Research Journal of Medicinal Plant

ISSN 1819-3455

Academic Journals Inc.

www.academicjournals.com

Research Journal of Medicinal Plant 9 (6): 284-292, 2015
ISSN 1819-3455 / DOI: 10.3923/rjmp.2015.284.292

Antioxidant Activity of Rambutan Honey: The Free Radical-Scavenging Activity *in vitro* and Lipid Peroxidation Inhibition of Oral Mucosa Wound Tissue *in vivo*

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ABSTRACT

Rambutan honey has been reported to be effective therapeutic in wound healing, may be due to it antioxidant activity, but research of rambutan honey antioxidant activity is still very limited. The purpose of this study is to determine rambutan honey free radicals scavenging activity *in vitro* and lipid peroxidation inhibition of oral mucosa wound tissue *in vivo*. The research method was experimental laboratory. The sample used for DPPH test was pure isolated rambutan honey with concentration of 0.5, 1, 2, 4 and 8 mg mL^{-1}. Experimental animals used were 64 male Wistar rats. All rats were injured except for the Negative Control group (NC), using the 4 mm-diameter punch biopsy on the palate and then divided into groups of Positive Control (PC), a group with a topical application isolate of Rambutan (RH) 1 mL and group with topical application of Vitamin C (VC) 0.1 mL, then sacrificed at day 0, 3, 7 and 14 and measured lipid peroxidation inhibition of wound tissue with TBARs method. Data were analyzed statistically, using Kruskal Wallis and Mann-Whitney post hoc. The results showed rambutan honey 1 mg mL^{-1} have DPPH inhibition of 45.3% and the lipid peroxidation inhibition activity in oral mucosa wound tissue significantly on day 3 ($p = 0.028$) and 14th ($p = 0.037$). The conclusions were rambutan honey has antioxidant activity *in vitro* and *in vivo* that these natural substances have the potential to be used as an antioxidant drugs in human oral mucosal healing.

Key words: Antioxidant, rambutan honey, wound healing

INTRODUCTION

Wound is damage to the structure and function of anatomical normal tissue due to mechanical and physical factors can be derived from internal and external regarding certain organs including the mouth tissue. Wound will become a problem, when there is interference on the healing process, such as bleeding, ulceration and infection. This can lead to chronic wounds and discomfort to the patients and can reduce quality of life. Handling wound should be good in order to accelerate wound healing. Wound healing process consists of three phases, namely inflammatory phase, the proliferative phase and remodeling phase (Peterson *et al*., 2004; Sjamsuhidajat and de Jong, 2010).

Wound healing influenced by several factors, namely age, nutrition, infection, hematoma, foreign object, systemic disease as well as free radicals (Sjamsuhidajat and de Jong, 2010). Free radicals in the wound can be mitigated by antioxidants of the (primary antioxidants) and outside the body (secondary antioxidants) (Kumar *et al*., 2009). The content of free radicals resulting from wound healing can lead to the emergence of Malondialdehyde (MDA) caused damage to the cell membrane. Free radicals are highly reactive molecules that can damage cells in the body (Papas, 1999). One of the mechanisms to investigate antioxidant activity is to study the scavenging effect on proton radicals. Investigation of the total antioxidant capacity was measured as the cumulative capacity of the compounds in the sample that can scavenge free radicals using 1,1-diphenyl-2-picrylhydrazyl (DPPH) reaction. *In vivo* antioxidant activity secondary to the process of wound healing can be seen from of wound tissue MDA levels (Hussein *et al*., 2011).

Measurement of antioxidant capacity using the DPPH is an application of radical-scavenging method. The method is the main mechanism of antioxidant activity in foods. The measurement of antioxidant capacity is a method to assess the antioxidant activity using the synthetic radical in polar organic solvents, such as; methanol at room temperature. The principle is to capture reaction of hydrogen by DPPH of phenol and flavonoid antioxidants. The 2,2-diphenyl-1-picrylhydrazyl is a stable radicals which can react with other radicals to form a stable compound or react with the hydrogen atom (derived from an antioxidant) form a reduced (-H) (Molyneux, 2004).

