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Effect of Topical Rambutan Honey Pharmaceutical Grade on Oral Mucosa Wound Healing Based on Tissue Wound Closure and Fibroblasts Proliferation *in vivo*

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ABSTRACT

Rambutan honey often used for topical treatment sores in the oral, because it has a good taste and fragrant. The use of rambutan honey empirically efficacious in wound healing has been scientifically proven yet, as a product or stimulant that serves to accelerate oral mucosa wound healing is still very limited. This study aimed to analyze topical Rambutan Honey Pharmaceuticals Grades (RHPG) in influencing wound closure and stimulation of the fibroblasts proliferation in the oral mucosa wound healing *in vivo*. The research method was experimental laboratory. Rats (n = 16/gp) were divided into negative control, positive control φ4±2 mm wound, 1 mL RHPG wound and 0.1 mL ascorbic acid. Oral mucosal wound (palatal region) were observed on day 0, 3, 7 and 14 administered topically twice. The data analysis are using the ANOVA and post hoc Tukey with $p<0.05$. The results show the RHPG effect on the wound width significantly at days 0 ($p = 0.002$), 3rd ($p = 0.005$) and the 7th ($p = 0.009$) and to the amount of fibroblasts significantly at days 0, 3rd and 7th with $p = 0.001$, fibroblasts cell amount per high power fields mean 101.75±8.99 (NC), 69±12.65 (PC), 83.25±25.83 (RHPG), 86.5±5.84 (AA), respectively. In conclusion, that topical administration of pharmaceuticals standard rambutan honeys effect to oral mucosa wound closured and to fibroblasts proliferation *in vivo*.

Key words: Fibroblast, rambutan honey, wound healing

INTRODUCTION

Wound is discontinuity of a tissue that can be caused by sharp objects or blunt trauma, changes in temperature, chemicals, explosion, electric shock, animal bites and contact heat (Barbul and Efron, 2010; Sjamsuhidajat and de Jong, 2010). In the human body is often exposed to injuries, including, the area of the oral mucosa. Injuries if not handled properly will lead to a long healing time, causing discomfort and complications include bleeding and infection and will ultimately lead to chronic wounds that can degrade the quality of life (Peterson, 2003). The incidence of injury had a prevalence reaching millions of cases per year. Impaired wound healing results, such as; the treatment of acute wounds and late chronic wounds will fail to progress to the normal stage of wound healing. The injury often enters pathological

inflammatory conditions because the process is delayed, incomplete or wound healing processes is uncoordinated (Menke *et al*., 2007). Good management and control on wound healing is very important. Wound healing is a process that involves the coordination of a complex relationship between cellular and humoral factors. The process of wound healing includes; the inflammatory phase, the proliferative phase and the remodeling phase (Kumar *et al*., 2004). Methods of wound healing have been progressing in recent years. The method was developed in the form of a product or a stimulant to compensate for the body's biological processes at every phase of the healing wound. Biological process targets, when the body compensates for injuries are the components that play a role in wound healing stages (Gosain and DiPietro, 2004). Fibroblast is one component of wound healing in the form of cell that is widely distributed in connective tissue. Fibroblasts produce collagen precursor substance, elastic fibers and reticular fibers (Marcovitch, 2005). In the stages of wound healing, fibroblasts play an important role in the process of fibroplasia. Fibroplasia is a wound repair process, involving the connective tissue, which has four components: Formation of new blood vessels, migration and proliferation of fibroblasts, deposition of ECM (extracellular matrix) and maturation, as well as the organization of fibrous tissue (remodeling). In the four components, fibroblasts play a role in the process of fibrosis that involves two of the above components, namely the migration and proliferation of fibroblasts and deposition of ECM by fibroblasts (Kumar and Clark, 2005). Honey is one of the products of natural materials are often used in the treatment of wound healing. Research reveals that honey containing flavonoids act as antioxidants, anti-inflammatory, antibacterial and have the effect of debridement (Perez *et al*., 2006; Suranto, 2007). Rambutan honey is produced by honey bees suck nectar from flowers rambutan tree (*Nephelium lappaceum*), often used for topical drugs sores in the oral, because it has a good taste and fragrant honey rambutan, so widely used by the community. The use of honey as a natural medicine rambutan empirically efficacious in wound healing has been widely known but scientifically, as a natural material products or stimulants in accelerating wound healing process is still very limited oral mucosa. Therefore, this study aimed to analyze topical administration rambutan honey standard pharmaceuticals (RHPG) in influencing wound closure and stimulate the proliferation of fibroblasts in the oral mucosal tissue wound healing *in vivo*.

