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Research Article
Effect of Rambutan-honey and its Flavonoid on TGF-β1 Induce Fibroplasia Oral Wound Healing

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Abstract

Background and Objective: The TGF-β1 is a multifunctional cytokine that contributes fibroplasia wound healing via mechanisms that involved free radicals. Honey has been succesfully used for treating wound healing. It's has an antioxidant, anti-inflammatory, antibacterial and debridement effects. The antioxidant substances such as flavonoid can reduce free radicals-malondialdehyde (MDA) on rats skin wound healing. However, the mechanisms of its action at stress oxidative oral mucosa wound healing have poorly understood. The purpose of this study are to investigate the effect of rambutan-honey topical induce fibroplasia wound healing by TGF-β1 inhibit MDA formation oral mucous rats wound healing and to identify the presence of flavonoid components responsible effect. **Materials and Methods:** The research methods were an experimental laboratory. The samples (n = 16 group^{-1}) were divided into negative control, positive control ϕ4±2 mm wound, 1 mL rambutan-honey wound and 0.1 mL ascorbic acid. Oral mucous wound were observed on day 0, 3, 7, 14. The production of TGF-β1 determined by ELISA reader. Bradford assay used for calculating protein concentration. The HPLC analysis was performed in order to identify rutin and myricetin on honey samples. The productions of free radicals were determined by MDA tissues level and fibroblast numbers that determined by histology examination and statistically analyzed (ANOVA, Tukey's, $p<0.05$). **Results:** The results showed that rambutan-honey induce fibroplasia significantly on day 0, 3 and 7 ($p = 0.001$) and TGF-β1 significantly on day 3 ($p = 0.008$) and inhibit MDA formation significantly on day 3 ($p = 0.028$) and day 14 ($p = 0.037$). Rutin was identified flavonoid in rambutan-honey, markedly induce fibroplasia TGF-β1 inhibit MDA formation. **Conclusion:** Rambutan-honey that contains rutin flavonoid potentially accelerating wound healing and prevents stress oxidative induces fibroplasia by TGF-β1inhibit MDA formation. Thus, our finding provide clear evidences that rambutan-honey topical have a potential as a natural treatment for human oral mucosa wound healing.

Key words: Wound healing, rambutan-honey, TGF-β1, MDA, oral mucosa

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Wound is a missing or partial destruction of body tissue often occur in the oral mucosa and when not treated immediately and properly will lead to chronic wounds that can degrade the quality of life[1,2]. Transforming Growth Factor β1 (TGF-β1) is a multifunctional cytokine that contributes fibroplasia wound healing via mechanisms that involved free radicals[3,4]. The TGF-β1 is found a lot in the early phases of wound healing[5]. The TGF-β1 stimulates the activity of NADPH oxidase which generates free radicals[6]. Malondialdehyde (MDA) is a marker for free radicals highly toxic to the cell membrane[7]. Free radicals can kill the bacteria that invade the wound and intracellular signaling pathways but can inhibit the migration and proliferation of cell[3]. One of the natural medicines that are often used in the treatment of wound healing is honey. Honey containing flavonoids act as antioxidants, anti-inflammatory, antibacterial and debridement. Rambutan-honey is honey produced from the nectar of rambutan flowers (*Nephelium lappaceum*). Rambutan-honeys are known in the community as a traditional medicine to accelerate wound healing including oral mucosa[8,9].

Many studies have supported that honey have antioxidant effect on cell and wound healing. Honey can be induced antiproliferative and apoptosis by oxidative stress, which is mediated by the generation of Reactive Oxygen Species (ROS) in tumor leukemia cell line (HL-60) as a model system according to the phenolic compound as antioxidant[10]. Effect of three type of Japanese honey on wound area in mice can reduces wound size in the inflammatory phase but some wound were not completely covered with new epithelium[11].