Lipid peroxidation is a complex biochemical reaction involving free radicals, oxygen, metal ions and body factors in biological systems. This cause's lipid peroxidation's become the focus in the emergence of a disease (Halliwell and Gutteridge, 2007). Malondialdehyde (MDA) is a biomarker of the condition of the increase in free radicals highly toxic to the cell membrane because it is considered as, the initiator of a reaction, as well as a mutagen compounds generated from lipid peroxidation. Malondialdehyde include; aldehyde group the most numerous and stable enough generated from arachidonic acid lipid peroxidation, eikosapentanoat and doksoheksanoat (Esterbauer *et al*., 1991). Free radicals have two opposite function, namely the positive effects can kill the bacteria that invade wound and play a role in intracellular signaling pathways, whereas the negative effects can inhibit the migration and proliferation of cells and tissue even neoplastic cell transformation (Kumar *et al*., 2009).

One of natural medicine, material are often used in the treatment of wound healing is rambutan honey, which come from bee sucking nectar of rambutan flowers (*Nephelium lappaceum*) is often used for topical drugs wound in the mouth, because it has a good taste and fragrant and also highly sought by community (Suranto, 2007; Yuslianti, 2014).

The use of rambutan honey as a natural remedy empirically efficacious in wound healing has become a trend, but scientifically including antioxidant activity in overcoming oxidative stress in oral mucosal wound healing is still very limited. This study aimed to analyze rambutan honey antioxidant activity by free radicals DPPH scavenging activity and topical rambutan honey in inhibiting the formation of MDA from lipid peroxidation oral mucosal tissue in wound healing.

MATERIALS AND METHODS

The study was conducted at Biochemistry and Molecular Biology of Medical Faculty, General Ahmad Yani University Cimahi Indonesia, which was held from May until June, 2015. Ethical approval was obtained from the Research Ethics Committee Hasan Sadikin Hospital Bandung with number of approval of 140/UN6.C1.3.2/IEC/PN/2015.

Experimental design: The research methods were experimental laboratory. The study consisted of pure isolated rambutan honey sample preparation, *in vitro* testing the reduction free radical DPPH *in vitro* and testing MDA level *in vivo* in rats wistar strain as seen in Fig. 1.

Rambutan honey pure isolated samples: Rambutan honey samples were taken from the National Beekeeping Centre (Pusbahnas) Perhutani Indonesia. Pure honey was isolated from honey nest with sterile technique as shown in Fig. 2. Samples of pure isolated rambutan honey were stored in the dark with temperature conditions of -20°C.

Free radical-activity analysis of rambutan honey *in vitro*: The scavenging ability of rambutan honey for the free radicals 1,1-diphenyl-2-picrylhydrazyl (DPPH) was measured, as described by Molyneux with some modifications. The DPPH assay performed with three times the measurement. The DPPH reagent solution was a solution with a concentration of 30 μg mL^{-1} in methanol. Reagent solution used to measure free radical scavenging activity of various concentrations of extracts isolate rambutan honey that was measured after 30 min and the absorbance was measured at a wavelength of 400-800 nm with form methanol pa, so that the reagent absorbance values obtained for 0.819 at a wavelength of 515.5 nm. Reference solution in the form of quersetin10.0 mg in 100 mL of methanol made a series of standard solutions. Pure isolate rambutan honey 100.0 mg with methanol in a flask made 100.0 mL concentrations of 0.5, 1, 2, 4, 8 mg mL^{-1}. Rambutan honey solution 1.5 mL in a test tube was added 3.0 mL of DPPH reagent solution, shake and incubated for 30 min at room temperature and then read the