MATERIALS AND METHODS

This research was conducted at Laboratory of Biochemistry and Molecular Biology, Medical Faculty of General Ahmad Yani University, Cimahi, Indonesia for the period of March–June 2015. Wistar strain rats were used in this study obtained ethical approval from the Research Ethics Committee Hasan Sadikin Hospital Bandung, Indonesia with number of approval of 140/UN6.C1.3.2/IEC/PN/2015.

Sample of Rambutan Honey Pharmaceutical Grades (RHPG): Samples were taken from the National Bee keeping Centre (Pusbahnas) Indonesia. Honey was purely isolated from the beehive with sterile technique and then standard setting pharmaceuticals were conducted. Samples of honey stored in the dark bottles and temperature conditions of -20°C (Ferreira *et al*., 2009).

Animal samples: Animal samples were taken from the rat population obtained from the Central Laboratory of Biological Sciences Bandung Institute of Technology as many as, 64 rats with inclusion criteria of male Wistar strain rats aged 3-4 months with a body weight of 200-300 g and healthy. Rat before hand adapted in the laboratory cages of animals for seven days at ambient conditions (temperature 22±3°C, relative humidity 30-70%, dark light conditions, during each 12 h, not noisy) and the maintenance of the same techniques as well as supervision. Prior to the making of the wound, the positive control and treatment were anesthetized by using, ketamine general dose of 10 mL/1000 g (0.1-0.2 mL). Rats were injected intraperitoneal at 2/3 posterior from the abdomen dextral previously undertaken aseptic action with 10% povidone iodine. Rats were randomly divided into 4 groups, each consisting of 16 rats, Negative Control (NC) without treatment injury, the Positive Control (PC) was given with punch biopsy injury in the palatal mucosa with a diameter of 4 mm and a depth of 2 mm and only given topical distilled water 1 mL, Rambutan Honey Pharmaceuticals Grade (RHPG) was given injury, then topical RHPG 1 mL and Ascorbic Acid (AA) given the injuries then topical vitamin C 0.1 mL. Test preparation administered topically twice, morning and afternoon during the 14-day study period. Dose calculation was based on the guidelines of empirical research conducted daily and the results of previous studies. Rats before and after treatments were weighed. Observation of experimental animals after treatment was observed on days 0, 3rd, 7th and 14th with terminated on 16 rats or each group of the 4 rats and then measured and taken the whole area of the rat palatal mucosa both injured and healthy (DiPietro and Burns, 2003; Yuslianti *et al*., 2013).

Oral mucosa tissues wound healing examination: The observation of oral mucosa tissue wound closure were done macroscopic with wound tissue clinical examination and wound width measurement through the average of four diameter of wound area by using the caliper and ruler on day 0, 3, 7 and 14 (Yuslianti *et al*., 2013).

Histology preparations of oral palatal mucosa tissue with a diameter of 10 mm from wound area and 2 mm depth were fixed using the normal buffer formalin (BNF) 10%, cut thin and dehydration process was carried out in alcohol-rise (80, 90 and 95) as well as clearing, using the xylol. The next stage was done embedding with paraffin that has a melting point of 58°C and printed in a special mold. Tissue was cut

with a thickness of 5 mm using the microtome, taken with a glass object and left clinging for overnight or heated at a temperature of approximately 60°C and given a dye Hematoxylin-Eosin (HE). Fibroblast cells counted with 40 X objective expansion light microscope by pathologist at Laboratory of Pathological Anatomy, Medical Faculty.

Statistical analysis: Data was analyzed statistically with SPSS 17.0. Data were statistically analyzed by one way ANOVA and Tukey's test, after affirming the normality and homogeneity of variances assumptions of the data sets. p-values <0.05 were considered to be significantly different.