Research suggests that the polyphenol constituents of honey can quench biological reactive oxygen species and counter oxidative stress while restoring the cellular antioxidant defense system. The ultimate biochemical impact of honey on mitochondrial dysfunction, apoptosis, necrosis, excitotoxicity and neuro-inflammation should also be explored. Furthermore, exploration of the actual cell signaling cascades that are associated with synaptic plasticity may provide more specific therapeutic interventions using honey[12]. Honey is an important source of phenolic compounds and the amount and the nature of phenolic acids and avonoids is of great interest because they are responsible for a number of functional and nutraceutical properties typical of this natural food. Moreover, several and recent studies have also confirmed that the phenolic profile is strictly related to the botanical and sometimes the geographical origin of uni oral honeys. The bioactive properties of new uni oral honeys worldwide correlate with to a number of health-promoting features. While recent studies have frequently been accompanied by at least a minimal validation, the application of chemometric instruments for the optimization of procedures of obtaining and managing the analytical data still appears insufficient, although the trend is in sharp increasing in the last years[13].

However, the mechanisms of its action on TGF-β1 mucosa wound healing have poorly understood. This study aimed to analyze topical administration rambutan-honey mechanism in stimulating the proliferation of fibroblasts by TGF-β1 which inhibit the formation of MDA oral mucosal tissue in wound healing as well as to identify the components of rambutan-honey responsible for these effects.

MATERIALS AND METHODS

Rambutan-honey samples: Samples were taken from the Indonesia National Beekeeping Centre (Pusbahnas). Pure honey was isolated with sterile technique then the experimental steps were carried out (Fig. 1).

Experimental animals: Animal samples of 64 rats were taken from the Central Laboratory of Biological Sciences ITB. Rats (n = 16 group^{-1}) were divided into Negative Control (NC), Positive Control (PC) ϕ4±2 mm wound, 1 mL rambutan-honey wound (RH) and 0.1 mL Ascorbic Acid (AA). Oral mucosal wound (palatal regio) was observed on day 0, 3, 7, 14 and administered topically twice[14].

Identification of flavonoid compounds: Identification of flavonoid bioactive compounds used HPLC UV-vis with myricetin and rutin standard[15].

Bradford protein assay and TGF-β1 assay: The production of TGF-β1 determined by ELISA reader at λ 450 nm and the Bradford assay used for calculating protein concentration[16].

			Sacrifice (days)				
Group (n =16)	RH	AA	0	3	7	14	Assay
Negative control	-	-	†	†	†	†	Wound healing
Positive control	-	-	†	†	†	†	Fibroblast cell
Rambutan-honey	√	-	†	†	†	†	TGF-ß1
Ascorbic acid	-	√	†	†	†	†	MDA

Fig. 1: Outline of experimental design

Fibroblast cells amount examination: Histology section stained with hematoxylin-eosin were made to examine fibroblast cell amount and counted in three fields of view using 40× objective expansion with light microscopy.

MDA assay: The productions of free radicals were determined by MDA tissues level with TBARs routine procedure perform absorbance readings at λ 532 nm.

Statistical analysis: 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. The $p<0.05$ were considered to be significantly different.

Research ethics aspects: Ethical approval was obtained from the Research Ethics Committee Hasan Sadikin Hospital (RSHS) Bandung with No. 140/UN6.C1.3.2/IEC/PN/2015. Ethical approval based on the principle of the '3R' American Veterinary Medical Association[17].

RESULTS

Analysis of antioxidant flavonoids rambutan-honey compounds: Based on the HPLC when rambutan-honey added flavonoids rutin standard it can be seen an increase in quantity peak retention times. Based on the results of the calculation pattern chromatogram peak area of 2.47 (rambutan-honey) from 2.226.762 increased to 2.961.557 (rambutan-honey plus rutin), so that there was an increase of 33% chromatogram area. Chromatograms area of rutin, rambutan-honey, rambutan-honey plus rutin can be seen in Fig. 2. It was clear that in rambutan-honey flavonoid compounds have been identified rutin.

