

Study of Different Propolis Extracts on Salivary Microorganisms' Glucose Consumption *in vitro*

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Abstract: In this study, it was assessed the *in vitro* inhibition of sugar consumption of human saliva microorganisms by propolis extracts. Saliva samples were collected from volunteers individuals after three mouth washes with distilled water. After an oral rinsed with propolis extract I, II and III, saliva samples were collected at one, two and three h after rinsing and glucose was added to samples. These aliquots were employed to determine glucose consumption at 0, 24 and 48 h after collection. Glucose levels were determined by the glucose oxidase method. It was verified statistically significant differences in glucose consumption when comparing patients treated with propolis to GC group, after 24 and 48 h. GExp_i presented glucose consumption inhibition at intervals from 0 to 24 h and 0 to 48 h at zero, one and two h after rinsing. Absence of glucose consumption was verified in GExp_{ii} and GExp_{iii} at 0 to 24 h and from 0 to 48 h in samples collected right after rinsing, while it was observed glucose consumption at one, two and three h after rinsing. Propolis extract inhibited of sugar consumption in saliva by microorganisms and can be faced as a suitable compound to be employed in dentistry.

Key words: Propolis, saliva, oral microorganisms, glucose consumption

INTRODUCTION

The oral cavity is an open growth system which shelters a great variety of microorganisms, repetitively introduced and removed from this system. It is an organic environment, where the only microorganisms that settle are those capable of adhering to the surfaces of their anatomic and physiologic constituents (Marsh *et al.*, 2009).

Compatibility between coexistence of this microbial population and human beings' individual health results from the development of immunologic mechanisms since birth and from continuous adaptation and re-adaptation processes responsible for biologic links established between man and microorganisms that usually shelter in oral cavity. Such link warrants these microorganisms a

saprophyt condition, in ecological balance, thus characterizing symbiosis and antibiosis situations (Marsh *et al.*, 2009). However, certain factors associated to a frequent and rich saccharosis diet may cause an imbalance to this microbial community, so as to favor growth and settlement of bacteria that are responsible for the major infection of the oral cavity, thus endangering the integrity of its tissues (Graf, 1983).

Man's concern for the beneficial action that may result from the use of natural products is growing and meeting significant popular acceptance. Among these products, propolis has been highlighted due to its applicability to food and cosmetic industry, as it is utilized as an active principle incorporated to several of these products, such as tooth paste, extracts and dermatologic creams. This is owed to the most diverse therapeutic

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properties attributed to propolis, such as antimicrobial, anti-swell, antioxidant, anti-carcinogenic and even anti-caries efficacy (Ikeno *et al.*, 1991; Park *et al.*, 1998). Despite the existence of other efficient antiseptics to fight oral pathogens, propolis appears as a natural alternative, with antimicrobial properties and active principles that are biocompatible with the human organism and its molecular diversity seems to be in harmony with mammiferous metabolism, a characteristic that dramatically reduces possible tissue aggressions (Marcucci, 1995).

Propolis is a resin of varied color and consistency, collected by bees from diverse parts of plants such as buds, floral buds and resin exudates, enriched with those insects' saliva secretions (Burdock, 1998). In chemical terms, propolis complex composition was revealed by gaseous chromatography technique coupled to mass spectrometry which allowed the detection of more than 150 components. Propolis major constituents are phenolic compounds characterized by the presence of at least one hydroxyl group straightly linked to an aromatic ring. These substances are represented by flavonoid aglycones, phenolic acids and ethers (Marcucci, 1995). Besides the phenolic compounds, propolis which originated from Brazilian Atlantic Forest has, in its composition, a high proportion of poorly studied apolar compounds which are been considered to have a potential antimicrobial action (Duarte *et al.*, 2003).

The presence of phenolic compounds, especially flavonoids, partially explains the important variety diversity of the therapeutic properties reported by several investigators. Propolis possible medical and odontologic applications increased the interest in its chemical composition and in its origin (Banskota *et al.*, 1998). However this all-healing characteristic with several biological activities, tends to inhibit its acceptance, as health professionals generally seem to question its efficacy due to many simultaneous biological activities attributed to it (Pereira *et al.*, 2002).

This study performed an *in vitro* assessment of glucose consumption inhibition of different propolis extracts towards microorganisms present in human saliva.