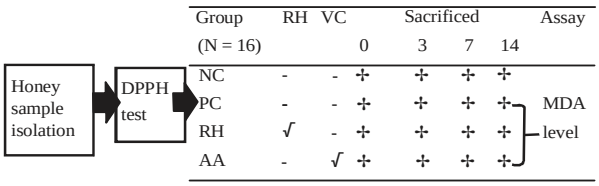

Fig. 1: Outline of experimental design

Fig. 2: Rambutan honey samples isolation technique

absorbance in the UV-Visible spectrophotometer at a wavelength of 515, 5 nm (Molyneux, 2004). The Radical-scavenging Activity (RSA) was calculated as a percentage of DPPH discolorations using the equation:

$$RSA\% = [(ADPPH-AS)/ADPPH]\times100$$

whereas was the absorbance of the solution when the sample extract has been added at a particular level and ADPPH was the absorbance of the DPPH solution (Ferreira *et al*., 2009). Free radical scavenging activity of pure isolate rambutan honey was determined by the percentage of concentration which can inhibit or reduce the intensity of absorption compared reagent solution.

Lipid peroxidation inhibition analysis of rambutan honey *in vivo*: Animal samples of 64 rats were taken from the Central Laboratory of Biological Sciences Bandung Institute of Technology. Rats beforehand were adapted in the laboratory cages 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 same maintenance techniques and strict supervision. Prior to the manufacture of wound, PC mice and treatment of common anesthetized using ketamine at a dose of 10 mL/1000 g, respectively 0.1-0.2 mL mice were injected intraperitoneal at 2/3 of the posterior abdominal dextral previously, undertaken aseptic actions with 10% povidone iodine. When, the rats had been under the influence of anesthesia, wound was made by using a biopsy punch with a diameter of 4 mm and a depth of 2 mm terminated by irrigation using sterile distilled water. Rats were randomly divided into 4 groups, each consisting of 16 rats, Negative Control group (NC) mice without treatment of the wound, the Positive Control group (PC) was given a punch biopsy wounds in the mucosa of the palate with a diameter of 4 mm and a depth of 2 mm and aquabidest given only topical, group Rambutan Honey (RH) given by punch biopsy injury in the mucosa of the palate with a diameter of 4 mm and a depth of 2 mm were given topical 1 mL RH, group Vitamin C (VC) were wound with topical vitamin C 0.1 mL. Test preparation administered topically in two doses in the morning and evening for 14 days of the research. Dose used in this research was based on empirical daily use and the results of previous studies. Rats before and after treatment were weighed (Yuslianti, 2014).

Observations on experimental animal after administration were observed at day 0, 3, 7 and 14 by the termination of the 16 rats or 4 rats each group. Palatal mucosa tissue was taken 5 mm from the wound area (Yuslianti, 2014). Tissues that had been taken were crushed with a drop of solution of Sodium Dodecyl Sulfate (SDS) and determined by MDA tissues level TBARs routine procedure perform absorbance readings at λ 532 nm.

Statistical analysis: Data of this study was analyzed statistically with SPSS 17.0. Samples were analyzed in triplicate. Data found will be presented with tables and related graph. Lipid peroxidation inhibition measurement (MDA tissues levels) were observed with Kruskal Wallis and Mann-Whitney post hoc with $p<0.05$.

RESULTS

Free radicals inhibition activity of rambutan honey *in vitro*: Free radical inhibition activity was measured by a decrease in the absorption of DPPH radicals after being treated rambutan honey as radical scavengers. Free radical activity measurement results of rambutan honey on

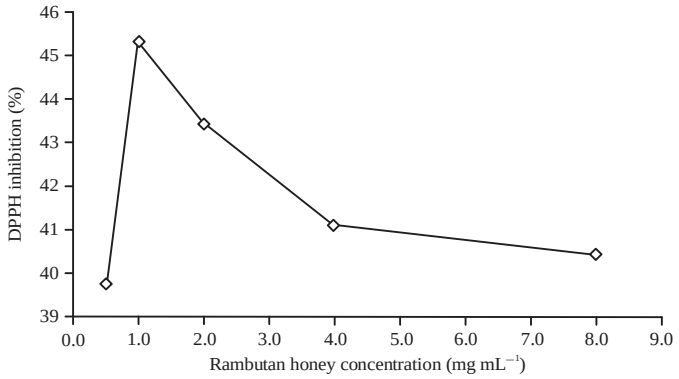

Fig. 3: Free radical scavenging activity (% inhibition) of rambutan honey concentration