Effect of rambutan-honey on TGF-β1 Wistar rat blood plasma levels: The rambutan-honey effect on TGF-β1 rat blood plasma levels significantly at day 3, $p = 0.008$, $p<0.005$. The TGF-β1 levels mean 103.13±22.38 pg mL^{-1} (NC), 123.04±13.96 pg mL^{-1} (PC), 218.03±42.27 pg mL^{-1} (RH) and 119.44±65.62 pg mL^{-1} (AA), respectively (Fig. 3).

Effect of rambutan-honeyon fibroblast cells amount: The rambutan-honey effect on fibroblasts cells amount significantly at days 0, 3rd and 7th, $p = 0.001$, $p<0.005$. Fibroblas cell amount per high power fields mean 101.75±8.99 (NC), 69±12.65 (PC), 83.25±25.83 (RH) and 86.5±5.84 (AA), respectively (Fig. 4). In histology examination on day 0 (Fig. 5), fibroblast cell in seen on normal connective tissue. Fibroblasts amount in the RH group and AA group were fewer than the PC group. Inflammatory cell were seen in each group. On day 3, fibroblast amount in rambutan-honey were less than other group and the inflammatory cell were seen each group, especially more common in PC group. On day 7, fibroblast amount and endothelial cells in rambutan-honey were higher than other group. On day 14, fibroblasts amount are less seen in rambutan-honey and new connective tissue formation more than other group, inflammatory cells were still prominent on the other group (Fig. 5).

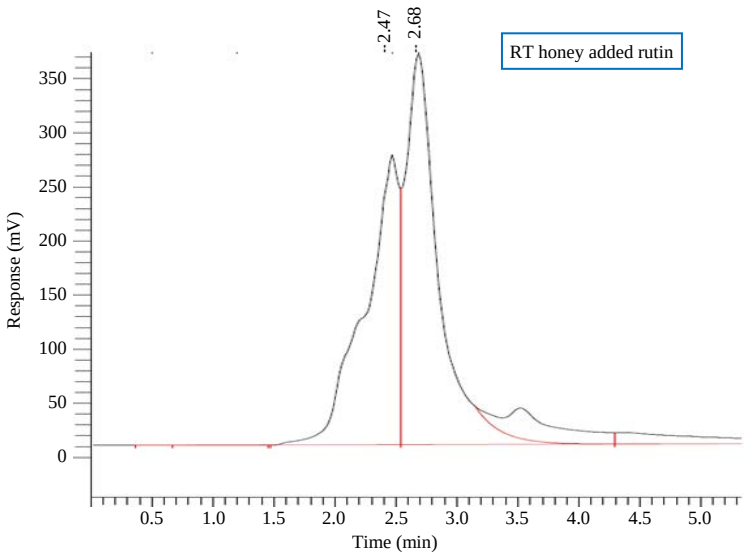

Fig. 2: Chromatogram of flavonoid rutin content using HPLC-UV absorption at λ 340 nm

Inhibitory effect of rambutan-honey on MDA oral mucosal tissues levels: Based on Kruskal Wallis test rambutan-honey effect on MDA tissues levels were significant at day 3, $p = 0.028$ and at day 14, $p = 0.0373$, $p<0.005$ 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} (RH) and 31.22±6.37 μg mL^{-1} (AA), respectively (Fig. 6).

DISCUSSION

Based on HPLC technique, peak that appears was related to flavonoid rutin[15]. Rambutan-honey with rutin flavonoids act as antioxidants that could inhibit free radicals (MDA) in oxidative stress oral mucosa wound healing[18].

