Formulation and Evaluation of New Biodegradable Periodontal Chips from Malaysian Propolis in Chitosan Base

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Abstract

This study aims to formulate periodontal chips from Malaysian propolis in chitosan base and to evaluate the physical, biological and antibacterial properties.

The propolis was extracted using ultrasonic method, diluted and mixed with chitosan to produce the propolis chips. Thickness and morphology of the chips were evaluated using SEM, the roughness was measured using profilometer. The chips were tested for biodegradability towards trypsin. Absorption test was done on artificial saliva and distilled water. In vitro release of propolis was evaluated by using spectrophotometer for 15 days. Antibaceterial effects of the propolis were analysed using minimal inhibitory concentration and minimal bactericidal concentration methods for gram positive and gram negative bacteria.

Propolis chips showed complete biodegredation on day 14. The release of propolis was up to 80% at day 6 and continued to increase within the experimental period. MIC values of extracted propolis, for gram positive and gram negative bacteria were found to be 0.15mg/ml and 1.25mg/ml respectively. While for the MBC the value was found to be 0.31 mg/ml and 0.25 mg/ml respectively.

Propolis chips were found to be biodegradable, exhibiting a high release rate and possessing antibacterial effects towards gram positive and gram negative bacteria.

Experimental article (J Int Dent Med Res 2017; 10(2): pp. 292-298)

Keywords: Propolis, Chitosan, Periodontal Chips.

Received date: 08 May 2017 **Accept date:** 19 June 2017

Introduction

Natural remedies are popular in preparation of drugs nowadays apitherapy, herbal medicine and homeopathy are several types of natural remedies.[1] Apitherapy is defined as the medicinal use of products made by honeybees like (honey, bee wax, venom, propolis, polen and royal jelly).

Honeybees collected the propolis from tree buds sap flows, shrubs or other botanical sources. Propolis is used to seal unwanted open spaces in the hives, protecting it from outside contaminants. It is resinous like, yellow to dark brown in colour.[2] Propolis is known to be the most important chemical compound of mgwem bees against microorganism. In the propolis, there are several compounds that has therapeutic effect like antimicrobial, antioxidant, immunostimulant and wound healing activities such as phenolics, flevonoids, flavones and fatty acids.[2] Due to these properties, propolis has been used since ancient time because of the wide range of biological activities.

Studies have shown that there plenty of usage of propolis based medication in dentistry. It is used as an oral aid in periodontology, restorative, orthodontic and oral surgery. In animal study, it is proved that propolis helps in reducing teeth caries.[3] This is because of multidirectional influence of bacterial flora; it

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reduces number of microorganism, decrease synthesis of insoluble glucans, and slow down activity of glucosyltransferase. In study done by koo et al, propolis was used to treat periodontal disease. It shows high effectiveness of propolis extraction on reducing growth of bacteria among red complex for example Porphyromonas gingivalis.[4] At some concentrations, propolis mouthrinse was found to be less effective than clorhexidine, however, it was found to be less cytotoxic on human gingival fibroblast . In one study by Toker et al, 2008, analyzed the morphometric and histopathologic changes associated with experimental periodontitis in rats in response to systemic administration of propolis. The result of the study found significant reduction on periodontitis-related bone loss when propolis is administrated systematically.[5]

Antimicrobial properties of propolis is widely supported by evidence.[6] It is confirmed that propolis extraction has shown an antimicrobial activity towards gram positive bacteria such as *streptococcus mutan* which is commonly found in human oral cavity and a main contributor of caries formation.[7-10] Some authors found that the antimicrobial activity of propolis was only active against gram positive bacteria and some fungi[11,12] however, there were some other authors found weak activity against gram negative bacteria.[7] Study by Ozan et al demonstrated significant effect of propolis on gram positive strain and sufficient effect on gram negative and candida strains.[13] One study by Sforcin et al, they verified that the growth of gram positive bacteria was inhibited at low propolis concentration which is 0.4%. antimicrobial properties of propolis observed in low concentration reveals bacteriostattic rather than bactericidal activity.[14] However, the susceptibility of microorganism towards propolis varies and depends on the genetic profile of specific individual microflora present in saliva.