MATERIALS AND METHODS

Patients' selection: To develop this experimental and laboratorial research, we selected thirty saliva

donors, who fulfilled requirements regarding general health satisfactory conditions, particularly regarding oral health, that is, absence of caries lesions and periodontopathies, a rigorous control of bacterial plaque and gum bleeding; not using orthodontic braces and having a minimum of twenty-four teeth. Once these conditions were met, the volunteers were submitted to a saliva test to assess its flow and tamponing capability (Krasse, 1998). Once the volunteers were selected, they were duly informed about the objectives and methodology designed for this research and after signing consent terms, they were requested oral prophylaxis during 12 to 14 h.

This study was approved by the Brazilian National Bioethics Committee.

Samples collection: Donors presented themselves at the Oral Biochemistry Laboratory of the Federal University of Bahia's Health Sciences Institute for saliva samples collection which was made to not perform any type of prophylaxis of the mouth after the last meal taken at 6:00 p.m. of the evening before the morning which saliva collection was scheduled. This condition guaranteed the absence of in sterilized tubes duly bedded in ice bath until the beginning of the analyses.

After reserving aliquots of this fluid for control group tests (GC), donors were divided into three groups, according to the propolis extract utilized in mouth rinse (Table 1) with ten patients in each group. After conclusion of the first collection, volunteers performed rinsing during one minute with its respective propolis extract diluted in water, according to each manufacturer's instructions. Immediately after rinsing, the second saliva sample was collected, followed by three sequential collections, one, two and three h after the initial propolis treatment, providing samples to form the experimental groups here identified as GExp_I, GExp_{II} and GExp_{III}, according to the propolis extract utilized. A 25% glucose solution in distilled water was added to all samples. All samples were incubated in Petri dishes, in a CO₂ incubator.

All samples were screened, before propolis addition or not and before incubation, for the glucose basal content. These results showed a mean of 2.15 mg dL⁻¹ with the standard deviation of ±0.55. Donors were allocated in each group considering this small variation, with the objective to enhance group homogeneity.

Table 1: Characteristics of the propolis extracts employed in the experiment

Identification	Brazilian Government Official Registry	Manufacturer	Geographic Origin and Active principle informed	Propolis Concentration (%)
Propolis extract I	Brazilian Ministry of Agriculture 0001/4675	NaturApi Products Naturais e Apicolas Ltd.	Dry Extract of Group 6 Propolis from Bahia State (Atlantic Rain Forest) Apolar compounds	11
Propolis extract II	Brazilian Ministry of Agriculture 0001/4675	Apis Flora Ind. e Com. Ltd.	Dry Extract of propolis mixture from Minas Gerais and Paraná States Flavonoids	20
Propolis extract III	Brazilian Ministry of Agriculture 0063/1204	Vita Hervas Ltd.	Dry extract of Propolis from Brazilian States from Southeast Region Flavonoids	30

Glucose consumption assay: All the samples collected as described before were submitted, after incubation, to a determination of glucose concentration, employing a commercial kit (Bioclin Inc., Belo Horizonte, Brazil), based on the glucose oxidase method, in order to determine the residual glucose in samples, that persists after bacterial consumption. Assays were performed right after collection and after 24 and 48 h of incubation.

Statistical analysis: Results are expressed in milligrams per deciliter (mg dL⁻¹) and represent the means of residual glucose in samples retrieved from patients and its respective Standard Deviations (SD). We compared the group's means through the variance analysis test (ANOVA) which was followed by the Bonferroni's parametric test.

RESULTS

This study performed *in vitro* assessed the efficacy of propolis extract I, II and III in the glucose consumption inhibition of microorganisms present in human total saliva.

In the samples which formed the control group with no action by the above mentioned propolis extract it was possible to verify that there were statistically significant differences in glucose residual rates after 24 and 48 h of incubation, as shown in Table 2. However, when comparing glucose rates of the experimental groups GExp_I, GExp_{II} and GExp_{III} in the same periods after incubation we were not able to see any statistical difference (Table 2). This result expresses that propolis extracts were able to prevent glucose consumption.

In GExp_I, when evaluating values of glucose residual rates corresponding to saliva samples collected 1, 2 and 3 h after the switch and after 0, 24 and 48 h of incubation, there was a significant decrease in glucose concentration only at the samples retrieved from the patients three h after the switch and incubated for three h (Table 3), representing that the switch with this propolis extract was able to avoid glucose metabolism by microorganisms even three h after the treatment.

In other hand, results for GExp_{II} (Table 4) and GExp_{III} (Table 5) presented a similar pattern. When results for samples that were incubated for 48 h and retrieved from the patient right after the switch were compared to those from samples retrieved one, two and three h after treatment and incubated for the same time it could be seen that these two extracts were not successful in prevent glucose consumption in a period longer than one h and with more than 24 h of incubation.