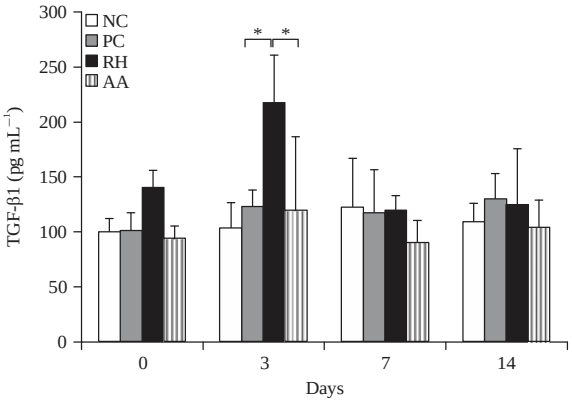

Fig. 3: Effect of rambutan-honey on TGF-β1 compared with negative control, positive control and ascorbic acid. Kruskal Wallis test, *p<0.05 significantly different, *Post hoc* Tukey, *p<0.05 significantly, NC: Negative control, PC: Positive control, RH: Rambutan honey, AA: Ascorbic acid

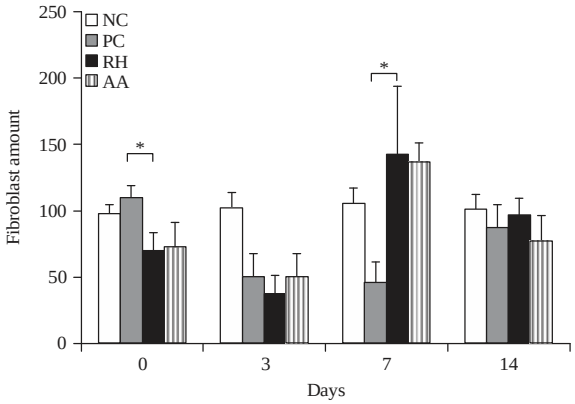

Fig. 4: Effect of rambutan-honeyon fibroblast amount compared with negative control, positive control and ascorbic acid. Kruskal Wallis test, *p<0.05 significantly different, *Post hoc* Tukey, *p<0.05 significantly, NC: Negative control, PC: Positive control, RH: Rambutan honey, AA: Ascorbic acid