RESULTS

Effect of topical rambutan honey on oral mucosa wound healing based on oral mucosa wound closure: Diameter wound area calculations were carried out on PC, RHPG and AA group at day 0, 3rd, 7th and 14th. Effect RHPG on wound area diameter compared with NC, PC and AA was shown in Fig. 1. Results of normality test (Kolmogorov-Smirnov) p-value was 0.000 ($p<0.05$), which indicated that the data was not normally distributed. This condition made ANOVA test was not applicable and Kruskal Wallis test was conducted instead. The research results of the RHPG effect on palatal mucosa tissue wound width obtained significant value in groups of rats day 0 ($p = 0.002$), 3rd ($p = 0.005$) and day 7 ($p = 0.009$), where $p<0.005$, as shown in Table 1.

From the result, to determine which groups were different from one another Mann-Whitney post hoc test shall be carried and showed that on day 0, 3rd and 7th there was a difference between the treatment groups PC, RHPG, AA to NC ($p = 0.029$), while the RHPG with PC and AA in all day there was no difference.

Palatal mucosa wound closure observation on day -0, 3, 7 and 14: The RHPG effect on clinical appearance of the palatal

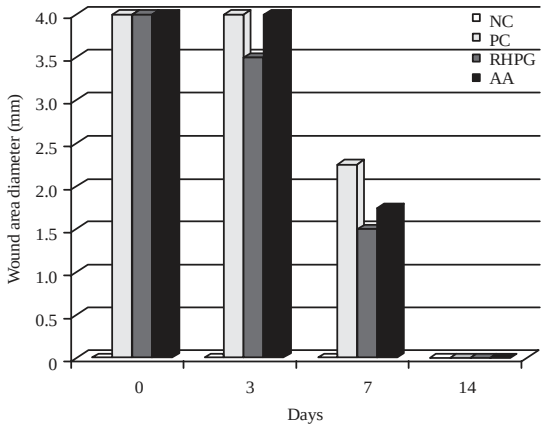

Fig. 1: Effect RHPG on wound width compared with NC, PC and AA

Table 1: Distributions of mean value, Standard Deviation (SD) and value significance effect RHPG on wound width on day 0, 3, 7 and 14

Groups	Days	N	Mean±SD (mm)	p-value
NC	0	16	0.00±0.00	0.002*
PC		16	4.00±0.00	
RHPG		16	4.00±0.00	
AA		16	4.00±0.00	
NC	3	16	0.00±0.00	0.005*
PC		16	4.00±0.00	
RHPG		16	3.5±0.580	
AA		16	4.00±0.00	
NC	7	16	0.00±0.00	0.009*
PC		16	2.25±0.50	
RHPG		16	1.50±0.58	
AA		16	1.75±0.50	
NC	14	16	0.00±0.00	0.093
PC		16	0.25±0.29	
RHPG		16	0.00±0.00	
AA		16	0.00±0.00	

Kruskal Wallis, *p<0.05 was significantly different

mucosa wound closure at day 3, 7 and 14 were seen significant compared with PC group seen in Fig. 2.

Clinically, there was no visible difference, appear in wound tissue of RHPG and PC groups on day 0 wound diameter of 4 mm and are still visible scars reddened scar tissue bleeding. On the 3rd day, wounds were seemed still open with a diameter wound on RHPG and PC groups have already started restriction but looked at the PC group wound area was still visible redness than RHPG group. On day 7, wound began to closure between PC and RHPG groups but can be seen in the PC group, there were granulations compared RHPG group. Observation on day 14 wound on RHPG group has begun to close completely, when compared to the PC group, can be seen also in the PC group still seems one of the signs of inflammation that tissue appear reddish (Fig. 2).

Effect of topical rambutan honey on oral mucosa wound healing based on the amount of fibroblast: Counting the number of fibroblasts was conducted on normal tissue (NC), PC, RHPG and AA then observations and counts were made on day 0, 3rd, 7th and 14th. Normality test result data by Kolmogorov-Smirnov test p-value obtained significantly for RHPG influence on the number of fibroblasts cells on day 0, 3, 7 and 14 were 0.565, 0.196, 0.708 and 0.104 ($p>0.05$). Distribution homogeneity test using Levene's test p-value obtained significantly for RHPG influence on the number of fibroblasts days 0, 3, 7 and 14 were 0.270, 0.777, 0.100, 0.479 ($p>0.05$). Test result for normality and homogeneity were the assumptions for ANOVA test was met.