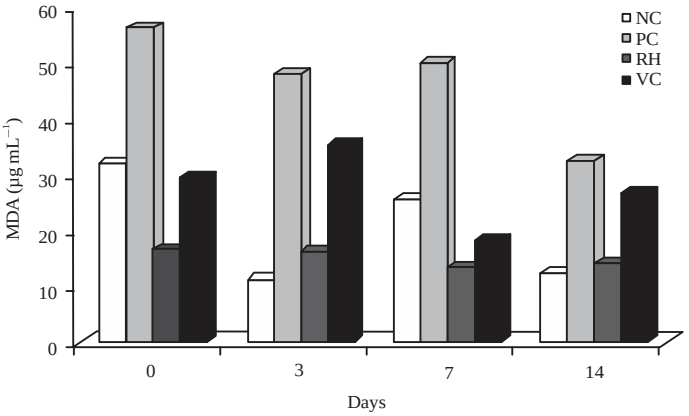

Fig. 4: Rambutan honey inhibitory effect on MDA levels compared with NC, PC and VC

DPPH *in vitro* compared with quercetin as a standard can be seen in Fig. 3. Rambutan honey at a concentration of 1 mg mL^{-1} had a visible reduction of activity amounting to 45.3% and the percentage reduction value became decreased when the rambutan honey concentration is increased.

Lipid peroxidation inhibition of oral mucosa wound tissue *in vivo*: The effect of rambutan honey topically on lipid peroxidation inhibition based on MDA tissues levels seen on day 0, 3rd, 7th and 14th of each phase of healing (the inflammatory phase, the proliferative phase and the remodeling phase). Normality test result data by Kolmogorov-Smirnov test p value obtained successively for RH influence on levels of MDA palate mucosal tissue of rat on days 0, 3rd, 7th and 14th were 0.001, 0.001, 0.806 and 0.001 where $p<0.05$ was that the data has not normal distribution. Results of homogeneity test using the test Levene's obtained p value consecutive to influence RH against MDA levels of mucosal tissue of the palate rat days 0, 3rd, 7th and 14th was 0.027, 0.02, 0.01 and 0.49, where $p<0.05$ so that the data variance between groups are not homogeneous. Results of tests of normality and homogeneity test was the assumption for ANOVA test was not met so using Kruskal Wallis test.

Based on Kruskal Wallis test rambutan honey effect on MDA tissues levels were significant at day 3 p = 0.028 and at day 14 p = 00373, $p<0.005$ with MDA level mean 11.95±4.67 µg mL^{-1} (NC), 40.49±34.61 µg mL^{-1} (PC), 15.37±4.19 µg mL^{-1} (RHPG), 31.22±6.37 µg mL^{-1} (AA), respectively also shown in Table 1 and Fig. 4. From the result were used to determine which

Table 1: Distribution of mean value, standard deviation (SD), 95% CI and effect of value of significance rambutan honey on MDA tissue levels on day 0, 3rd, 7th, and 14th

			95% CI		
Days and group	N	Mean±STD	Lower	Upper	p
0					
NC	16	32.33±15.45	7.74	56.91	0.092
PC	16	56.55±29.21	10.06	103.04	
RH	16	16.83±5.91	7.41	26.24	
VC	16	29.65±9.83	14.01	45.29	
3					
NC	16	11.40±4.53	4.19	18.62	0.028*
PC	16	48.13±36.44	9.87	106.12	
RH	16	16.43±4.33	9.54	23.31	
VC	16	35.55±5.19	27.28	43.82	
7					
NC	16	25.78±15.78	0.66	50.89	0.098
PC	16	50.28±33.88	3.638	104.19	
RH	16	13.45±2.64	9.24	17.66	
VC	16	18.43±7.75	6.10	30.75	
14					
NC	16	12.50±4.80	4.86	20.14	0.037*
PC	16	32.68±32.82	19.55	84.9	
RH	16	14.30±4.06	7.84	20.76	
VC	16	26.88±7.54	14.88	38.87	