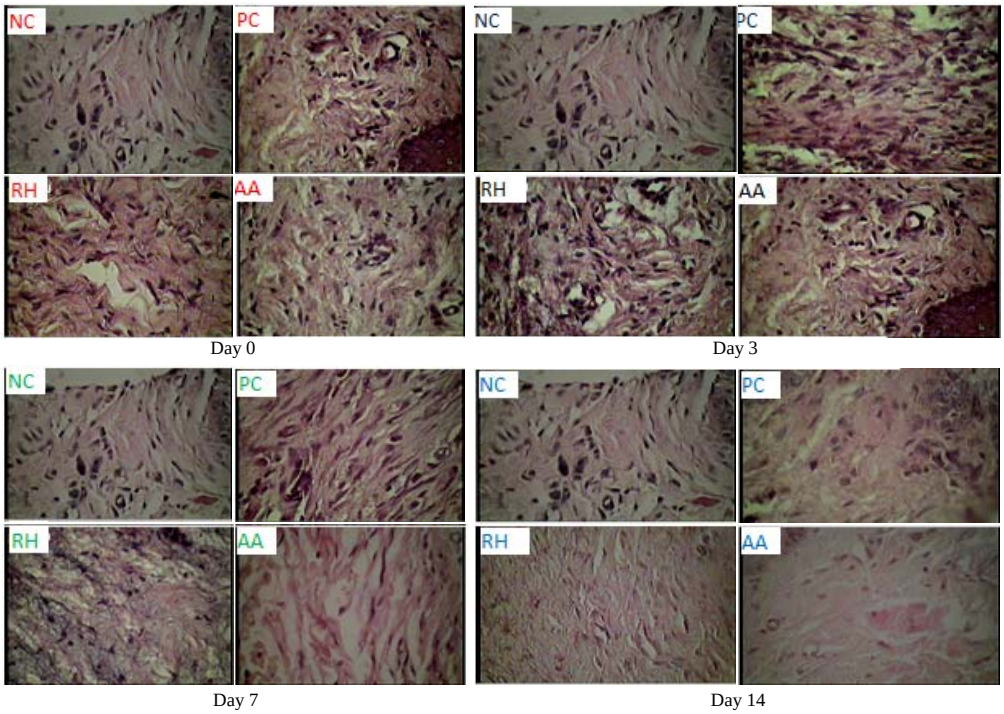

Fig. 5: Histology section stained with hematoxylin-eosin of wound area using 40× objective expansion with light microscopy, NC: Negative control, PC: Positive control, RH: Rambutan honey, AA: Ascorbic acid

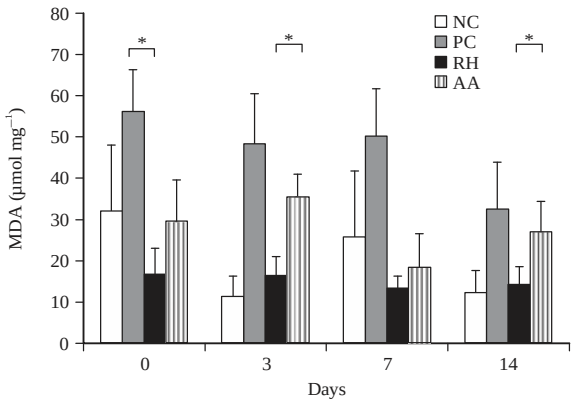

Fig. 6: Effect of rambutan honeyon MDA level compared with negative control, positive control and ascorbic acid. Kruskal Wallis test, *p<0.05 significantly different, *Post hoc* Tukey, *p<0.05 significantly, Mann-Whitney test, *p<0.05 significantly, NC: Negative control, PC: Positive control, RH: Rambutan honey, AA: Ascorbic acid

Flavonoids consist of compounds flavonols, flavanones, anthocyanidins and isoflavones. Honey produced monofloral plants known to contain flavonoids flavonols quercetin, rutin and mirycetin. In this study identified classes of compounds flavonols rutin.

Identification HPLC techniques class of compounds consisting of two techniques; first, the comparison of retention times/RT patterns between standard chromatography flavonoids with honey. Second, the additions of flavonoid rutin standard to rambutan-honey sample. The result showed that standard peak retention time increased, it was clear that the sample of the honey contains flavonoids rutin[19]. The results showed that was not detected mirycetin in rambutan-honey. Rambutan-honey was natural materials that have the potential act as an antioxidant and antibacterial compounds that contain flavonoids rutin. Rutin was a flavonoid compounds flavonols. Flavonoids were antioxidants that can reduce free radicals (MDA) and flavonoids also known as vitamin C extender activity through increased absorption and protection from oxidative stress as well as through the replacement of some biological function. Rutin was a secondary metabolite bioflavonoid natural material and known as glycosylated flavonoids, including flavonols subgroups. In this study, possible sources of rutin honey was not only derived from the rambutan flowers nectar but could derived from *Apis mellifera* bees wax. Rutin compound in rambutan-honey has glycosylated because of temperature and age. These results were consistent with study of Pimentel *et al*.[19] that identified high levels of rutin in Brazilian honey. Rutin compound in honeys have meaningful relationships with antimicrobial activity.

Rutin flavonoid molecules (rutosid, soforin or quercetin-3-rutinosid) have antibacterial, anti-inflammatory, allergy, antithrombotic and many biological effects. Epidemiologically studies indicated that flavonoids have a role in the prevention of cardiovascular disease and cancer. High doses flavonoids have interesting pharmacological effects to biological systems in experimental animals. A study reported that the antioxidant effect of flavonoid intake *in vivo* in mice can reduce lipid peroxidation[20].