The research results on effect RHPG to the amount of palatal mucosa fibroblasts tissue wound healing NC, PC, RHPG and AA obtained significantly in the groups of rats days 0, 3rd and 7th with $p = 0.001$ ($p<0.005$) with the mean and standard deviation of fibroblasts cells amount sequentially 101.75±8.99, 69±12.65, 83.25±25.83 and 86.5±5.84 cells with minimum-maximum value of 95% confidence intervals are shown in Fig. 3 and Table 2. The results to determine,

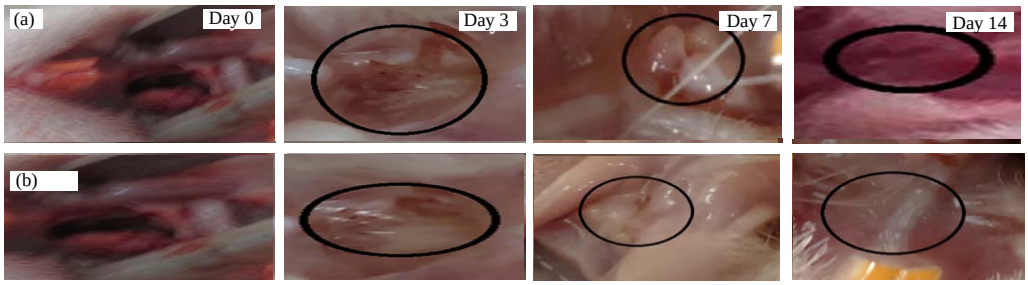

Fig. 2(a-b): Clinical appearance of the palatal mucosal wound closure PC and RHPG, (a) PC and (b) RHPG

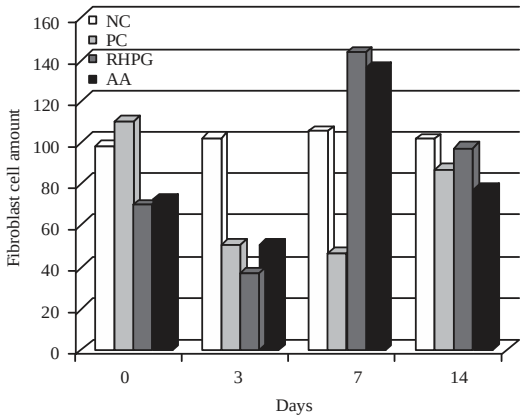

Fig. 3: Effect RHPG on fibroblast amount compared with NC, PC and AA

which groups were different from one another with Tukey *post hoc* test shall be carried and obtained the results as in Table 3.

Fibroblasts observation around wound area: The number of fibroblasts cells was calculated from three fields of view by using, a tally counter under a light microscope. Result of fibroblasts amount, can be seen in Fig. 3, showed that the fibroblasts in the RHPG group day 0 and 3 were less but at day-7 more than other groups, while day 14 almost equal to the number of fibroblasts NC group.

Fibroblasts observation at days 0, 3, 7 and 14 were conducted on histological preparations in each group (Fig. 4). On day 0, fibroblasts were slightly found more in group NC, PC than RHPG group and AA. Inflammatory cells already existed in the PC group, RHPG and AA compared with NC group. On day 3, fibroblasts were seen least spreading in RHPG group than the other group. Inflammatory cell infiltration can be seen in the PC group, RHPG and AA, which signifies the inflammatory phase was still going on day 3. In the PC groups found more inflammatory cells and endothelial cells with a round shape and thick cell periphery area containing erythrocytes. Reddish colors were found in the PC group, RHPG, AA compared with NC group, which indicates the persistence of bleeding due to trauma from injury. On day 7, in the RHPG group fibroblast cells were found more and young collagen are seen thick but not meeting the endothelial cells were seen scattered in the area around the wound well and epithelial cells already begun composed, while in groups of cells found fibroblasts and collagen much younger seen but endothelial cells were seen in the area around the wound slightly with varying the size of the endothelial cells. The PC group fibroblasts distributions were few in number and the young connective tissue were still seen thin and dominance of inflammatory cells and granulation tissue seen. On day 14, in the RHPG group, fibroblasts density began to decrease and connective tissue density increase almost equal to the NC group. In the AA group, fibroblasts

Table 2: Distributions of mean value, standard deviation (SD), 95% CI and value significance effect RHPG on fibroblasts amount on day 0, 3, 7 and 14