Other than antimicrobial properties, propolis extraction also contain anti-inflammatory agent. Propolis stimulates the immune system by promoting phagocytic activity and cellular immunity.[14,15] and improves the healing effects in epithelial tissues.[13]

In this research, we also focusing on the other important biological compound called Chitosan. Chitosan is a sugar that is obtained from the hard outer skeleton of shellfish. It has widely usage in medicine. Chitosan is one of the most valuable polymer for biomedical and pharmaceutical applications due to its biodegradability, biocompatibility, antimicrobial, non-toxicity, and anti-tumor properties, examples of such applications include nasal, ocular, oral, parenteral and transdermal drug delivery.[16, 17]

Materials and methods

Extraction of *propolis* by sonication technique

Raw propolis purchased from Ayer Keroh, Malacca had been used in this research. 5 grams of propolis was measured and cut into small pieces and frozen at -80°C overnight. After that, propolis was ground (Moulinex LM 2211 uno blender with mill) and extracted with 70% of ethanol with the ratio of 1:20.

The solution was sonicated using ultrasonic bath at 25°C for 30 minutes with high sonic in the dark room. After sonication, the solution was filtrated through Whitman filter paper No.1 and evaporated by using Rotary evaporator and left for overnight to remove the organic solvent. The pH value of the extraction was measured prior dilution.

Preparation of the *propolis* incorporated biodegradable chitosan chips.

1% of acetic acid was prepared to immerse with the 1g of chitsan overnight. Then, it was dissolved in 26ml of water followed with 1ml of propolis (100% w/w) and vibrator was used to provide a homogenous mixture and poured into a sterilized petri dish lined with aluminium foil. After drying overnight in the oven with 40°C by uncovered the petri dish the resultant film was cut into small round film chips of size 6 x 6 mm in diameter.[16, 17]

Preparation of *propolis* dilution

For preparation of propolis dilution, this formula was used; M 1 V 1 = M 2 V 2 . (M=molarity, V=volume). 50% and 25% of dilution were prepared by this formula.

Assessment of thickness and surface morphology

Thirty five propolis chips in chitosan base were used to evaluate their surface morphology and thickness by using scanning electron microscope (SEM).

Assessment of the surface roughness

Propolis chips was tested using profilometer device to measure their surface roughness.

In Vitro Release study

A 'vial' method was used for the in-vitro release study. Ten propolis chips containing bigodegradable chitosan of size 0.6 x 0.6 cm^2 were placed in universal bottle vials which contained 10ml of distilled water. Samples (1.0ml) were witdrawn occsionally at an interim of 2 up to 12 hour and at 1, 6, 10 and 15 days. Every time trading the specimen with the equivalent of distilled water as a standard value to maintain sink conditions. The specimens were analyzed using spectrophotometer at 350nm. The propolis concentration was calculated from the calibration curve set in standard value of distilled wate. An in-vitro release was constructed from the data obtained.[18,19]

Biodegradable test for propolis chips

Aqueous trypsin (15ml 2.5%) was added to 15 eppendrof tubes. Then, 15 chips were weighed in vacuum weight scale and added into these eppendrof , one in each tube. It was incubated at 37°C for 8 days till achieve the maximum resorption. After that the chips were removed and dried with a filter paper and weighed for 8 days.

Artificial saliva absorption by the chips

For the saliva absorption test, 15 chips were weighed and put into 15 eppendrof tubes cotaining artificial saliva. The weighed chips were emerged into these tubes, one in each eppendrof tubes at room temperature 23°C for 8 days. After that the chips were removed and dried with a lane free cloth, and weighted for 8 days. Absorption was expressed as increase in weight percent.

Percent absorption = [(Wet weight – Dry weight)/ Dry weight] x 100.[20]

Swelling test / water absorption

For the distilled water absorption test, 15 chips were weighed. 15 eppendrof tubes containing distilled water were prepared. The weighed chips were emerged into thee tubes,one in each eppendrof tubes at room temperature 23°C for 8 days. After that the chips were removed and dried with lane free cloth and weighted for 8 days. Absorption was expressed as increase in weight percent.

Percent absorption = [(Wet weight – Dry weight)/ Dry weight] x 100.[20]

Determination of antimicrobial activity

Antimicrobial sensitivity test was performed on the propolis extract using agar diffusion method against *Staphylococcus aureus* and *Escherichia coli*, representing gram-positive and gram-negative bacteria, respectively. The strains used in this study were ATTC culture, *S. aureus and E. coli*. Briefly, each of the bacterial suspension with turbidity equivalent to 0.5 MacFarland Standard, was homogenously spread onto Muller-Hinton agar, according to the method of Clinical and Standards Laboratory Institute (CLSI, 2005). Three wells were made on the agar medium using cork-borer (60 mm in diameter), and each well was filled with 20 µl of the extract, chlorhexidine (0.2%, w/v) and ethanol (20%, w/v), the latter two samples served as controls. Following overnight incubation at 37°C, the diameter of the size of zone of inhibition was measured.