Flavonoids also act as hydroxyl free radical scavengers (pyrogallol) in ring B flavonoid structure. Reactive Oxygen Species (ROS) produced in the process of phagocytosis wound healing can damage cells. Reactive oxygen species metabolites generated by neutrophils and macrophages can cause an inflammatory reaction that ends in cell damage. Flavonoids were known to inhibit the release of ROS in cells neutrophils, inhibit the enzymatic and non-enzymatic reaction of lipid peroxidation and actively inhibit free radicals in the initiation and termination stage. Flavonoids may inhibit the auto-oxidation reaction routine lipid peroxidation in the termination stage. Flavonoid rutin (quercetin-3-rutinosid) can regenerate ascorbic acid in the body, stimulates the formation of collagen and had a good antimicrobial activity[20,21].

Fibroblast cell amount can be a marker on wound healing that required connective tissues. Fibroplasia found in the rambutan-honey and ascorbic acid group at day 7 higher than other groups and it proved that rambutan-honey had potential to stimulate fibroplasia. Newly formed fibroblast by activation of growth factor such as TGF-β1 was induced by rambutan-honey. Effect of rambutan-honey on TGF-β1 blood plasma levels was found slightly higher than other groups on the inflammatory phase. This result supports the effect of rambutan-honey to fibroplasia significant on day 3. Recent studies showed that TGF-β1 serves as a factor that stimulates fibroplasia and have strong anti-inflammatory effect through inflammatory cells signal around the wound area[3]. The TGF-β1 stimulates the activity of NADPH oxidase which produces free radicals[5,6]. Effect of rambutan-honey was significant in suppressing free radical resulted from wound healing process especially in inflammation and proliferation phase, so that it can comprehend oxidative stress condition.

In this study, effect of rambutan-honey against one of the growth factors that play a role in wound healing, namely TGF-β1 because this cytokine was a hormone that cellular and humoral important for wound healing including fibroplasia in forming collagen tissues. The formation of fibroblasts during injury and inflammatory phase increases because of the TGF-β1 produced by macrophages, platelets and fibroblasts

alone[22]. The results explain that TGF-β1 was found a lot in the early phases of wound healing[5]. Also interesting, TGF-β1 has two roles opposite for the wound healing process depends on the concentration of the resulting molecules. Roles associated with signaling processes in NADPH oxidase activity that generates free radicals. Free radicals at low concentrations were signaling messenger to attract leucocytes, chemokines and cytokines in the inflammatory phase that plays important to the wound healing process. Free radicals at high concentrations can cause cell damage that inhibits wound healing process[6]. In accordance with Shumin Li in 2007 study which explained that the levels of TGF-β1 1 ng mL^{-1} have the effect of prooxidant-producing ROS 90% in myocardial cells of mice causes dysfunction of the heart muscle contraction that inhibition of this hormone will prevent the occurrence of fibrosis myocardial remodelling phase. At the right levels and the right time were the initial phases of the healing effects of TGF-β1 expected to accelerate wound healing by stimulating the fibroplasia process[23].

In this study found that rambutan-honey stimulate TGF-β1 levels in rat blood plasma on the 3rd day of the inflammatory phase ($p = 0.008$), which supports the results of the rambutan-honey to the number of fibroblasts significantly on the 3rd day. Clinical observations found that the area of the wound at day 3 has begun to decline/shrink and at the 7th day began to close due to the proliferation of fibroblasts stimulated by TGF-β1 in early wound healing. This was consistent with the theory and study results of Penn *et al.*[24] that TGF-β1 levels in plasma high in the early phase of wound healing to the fibroblasts proliferation in the proliferative phase. The TGF-β1 levels in the blood plasma of proliferative phase and remodelling was not increased and it can be seen that in the group with rambutan-honey wound closes completely without scarring and granulation. At the end of the healing phase, the levels of TGF-β1 which will lead to increased fibroplasia which can affect scar tissue hypertrophy[24]. Wang *et al.*[25] suggested that TGF-β1 in addition to having a positive role in wound healing and anti-inflammatory but the keratinocyte cell skin wounds can be pro-inflammatory so the effects on skin that has injury the TGF-β1 persistent unfavorable for the wound healing process.