Groups	Days	N	Mean±SD	95% CI lower	Upper	p-value
NC	0	16	98.00±5.420	89.38	106.62	0.001*
PC		16	110.00±7.830	97.54	122.46	
RHPG		16	69.75±12.82	49.36	90.14	
AA		16	72.50±17.48	44.68	100.32	
NC	3	16	101.75±11.15	84.01	119.49	0.001*
PC		16	50.50±16.11	24.86	76.14	
RHPG		16	37.00±14.35	14.16	59.84	
AA		16	50.75±16.44	24.59	76.91	
NC	7	16	105.75±10.40	89.19	122.31	0.001*
PC		16	46.50±14.01	24.20	68.80	
RHPG		16	143.00±50.31	62.94	223.06	
AA		16	136.25±13.69	114.46	158.04	
NC	14	16	101.25±10.31	84.85	117.65	0.155
PC		16	87.00±16.79	60.28	113.72	
RHPG		16	97.25±11.15	79.51	114.99	
AA		16	77.75±18.52	48.28	107.22	

ANOVA test, *p<0.05 was significantly different

Table 3: Fibroblast amounts value significance differences between treatment groups

Days	Groups	p-value
0	RHPG	
	Negative control	0.025*
	Positive control	0.020*
3	RHPG	
	Negative control	0.000*
	Ascorbic Acid	
	Negative control	0.020*
7	RHPG	
	Positive control	0.002*

Tukey *post hoc* test, *p<0.05 significantly

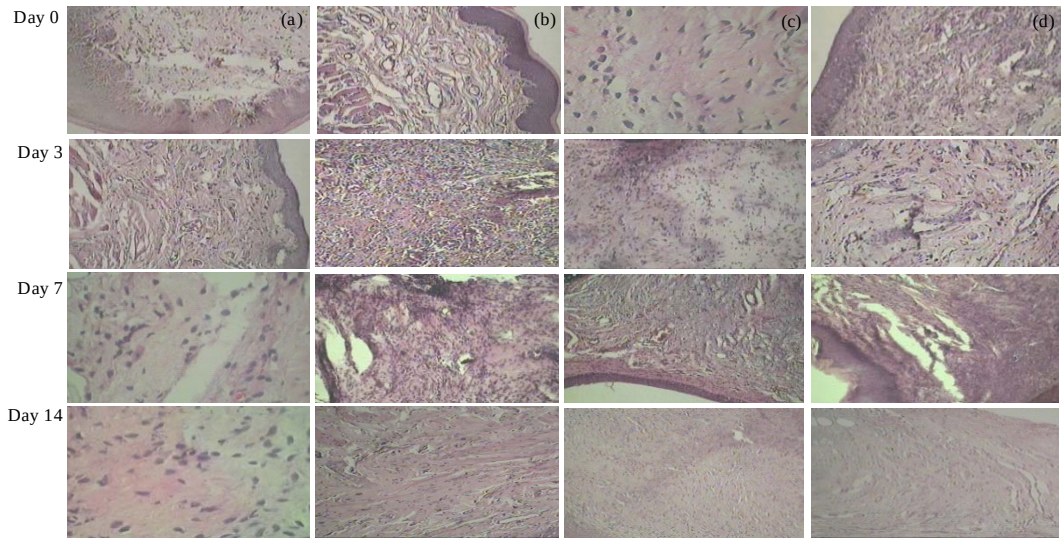

Fig. 4(a-d): Hematoxylin eosin staining of wound area (a) NC, (b) PC, (c) RHPG and (d) AA

density seen began to decrease almost the same as the group of RHPG and young connective tissue looks thin and sparse. In the PC group fibroblasts both active and inactive invisible, inflammatory cells spreading were still visible on the PC group than other groups. Day 14 indicates the end of the proliferative phase and remodeling phase commencement.

