honey propolis candy in comparison with the MPO activity after the consumption of honey candy was allegedly caused by propolis. The metabolism of honey and glucose produces H_2O_2 as a by-product, which can increase the amount of H_2O_2 for use as a substrate by MPO. The increase in MPO activity after the consumption of honey propolis candy was more significant than that of honey candy, which was likely due to the addition of H_2O_2 from propolis, honey, and glucose in honey propolis candy. In contrast, the addition of H_2O_2 after the consumption of honey candy was obtained from the metabolism of honey and glucose. These results are in agreement with those of previous studies that found that glucose contained in the honey candy and honey propolis candy produces H_2O_2 as an end product of glucose oxidation [17]. Another study reported that the formation of H_2O_2 by propolis could occur extracellularly, but required the presence of a transition metal, such as ferric ions. In this proposed reaction, a flavonoid acts as a temporary carrier of electrons produced by the oxidation of ferric ions (Fe^{2+}◊ Fe^{3+}). The released electrons are received by oxygen in the formation of superoxides (O_2◊O_2^-) that bind with hydrogen (H^+) and form H_2O_2, which is used to oxidize and subsequently destroy bacterial DNA [6]. Likewise, the metabolism of honey and glucose also produce H_2O_2 through enzymatic glucose oxidation [20.26] in the reaction of $C_6H_{12}O_6 + H_2O + O_2 \rightarrow C_6H_{12}O_7 + H_2O_2$ [16,18].

Table 3: Mean differences in MPO activity before and after treatment

Group	Mean difference in MPO activity before and after treatment
Honey propolis candy, (n=80) Mean±SD	−0.0578±0.015365
Honey candy (n=80) Mean±SD	−0.0595±0.015198
X brand candy, (n=80) Mean±SD	0.00040±0.008539
Kruskal–Wallis (Sig.)	0.027

MPO: Myeloperoxidase

The change in MPO activity after the consumption of X brand candy, which contains propolis, was insignificant because the OD value had decreased by 0.001 (Table 2). As a possible explanation, honey can add H_2O_2 as a substrate to the MPO system reaction, but the candy may contain artificial sweeteners, such as polydextrose, lactitol, licorice, and acesulfame-k, which contain synthetic glucose that does not participate in the oxidative reaction; therefore, no H_2O_2 is produced. The production of H_2O_2 by propolis is allegedly proportionate with the decrease in H_2O_2 production due to the inhibition of *S. mutans* reproduction caused by the antibacterial effects of propolis [13].

The results of the Kruskal–Wallis test revealed a significant difference in MPO activity before and after the consumption of honey propolis candy, honey candy, and X brand candy (Table 3), whereas the results of the Mann–Whitney U-test showed significant differences between the honey propolis candy group and X brand candy group (p=0.008), which was likely due to differences in the components of the candies.

This finding is supported by the results of previous studies that honey propolis candy contains honey, propolis, and glucose (which produces H_2O_2 as an end product) [6,16,18].Meanwhile, the X brand candy only contains propolis, which can produce H_2O_2, although artificial sweeteners do not produce H_2O_2 [6].

Propolis has been studied for almost a decade in order to find alternative medications for fighting caries. Recently, several studies have shown promising alternative propolis combinations, such as anticariogenic agents [19, 20]. However, further studies should be performed in order to investigate other properties of propolis to fight caries.

CONCLUSION

The results of this study showed that the consumption of honey propolis candy increased MPO activity in stimulated saliva. The X brand candy most effectively decreased MPO activity in stimulated saliva as compared with honey propolis candy and honey candy.

CONFLICTS OF INTEREST

There are no conflicts of interest to declare.

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