Minimum inhibitory concentration of the extract was carried out using broth microdilution, method according to CLSI, 2005. Briefly, the extract was serially double-diluted to eight dilutions with one well served as positive growth control (broth plus inoculum), one well served as negative control (broth only). Each row of the 96-well microtitre plate was seeded with 5µl of the bacterial suspension containing approximately 10^5 CFU/ml of each bacterium. The plate was incubated at 37°C for 24 hours and the broth was observed for turbidity. The concentration of the extract that showed bactericidal activity was investigated by culturing broth from wells which the broth did not turn turbid, onto nutrient agar.

Data Analysis

Data collection was analyzed by using Statistical Package for the Social Sciences (SPSS) version 22.0 by using paired t-test and ANOVA. The confidence interval (CI) is 95% confidence level. A p-value of ≤ 0.05 is considered significant.

Results

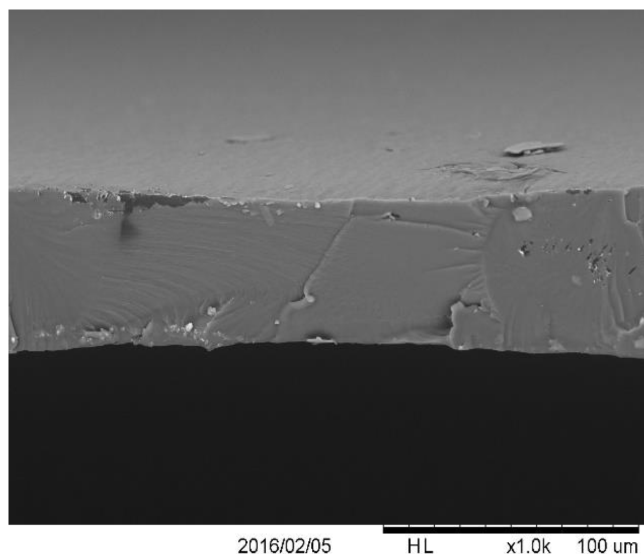
Determination of antimicrobial activity of propolis extract

The extracted propolis showed antimicrobial activity to both the gram-positive and gram-negative bacteria. The propolis extract produced larger zone of inhibition against *S. aureus*, compared to the inhibition zone produced against *E. coli*. Its antimicrobial activity was relatively comparable to chlorhexidine. A concentration of 0.15 mg/ml was able to inhibit growth of *S. Aureus* while concentration of 1.25 mg/l was required to inhibit growth of *E. coli*. It

showed bacteriostatic activity at 0.31 mg/ml and 0.25 mg/ml against *S.aureus* and *E. coli*, respectively.

Assessment of propolis chips morphology

The diameter of chips were measured by using SEM, it was 6.09 mm.(6.1± 0.025). Figure 1 showed thickness of propolis chips with thickness is 58 µm. Lower and upper bound for this thickness are 54.848 µm and 62.112 µm respectively.



propolis

Figure 1. Thickness of propolis.

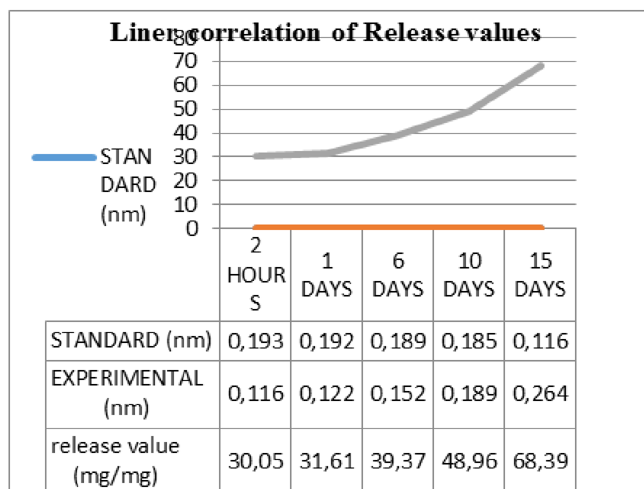


Figure 2. Value of propolis release from second hour to day 15.

Roughess of chitosan-propolis chips was tested using profilometer device.