DISCUSSION

Wound healing methods have been developed, either a product or a stimulant to the body's biological processes in wound compensation. The main target of oral mucosal wound treatment was to restore the function and to form the oral mucosal tissue back to normal with minimum local complications. Fibroblasts were one component that plays an important role in the healing process of fibroplasia. Rambutan Honey Pharmaceutical Grades (RHPG) were pure isolate honey standardized for pharmaceutical product with good taste and fragrant have been made as a natural product or stimulant for wound healing treatment. These study results indicate that the topical RHPG effect on diameter palate mucosal excision wounds of mice on day 0, 3rd and 7th (Table 1 and Fig. 2) were adequately. The tissue will undergo a process healing was a complex phenomenon and involves multiple cellular and humoral processes. During the later stages of wound healing, fibroblasts itself contribute to the synthesis. The RHPG topical administration to the number of fibroblasts showed significant results than other groups (Table 2). Observation of day 3rd and 7th (Fig. 4) shows the fibroplasia area around the wound was found in RHPG group and AA group compared to the PC group. Distribution of fibroblasts in the area around the wound at day 3, which was an inflammatory phase showed significant difference in the groups of RHPG and AA compared to PC group at day 7, which was a proliferative phase, where significant increase in the number of fibroblasts. Observation of number of fibroblasts in the area around the wound for the entire group at day 7 (Fig. 3) shows that the number of fibroblasts in the group of RHPG higher than other groups. These results demonstrate the potential of RHPG in stimulating the fibroplasia. Fibroblasts are components in the process of wound healing and acts as, a marker in wound healing, which requires excision repair connective tissue. Fibroblasts can synthesize and secrete extracellular molecules with autocrine and paracrine including extracellular matrix, components of the basic substance and other biologically active molecules are proteinase, cytokines and growth factors. The formation of fibroblasts is activated by TGF-β and FGF of macrophages generated especially, during the inflammatory phase until the phase of remodeling (Nanci, 2013).

Based on the results of research on the wound by topical application of RHPG have potential fibroplasia effect. This was probably caused by the content of flavonoid that play an antioxidants role in enhancing the hormonal activation of fibroblasts and connective tissue that formed well without granulation tissue (Middleton *et al*., 2000).

In addition, RHPG flavonoids protect the cells of Reactive Oxygen Species (ROS) produced in the inflammatory process, so that the cells around the wound were not damaged. Flavonoid inhibits degradation of connective tissues by ROS, which usually take part on wound healing and inflammation process (Eming *et al*., 2007). This was supported on broad observation of the wound, where day 7 broad wounds of RHPG group began to close compare the PC groups with formed granulation tissue. Many studies have suggested that flavonoids exhibit biological activities, including; anti-allergenic, antiviral, anti-inflammatory and vasodilation actions. However, most interest has been devoted to the antioxidant activity of flavonoids, which was due to their ability to reduce free radical formation and to scavenge free radicals. The capacity of flavonoids to act as antioxidants

in vitro has been the subject of several studies in the past years and important structure-activity relationships of the antioxidant activity have been established (Pietta, 2000). This anti-inflammatory influence was linked to the antioxidant content of antioxidant which was owned by honey, whereas honey can inhibit the release of Reactive Oxygen Species (ROS), which cause an inflammatory process lasting or abnormal (Dem Keller *et al*., 2006).

In addition, the reduction of the inflammatory process may be due to the antibacterial effect of honey or direct anti-inflammatory effects. Results of the study on day 14 showed significant results from of RHPG the effect on the number of fibroblasts and by observation histology (Fig. 4) density of fibroblasts was perfect on RHPG groups and did not differ with NC group which were not injured but both were slightly different from the AA group that has good density, although, there are cavities. This suggests that the process of re-epithelialization faster in the group of RHPG, which indicates the process of wound closure faster than the other groups. This was due to the remodeling phase changes the phenotype of fibroblasts into myofibroblasts, which serves to retract the wound caused by the increasing number of connective tissue in the wound, the greater the contraction power wound, so that the wound will be attracted and cause large wounds become smaller. Topical administration of RHPG can accelerate wound closure and stimulate fibroplasia especially in the proliferative phase showed the presence of both the cellular mechanisms of RHPG on wound healing, which was expected, when it applied a standardized natural ingredients will help the healing process towards the regeneration of wound tissue not only repair that will have an impact on optimal wound healing results.

CONCLUSION

Based on the results of this study concluded that topical administration of pharmaceuticals standard rambutan honey effect to oral mucosa wound closured and fibroblasts proliferation *in vivo*. This was clear evidence that potential of rambutan honey as a natural product or stimulant in treating human oral mucosal wound healing that was safe, effective and delicious flavor to be applied. Further research is needed to determine the role RHPG in stimulating wound healing through hormonal mechanisms.

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