Table 2: Residual glucose concentration right after treatment with different propolis extracts

Time groups	Time after incubation (h)		
	0	24	48
GC (n = 30)	128.8±1.22	110.6±1.32*	97.0±1.32*
GExp _I (n = 10)	128.8±1.90	129.6±1.45	113.5±5.55
GExp _{II} (n = 10)	128.8±2.52	125.4±3.33	124.2±3.47
GExp _{III} (n = 10)	126.8±1.05	131.4±3.41	113.4±3.13

*Significant statistic difference between glucose rates determined at 0, 24 e 48 h after incubation, at p<0.05. Results obtained for residual glucose concentration, in the glucose oxidase method, with saliva samples retrieved from thirty healthy patients submitted to switch with different propolis extracts, right after the treatment. Results are expressed in mg dL⁻¹ and represent the means and standard deviations obtained

Table 3: Residual glucose concentration after treatment with propolis extract at 11% concentration

Time after switch	Time after incubation (h)		
	0	24	48
0	128.8±1.90	129.6±1.45	113.5±5.55
1	129.9±2.59	131.3±1.82	114.6±5.68
2	129.1±3.02	131.7±2.55	110.9±6.31
3	129.0±2.80	129.2±1.76	105.4±4.04*

*Significant statistic difference between glucose rates determined at 24 and 48 h after incubation, at p<0.05. Results for residual glucose concentration obtained in the glucose oxidase method with saliva samples retrieved from ten healthy patients submitted to switch with propolis extract at 11% concentration (GExp_I). Results are expressed in mg dL⁻¹ and represent the means and standard deviations obtained

Table 4: Residual glucose concentration after treatment with propolis extract at 20% concentration

Time after switch (h)	Time after incubation (h)		
	0	24	48
0	128.8±2.52	125.4±3.33	124.2±3.47
1	128.6±2.50	115.2±5.24	105.5±7.12*
2	128.1±2.21	115.5±4.98	107.6±6.38*
3	126.8±2.14	114.1±4.57	102.8±4.79*

*Significant statistic difference between glucose rates determined at 0 and 48 h after incubation, at p<0.05. Results for residual glucose obtained in the glucose oxidase method with saliva samples retrieved from ten healthy patients submitted to switch with propolis extract at 20% concentration (GExp_{II}). Results are expressed in mg dL⁻¹ and represent the means and standard deviations obtained

Table 5: Residual glucose concentration after treatment with propolis extract at 30% concentration

Time after switch (h)	Time after incubation (h)		
	0	24	48
0	126.8±1.05	131.4±3.41	113.4±3.13
1	128.4±1.59	129.5±1.23	107.5±4.44*
2	126.6±1.32	129.4±1.63	111.2±3.64*
3	127.5±1.30	126.7±1.25	106.5±2.28*

*Significant statistic difference between glucose rates determined at 0 and 48 h after incubation, at p<0.05. Results for residual glucose obtained in the glucose oxidase method with saliva samples retrieved from ten healthy patients submitted to switch with propolis extract at 30% concentration (GExp_{III}). Results are expressed in mg dL⁻¹ and represent the means and standard deviations obtained

DISCUSSION

The determination of glucose rates rendered possible the assessment of the control group efficacy, through the fall in this carbohydrate level, when contrasting the values detected in time zero with those dosed after 24 and

48 h of incubation (0 h: 128.8±1.22; 24 h: 110.6±1.32; 48 h: 97.0±1.32). Such differences indicate glucose consumption in total saliva which did not suffer the influence of rinses with propolis extracts. The results obtained prove the reliability of the methodology adopted in this study, through glucose consumption in the maintenance of the energetic metabolism of the microorganisms present in total saliva.

The control and experimental groups analyses permitted to verify that, at zero time, the differences are not considered as statistically significant, as it is in this time that saliva enters in contact with glucose, both in control and experimental groups, in the various times of collection.

The assessment of the results of the experimental group in which propolis extract I (GExp_I) was used, reveals that there was no glucose consumption at 24 h of incubation by total saliva collected right after rinse and after one, two and three h of the swish using this extract, since glucose rates corresponding to this experimental group, when compared to residual rates found in the control group, characterize propolis extract I efficacy, according to Table 3. This extract was original from Brazilian Rain Forest region, classified as pertaining to group 6, rich in apolar compounds, according to the manufacturer. Probably, such apolar compounds were the responsible elements for inhibiting the activity of microorganisms present in saliva (Burdock, 1998; Park *et al.*, 2002; Salomo *et al.*, 2004). However, the antimicrobial action mechanism of type 6 propolis is still not clear. It is apparently very complex and may result from the synergic effect with other compounds (Burdock, 1998).