The roughness surface of the propolis chips are within range of 1.346 to 4.206 µm (m= 2.776µm (2.637 ± 2.637).

In Vitro Release study

The release of propolis in vitro study increased gradualy through out the experiment. The highest release was on day 6 which was about 80%. Figure 2 showed the value of propolis release from second hour to day 15.

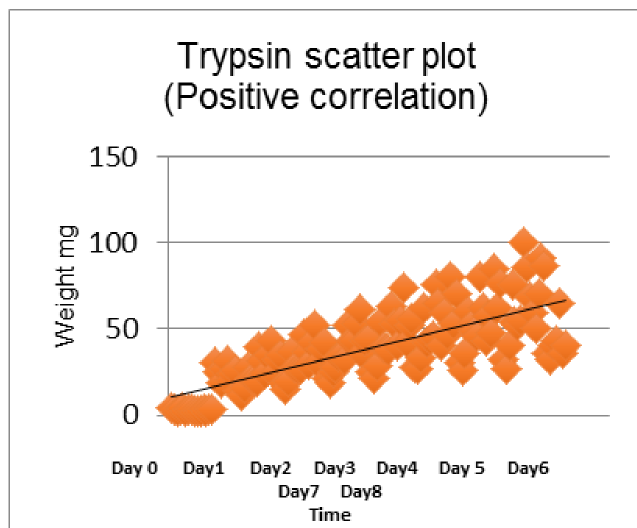


Figure 3. Biodegredation of the chips in Trypsin.

	N	Mean	St.-Dev. (Weight mg)	Std. Error	95% Confidence Interval for Mean Lower Bound	Upper Bound
Artificial Saliva	135	47.10	25.685	2.2	42.73	51.47
Distilled water	135	48.79	26.471	2.3	44.28	53.28
Trypsin	135	38.55	21.664	1.9	34.86	42.73
Total	405	134.4	73.82	1.2	121.87	147.48

Table 1. Standard error and confidence interval in each media.

Biodegradation test of propolis chips

In vitro biodegradation study done by using trypsin. Absorption test was done using artificial saliva and distilled water. Weight of the chip immersed in the solution was measured everyday for 8 days. All the chips changed their shape after several days. Statistical analysis showed signficant diffrence in chips weight. One way anova showed that the average weight (mg) varies across values of bioderadation media. Post Hoc t-test of weight by biodegradation

media within groups showed clear significant diffrences of the trypsine coparing to other media (Figure 3,table 1,2 and 3).

One-way ANOVA				
Weight by Biodegradation Media (between groups)				
Test Statistic F	Critical Value @5 % C	St. Error	Significant F>C	P-value
25.606	3.014	1.2	**Yes**	<0.05

Table 2. Average weight (mg) varies across values of bioderadation media.

Post Hoc t- test						
Weight by Biodegradation Media (within groups)						
		Test Statistic t	Critical Value @5 % C	St. Error	Significant t>C	P-value
***Artificial Saliva**	Distilled Water	0.531	1.969	1.6	**No**	0.596
	Trypsin	2.959	1.969	1.5	**Yes**	<0.05
***Distilled Water**	Artificial Saliva	0.531	1.969	1.6	**No**	0.596
	Trypsin	3.479	1.969	1.5	**Yes**	<0.05
*** Trypsin**	Distilled Water	3.479	1.969	1.5	**Yes**	<0.05
	Artificial Saliva	2.959	1.969	1.5	**Yes**	<0.05

Table 3: Post Hoc t-test of weight by biodegradation media within groups.

Discussion

Periodontal disease is a chronic infectious disease resulting from a response towards a complex dental biofilm containing various type of pathogenic bacteria. Periodontal disease caused destruction of tooth-supported tissue such as gingiva, periodontal ligament, alveolar bone and cementum. Failure of getting treatment for periodontal disease will lead to tooth loss. Treatment of periodontal disease can be non-surgical and surgical. For the non-surgical technique, it includes scaling and root debridement. While for the surgical technique, it includes bone graft, guided tissue regeneration, soft tissue graft, and flap surgery or pocket reduction surgery. Most of the prophylactic and therapeutic interventions done is to reduce the amount of bacteria in such a way that optimum oral health can be obtained and maintained. Antimicrobial agents are useful to support these effort by effectively inhibit the formation of dental biofilm and removing established dental biofilm.