Some study explains that the TGF-β1 and TGF-β2 can be found a lot in the early wound healing phases, while TGF-β3 can be found at the end wound healing phases[5]. Proliferation of cells in wound healing and epithelialization were mediated by the activity of TGF-β1 expressed by the epidermis, in addition to other growth factors such as PDGF, as well as cytokines, especially IL-1 and TNF-α[3]. From various studies in animals and clinical trials were known that TGF- β1 plays an important role in the development of inflammatory diseases and fibrosis. The TGF-β1 generated by macrophages in the inflammatory process of wound healing. In experiments using cell cultures, TGF-β1 was known to have inhibitory effects on the growth of endothelial cells, epithelial cells and lymphocytes[6].

The TGF-β1 serves as a factor that stimulates the fibroblasts and has strong anti-inflammatory effects such as through signal for inflammatory cells function in the injured area[3].

The NADPH oxidase activity that generates free radicals stimulated by TGF-β1 in addition to the body's cells have a primary antioxidant form of the enzyme superoxide dismutase and glutathione in reducing free radicals of NADPH oxidase activity to prevent conditions of oxidative stress and cell damage[5,6]. The role of rambutan-honey significantly reduce free radicals that produced in the wound healing process, especially in the inflammatory phase ($p = 0.028$) and the proliferative phase ($p = 0.037$), so that it can cope with conditions of oxidative stress from inflammatory cells and the results of stimulation of TGF-β1 because free radicals. The TGF-β1 signaling acts as a messenger to attract leukocytes, chemokines and cytokines in the inflammatory phase of healing response while free radicals biosinyal results can cause damage to cells, inactivate antiproteases and damage the surrounding cells that can inhibit wound healing process. Application of rambutan-honey in humoral molecular TGF-β1 was responsible for signaling the result of direct oxidation by protein kinases can stimulate fibroplasia especially in the inflammatory phase but reduce free radicals (MDA) so it was very clear that there mechanisms of cellular and humoral rambutan-honey against wound healing that expected when standardized natural material applied will help the healing process in the direction of the wound tissue regeneration not only reparations that will have an impact on the results of the optimal wound healing without scarring.

The main targets of oral mucosal wound treatment were to restore the function and form of oral mucosal tissue back to normal with minimum local complications. These results showed the effect of topical rambutan-honey and its flavonoid rutin to accelarete oral mucosa wound healing on days 0, 3rd and 7th. In the event of injury, the tissues will experience the healing process was a complex phenomenon and involves multiple cellular and humoral process.

Therefore, we assume that rambutan-honey can act as an antioxidant with both inhibitory and stimulatory properties which stimulate the production of TGF-β1 cytokines induce fibroplasia and suppress MDA free radicals formation.

CONCLUSION

The rambutan-honey effects on TGF-β1 and MDA on different wound-healing phases, including inflammation, tissue proliferation and tissue remodeling. These studies will provide unique insights into a better understanding of phases and antioxidant effects of rambutan honey on TGF-β1 in oral mucosa wound healing, which will facilitate the development of new therapies for impaired wound healing through modulating TGF-β1 elicited signaling and inhibit MDA formation.

The flavonoid rutin was identified by HPLC. This is the first study of the presence of this flavonoid in rambutan-honey produced in Indonesia. However several peaks were observed in the chromatogram regions considered to be associated other flavonoid compounds.

Based on the results of this study it was concluded that rambutan-honey and its flavonoid (rutin) compounds have the ability to counteract destructive cell damage (MDA) and re-establish normal wound healing induce fibroplasia by TGF-β1. Thus our finding provides clear evidences that rambutan-honey topical have a potential as a natural treatment for oral mucosa regeneration wound healing. Further studies are needed to determine whether rambutan-honey would also be able to apply directly in oral mucosal human wound environment for optimal wound healing.

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