NC: Negative control, PC: Positive control, RH: Rambutan honey, VC: Vitamin C, Kruskal Wallis test, *p<0.05 significantly different

Table 2: Rambutan honey inhibitory effect on MDA levels value significance of differences between treatment groups

Group	p-value
NC	
PC	0.901
RH	0.009*
VC	0.942
PC	
NC	0.901
RH	0.030*
VC	0.999
RH	
NC	0.009*
PC	0.030*
VC	0.024*
VC	
NC	0.942
PC	0.999
RH	0.024*

NC: Negative control, PC: Positive control, RH: Rambutan honey, Mann-Whitney *post hoc*, *p<0.05 significantly different

groups are different from one another then tested the dual comparative (Post Hoc) with the Mann-Whitney and obtained in Table 2.

DISCUSSION

Rambutan honey with flavonoid compounds were natural antioxidants, exhibits various biological effects, such as; anti-inflammatory, antibacterial and vasodilator actions. Chemical structure of phenolic compounds has an important impact on radical scavenging activity. Antioxidant capacity was directly related to hydroxyl moieties. Phenolic compounds also help to keep cell membranes in normal state by decreasing lipid peroxidation and free radical scavenging. They also enhance membrane integrity to protect from chemical and physical stress conditions. Free radicals were scavenged by phenolic compounds of honey by thwarting production of malondialdehyde which was a biomarker of oxidative damage (Shobhna *et al*., 2012). Animal

studies revealed that phenolic compounds exhibit antidiabetic effect, when they are administered in higher doses and moderate antidiabetic response in lower doses by regenerative changes in islet cells of the pancreas (Benkhayal *et al*., 2009).

Antioxidant activity testing of UV-visible spectrophotometry to rambutan honey pure isolate was based on ability of rambutan honey pure isolate to capture free radicals DPPH were determined by measuring the decrease in the wavelength of maximum absorbance at 515.5 nm. DPPH absorbance measurements carried out after the test solution was reacted with DPPH, where H^+ is the hydrogen atom contains one proton and one electron that comes from free radical reducer compound that will react with DPPH compound to form a stable DPP-hydrazine (Molyneux, 2004). Free radical inhibition activity of rambutan honey shown in a concentration of 1000 μg mL^{-1} with reduction percentage of 45.3% (Fig. 2). At greater concentration, reduction percentage did not show greater results so that it can be concluded that the optimum concentration to produce a high percentage reduction was 45.3% contained at a concentration of 1000 μg mL^{-1}. The possibility of the percentage reduction will be maximized if the content of antioxidants, such as; flavonoids or polyphenols of rambutan honey were extracted and tested. Data in a study by Ferreira *et al*. (2009) on Portuguese honey support the concept that phenolic antioxidants from the entire honey have a better scavenging activity and lipid peroxidation inhibition showed by the phenolic extracts in comparison to the entire honeys proved once more that other substances than phenols might be present in the entire samples. Nevertheless, these tests were just based on chemical reactions between honey and artificial free radicals and have no similarity with biological systems, but it was important to be aware that rambutan honey antioxidant compound may augment defenses against oxidative stress and that they might be able to protect humans from oxidative stress (Ferreira *et al*., 2009).

Rambutan honey topical administration of 1 mL has effect of reducing the MDA levels on day 3 (inflammatory phase) and 14 (remodeling phase) (Fig. 4 and Table 1). This was consistent with previous studies that honey rambutan *in vitro* proven to reduce levels of MDA (Yuslianti, 2014). Rambutan honey influence compared to other treatment groups seen (Table 2). Research in test animal with the complex cellular and humoral, which may help explain the mechanism of antioxidant activity of rambutan honey seen from decreased levels of free radicals in wound healing. Recent studies revealed that manuka honey has been shown to promote synthesis of cytokines by monocytes that have the potential to mediate the immune response (Tonks *et al*., 2003, 2001). In responding to challenges, such as; infected wounds, neutrophils and macrophages utilize the presence of free radicals, such as; superoxide and hydroxyl to modulate the activity of other cells such as monocytes and platelets. These in turn produce specific cytokines to signal activation of other cells (Burdon, 1995). However, in a prolonged insult, such as; a chronically infected wound, such stimuli may give rise to an excessive response and the ability to dampen free radicals may therefore contribute to the complex interaction that helps to resolve the state of chronic inflammation typifying these wounds (Henriques *et al*., 2006).