In Brazil, the Ministry of Agriculture which is responsible for regulating propolis identification and quality, requires that the propolis extract composition contain a 0.25% minimum concentration of flavonoids which are one of the most important antimicrobial elements of propolis. But such regulation does not include apolar compounds which does not possess aglycon flavonoids (Bankova, 2005). It must be emphasized that the composition of these natural products from different origins is not specified in details by propolis extract manufacturers.

The prolonged antimicrobial action of propolis extract I tested in saliva samples collected in zero, one, two and three h after rinsing demonstrates this product's pharmacological potential, as well as its extension. In order to have an oral antiseptic cleanser effect prolonged it is necessary that its principles keep adhered to oral surface and be slowly liberated. As propolis extract I (rich in apolar compounds) had a more long-lasting

antimicrobial action than propolis extract II and III (rich in flavonoids and caffeic acid) we can infer that the apolar compounds probably have a more lasting adherence to oral tissues than flavonoids and caffeic acid and that their antimicrobial effect also lasts longer. Such possibility results in a gradual liberation of these compounds from their respective connecting sites, assuring a longer period of adherence of these products in the oral environment and prolonging the contact between these chemical agents and target microorganisms (Thylstrup and Fejerskov, 2005).

When assessing the same experimental group (GExp_I) after 48 h of incubation, we verified that there is no glucose consumption in the saliva samples collected at zero, one and two h after rinse. However, when contrasting results obtained for saliva samples collected three h after the swish with glucose rates determined at zero time and after 48 h of incubation, we could observe that some consumption of this sugar had occurred. The decrease of this subtract residual rates showed that, at three h after rinse, propolis extract substantivity undergone a reduction, probably due to a constant renewal of saliva fluid in the oral environment, followed by a gradual reduction in concentration of this natural product, corroborated by the natural saliva swallowing. This allows the microorganisms to return and multiply, recovering their energetic metabolism slowly, at three h after rinse. The antimicrobial activity of the oral environment certainly returns to its regularity and it begins to happen a gradual adaptation and multiplication, thanks to the gradual reduction of propolis rates to levels considered as close to zero (Thylstrup and Fejerskov, 2005).

Results assigned to experimental groups in which the volunteers swished propolis extract II or III in the mouth (GExp_{II} and GExp_{III}, respectively) demonstrate that there was no glucose consumption after a 24 h incubation of total saliva collected right after rinse, as well as after one, two and three h. This phenomenon suggests effective antimicrobial activity. However, upon confronting results obtained after 48 h of incubation, we could observe that there was a glucose consumption referring to saliva collection performed at one, two or three h after rinsing with these extracts. This finding seems to demonstrate less potentiality/substantivity of propolis extract II and III as compared to propolis extract I which only showed such reduction in the saliva samples collected three h after rinse.

Propolis extract II stems from propolis collected in the Brazilian states of Minas Gerais and Paraná, while the 30% propolis extract comes from the southeast region of Brazil. Both contain in their composition the aglycon flavonoids

(Salomo *et al.*, 2004), considered as a responsible element for propolis antimicrobial activity together with caffeic acid phenethyl ester (CAPE), according to the information of manufacturers, who do not inform their classification according to propolis type or the flavonoids contained in the extracts and their respective concentrations. So far, the effective scientifically accepted antimicrobial action is credited to pinocembrin, galangin, quercetin, procianin, naringenin and andgonidin flavonoids (Burdock, 1998; Mirzoeva *et al.*, 1997). But it is necessary to perform refined chemical tests combined with biological assays, especially microbiological, in order to assess and consolidate the pharmacologic efficacy of propolis from different origins, aiming at the quality control of these products (Bankova *et al.*, 1983, 1999; Park *et al.*, 2002).

The fact that manufacturers provide so scarce information on the composition of these products points to the importance of performing several studies on this subject, in order to clarify its composition, so that we can enjoy the benefits of propolis, whether it is prescribed in its lowest concentrations, or recommended in more concentrated extracts. Therefore it is necessary to conduct a rigorous evaluation of the composition of propolis to be commercialized, including also its origin, concentration of its active principles and its potentiality and substantivity, with the aim of warranting its therapeutic properties, particularly in Dentistry.

CONCLUSIONS

Considering the results obtained in this *in vitro* experimental biochemical study it can be concluded that although propolis extract I, II e III reveal inhibition of glucose consumption by oral microorganisms, propolis extract I is the most suitable to be prescribed in Dentistry products, due to its long-lasting action, when compared to extracts II and III.

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