Physical properties like thickness, roughness and diameter of the propolis chips in chitosan based was analyzed using s electron microscope, profilometer and digital caliper. The average thickness of the propolis was calculated to be 58.58µm. With this measurement, it is very applicable to be used inside the periodontal pocket as the guided tissue regeneration material. In addition, the diameter of the chips is 6.09mm, so it will be easily handled by the operator during the procedure.

Due to its antimicrobial, anti-inflammatory, and anti-oxidant properties, propolis has been used since ancient time as a remedy for the treatment of many diseases. To obtained all of this properties from the active ingredients in the raw propolis, extraction with ethanol was done. Propolis extraction can be performed by several techniques such as maceration technique, sonication technique and microwave technique. However, in this research sonication method was used. Although the extraction of propolis using maceration technique had the percentage of yield higher than sonication, sonication technique is faster and it showed antioxidant activity, total phenolics and flevonoids compound content higher than maceration technique.[21] Percentage of yield of the extraction with sonication technique in this research was 60%. The ratio of propolis to organic solvents can be 1:10 or 1:20. It does not affect the extraction of active compound in propolis.[22] We used ratio of 1:20 for this research. 70% of ethanol was used as the organic solvent, this is to make the extracted propolis soluble in water for dilution and more active compound of the propolis being extracted. As in this research, initially we use 20% of ethanol for the organic solvent. However, in antimicrobial test, it failed to show any antimicrobial activity against both gram positive and gram negative bacteria. The first objective of this research is to formulate periodontal chips from Malaysian propolis in chitosan base. As the extracted propolis is diluted, the dilution was used to be mixed with chitosan to produce a chitosan based chip.

In vitro biodegradation study done by using trypsin. Absorption test was done using artificial saliva and distilled water. Weight of the chip immersed in the solution was measured everyday for 8 days. All the chips changed their

shape after several days and become a gelatin form like. There was an increment of weight in each day for each chip in all the three solution. However, the absortion rate of the propolis chips were inconsistent throughout the experiment. This is because of the structure of the chips where some of the chips contain bubbles. In comparison to study by N. Tabary et al regarding chlorhexidine-loaded chips, the chips displayed a rapid degeneration within the first 3 days.[23]

A possible explaination of this result was that chitosan based chips has the pore morphology that provide a less surface area for the solution to acces the polymer chains of chitosan.[24] However, it shows that the chips will release an active molecules at a constant rate over an extended period, compared to a system that would rapidly degrade and provide a burst release effects of active molecules. Such a degradation study should be done under in vivo condition where the complex factors in the GFC of the periodontal pockets such as enzymes, inflammatory factors and subgingival bacteria should enhance the degradation rate compared to in vitro condition.[23]

Drug released was done by dipping 10 propolis chips in chitosan based into 10ml of distilled water under stirring for half an hour. Then the released of propolis was measured using spectrophotometer at 350nm for 2h, 1day, 6days, 10days and 15 days. The result showed that the released of propolis was up to 80% in 6 days. Based on the study by N.Tabary et al, the in vitro release from periochips occured with a burst effect as they released 80 % of their active component within 2, 4 and 6 days then levelled of in the following days.[23] If we compare the released of propolis to the periochips, propolis was progressively released within the whole experiment. In addition, the propolis extracted in this research was water soluble, so we can conclude that water soluble propolis extraction showed higher amount of released in compare to lipid soluble propolis extraction.[25]

There are a lot of study found that propolis has antimicrobial properties.[25,26,27] In this research we tested the propolis extraction with gram positive and gram negative bacteria species. Extracted propolis showed antibacterial activity against gram positive and gram negative species. However, there was weaker antibacterial activity against gram negative bacteria. The MIC value of the propolis extraction against gram positive bacteria was 0.152 mg/ml while against the gram negative bacteria was 1.25 mg/ml. This was the same as study done by silici S and Kutluca S, 2005, they found that ethanolic extract of propolis sample showed high antibacterial activity against gram positive cocci, but had a weak activity against gram negative bacteria.[28]

Conclusions

As a conclusion, Malaysian propolis can be evaluated into a chip and to be used in treating patient with periodontal disease. It was found to be biodegradable,have high release rate, and have antimicrobial activity against gram positive and gram negative bacteria.

Acknowledgements

The authors express gratitude to Faculty of Dentistry UiTM, Institute for Medical Molecular Biotechnology (IMMB) and Universiti Teknologi Mara Sg. Buloh, Selangor, Malaysia. All authors have made a substantive contribution to this study and/or manuscript, and all have reviewed the final paper prior its discussion.

Declaration of Interest

The authors report no conflict of interest.

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