Previous research explained that flavonoids in honey were a substance that acts as an antioxidant and is capable of capturing free radicals to help cell regeneration. These results indicate, decreased levels of free radicals in tissue wound healing process, especially, in the inflammatory phase and remodeling. Antioxidants can react through: (1) Cleaning compounds or a decrease in the concentration of reactive oxygen locally, (2) Cleaning of the catalytic metal ion, (3) Cleaning free radical which serves as an initiator such as hydroxyl, peroxyl and aloksil, (4) Termination of chain reaction sequence is initiated by free radicals and (5) Reduction reaction and cleaning singlet oxygen (Middleton *et al*., 2000). Antioxidants can inhibit lipid peroxidation

reactions through the mechanism (1), (2) and (4). The mechanism (3), serves as, an antioxidant deterrent but the mechanism is carried out by enzymes, such as; superoxide dismutase (SOD), glutathione peroxidase and catalase. Antioxidants reaction breaker, singlet oxygen absorbers and metallic binder can contribute to react in performing its function (Papas, 1999). Antioxidant effects of honey played by flavonoids. Honeys prevent the oxidation process of the cell membrane and prevent cell damage. Results of previous studies in the inflammatory phase of wound healing process, free radicals produced by inflammatory cells in addition act, as killing agent of the bacteria that invade the wound and play a role in intracellular signaling pathways as free radicals plus side having such a negative effect on certain concentration of ROS and H_2O_2, can inhibit the migration and proliferation of some cells, including keratinocytes cells (Kumar *et al*., 2009).

It was known that reduced levels of ROS in skin wound tissue of mice were characterized by reduced levels of SOD, catalase and GPx plays a role in wound healing of the wound repair process. The provision of Mn-SOD antioxidant preparations on chronic wound area of diabetic foot ulcers of mice can accelerate wound healing (Wlaschek and Scharffetter-Kochanek, 2005). The MDA free radicals were generated by wound with the formation of free radicals superoxide and formed hydroxyl radicals, which causes damage to the cell. Wound condition is indirectly explained that an increase in ROS in animal experiments.

Different act with rambutan honey antioxidant activity, dry products like gutkha and pan masala were known will increase, the ROS concentration in the oral cavity of chewers in India, as soon as the areca nut and catechu polyphenols together with slaked lime dissolve in the saliva, similar to the reaction observed *in vitro*. High copper and iron content in these products would further add to the load through their action as catalysts via the Haber-Weiss and Fenton reactions. This has been demonstrated in primary human oral keratinocytes, where pan masala induces superoxide radical production and lipid peroxidation and provides the evidence that gutkha and pan masala chewers may be at high risk for developing oral cancer because Reactive Oxygen Species (ROS), implicated in multistage carcinogenesis, are generated in substantial amounts in the oral cavity during chewing (Fareed *et al*., 2011).

It was clearly shown that there was a good mechanism of antioxidant activity of rambutan honey on wound healing *in vitro* and *in vivo*, which was expected, when this was applied, a standardized natural ingredients will help the healing process towards the regeneration of injured tissue not only repair that will have an impact on the outcome of an optimal wound healing.

CONCLUSION

Based on the results of this study concluded that rambutan honey has an antioxidant activity *in vitro* and *in vivo* studies whereas topical administration in the rat wistar strain oral mucosa wound prevents the formation of free radicals MDA tissue injuries. This was clear evidence that rambutan honey has a potential as a natural ingredient in the treatment of human oral mucosal wound healing, which were safe, nutritious and delicious. Further studies are needed to determine rambutan honey flavonoids components and other antioxidant components and whether it would also be able to apply directly in human oral mucosal wound tissues for optimal wound healing.

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