RESEARCH ARTICLE

The effect of papaya leaf extract (*Carica papaya L*) on healing process of buccal traumatic ulcer in wistar rats

Afryla Femilian*,, Dewi Agustina***, Goeno Subagyo***

*Department of Oral Medicine, Dental school, Faculty of Medicine and Health Science, Universitas Muhammadiyah Yogyakarta, Yogyakarta, Indonesia
**Master of Clinical Dental Sciences, Faculty of Dentistry, Universitas Gadjah Mada, Yogyakarta, Indonesia
***Department of Oral Medicine, Faculty of Dentistry, Universitas Gadjah Mada, Yogyakarta, Indonesia
*Jl Lingkar Selatan, Tamantirto, Kasihan, Bantul, Yogyakarta, Indonesia; e-mail: lafryla@yahoo.com

ABSTRACT

Indonesians have been using herbal medicines for a long time to cure some illnesses. *Carica papaya L* is an example of an herb that contains papain enzymes, saponins, lysozymes, lipases, flavonoids, polyphenols and vitamin C. These ingredients are believed to be beneficial for the wound healing process. The purpose of this study was to determine the effect of topical application of ethanolic extract of *Carica papaya L* to the healing process of rat mouth ulcer. Subjects were 32 Wistar rats divided into 2 groups (control group and treatment group), each group containing 16 rats. All subjects were made to suffer from ulcers using glacial acetyl acid applied for 40 seconds in the buccal mucosa. Treatment group was treated with papaya leaf ethanolic extract on ulcers twice a day using microbrush, while the ulcer in the control group was not treated with the extract. The ulcerated tissue was biopsied and stained with H&E. Observations were performed on the day 0, 3rd, 7th and 12th on HE slides. Data were observed by looking at three indicators of wound healing i.e. macrophage, angiogenesis and re-epithelisation. Number of macrophages and angiogenesis were analyzed using two-way ANOVA. Data of epithelial thickness were analyzed using Kruskal-Wallis test. The post hoc test in the treatment group and the control group on day 0 showed results of p = 1.00. On day 3, day 7 and day 12, the result of p was <0.05. In the treatment group on day 0 compared to the treatment group on the 3rd, 7th, 12th day, the results were p<0.05. In the treatment group on the 3rd day compared to the 7th and 12th days the results were p>0.05. The treatment group on the 7th day compared to the treatment group on the 12th day had p>0.05. In the control group, on day 0 compared to day 3, the results of p>0.05, while the control group day 0 with day 7, 12 had a result of p<0.05. The control group on the 3rd day was compared with the 7th day. The 12th also had a result of p<0.05. The comparison between the 7th and 12th day control groups showed p of >0.05. These data suggest that the papaya leaf ethanolic extract could accelerate the healing of oral ulcer on the buccal mucosa of wistar rats.

Keywords: angiogenesis; macrophage; oral ulcer; papaya leaf ethanolic extract; re-epithelisation

INTRODUCTION

One type of medicinal plant that is often used in traditional medicine is papaya (*Carica papaya L*). Papaya plant is one of the plants that almost all its parts are useful. One part of papaya plants that has many benefits is the leaf. Papaya leaf contain papain enzymes, saponins, lysozymes, lipases, flavonoids and vitamin C that are believed to be beneficial to the wound healing process, including in oral ulcers.[1]

Papain enzyme is an endoprotein similar to the enzyme pepsin in humans that are anti-bacterial, anti-inflammatory and eliminate debris function. Topically proven papain proved to be useful as wound debridement, anti-inflammatory and anti-edema.[2] Papain enzymes have anti-inflammatory and analgesic effects by neutralizing inflammatory mediators such as quinine and prostaglandins that inhibit directly on pain receptors.[3]

Papaya leaf contain a variety of nutrients including vitamin A, vitamin B1, and vitamin C. In 100 grams of fresh papaya leaf, there are 140 mg of vitamin C. Vitamin C can form as L-dehydroaskorbat acid, both of which are active substances of vitamin

C. Vitamin C is included in water-soluble vitamins, and it has an important effect in the formation of collagen, an important component of forming connective tissue in the body. Adequate collagen synthesis is necessary for strong ligaments, tendons, skin dentine, blood vessels, and bones, and for wound healing processes. Vitamin C can protect phagocytic activity from auto-oxidation, increase the production of interleukin-1 and TNF-α, and increase phagocytosis of NK cells and macrophage cells. In addition, vitamin C also inhibits tissue damage by inhibiting excessive production of reactive oxygen speciesi (ROS).[4]

Saponin stimulates the activity of TGF-β that is capable of affecting the perisite that governs the proliferation, migration and differentiation of cells.[5] The presence of endothelial cell activation, platelets, macrophages, fibroblasts, and local cytokine release will trigger endothelial cells to enter and migrate to the extracellular matrix, which then proliferates and forms new young tubules. Saponins can also trigger the formation of collagen that plays a role in the wound healing process.[6]

Mucosal ulcers are the most commonly found mouth disease. Ulcers in the mucous membranes are common due to trauma. Oral cavity ulcers can cause a variety of complaints in patients, which can affect mouth activity ranging from pain complaints, mastication, ingestion and affect the quality of life of patients so that the process of rapid healing of mouth ulcers is needed. The process of wound healing normally follows a certain pattern, which is divided into several overlapping phases, characterized by cellular, vascular and biochemical changes. These phases include hemostasis and inflammation, proliferation, and maturation or remodeling. This phase runs from wound to healing. All types of wounds must pass through these phases in order to create the restoration of scar tissue integrity as expected.[7]

The aims of this study is to examine the effect of topical application of papaya leaf ethanolic extract (Carica papaya L) to the process of healing mouth ulcers in mice. The healing process was observed with three indicators namely macrophages, angiogenesis and re-epithelization.

MATERIALS AND METHODS

This study was an experimental study with randomized control group. The independent variable was papaya (Carica papaya L) leaf ethanolic extract. The dependent variables was the number of macrophages, while angiogenesis was expressed as the number of blood cell and re-epithelialization thickness.

The papaya leaves were dried and mashed with a grinding machine. Papaya leaf powder of 100 grams was poured in a dark colored container, added with 70% ethanol of 750 ml, and was stirred until homogeneous. It was closed immediately and then stored in a room, which was protected from sunlight for 5 days and was often shaken. The immersion was filtered with a flannel cloth, and the pulp was washed with solvent to a volume of 750 ml. The results were concentrated with a vacuum evaporator until thick extracts were obtained.

Subject of this study were 32 Wistar rats divided into 2 groups (control and treatment group). The 16 rats were included in each group. All subjects were made to suffer from ulcers using glacial acetyl acids applied for 40 seconds in the buccal mice of Wistar rats using 4 mm diameter whatman paper, which had been soaked for 1 minute. Treatment group was applied with papaya leaf ethanolic extract on ulcers twice a day using microbrush, at 8 am and 4 pm, while the ulcer in the control group was not applied with the extract. The ulcerated tissue was biopsied and stained with HE. Observations were performed on the day 0, 3rd, 7th and 12th at three indicators of wound healing (macrophages, number of blood cell and re-epithelialization thickness).

RESULTS

This research has been declared ethically feasible by the ethics and advocacy unit of the Faculty of Dentistry, Universitas Gadjah Mada. Ethical Clearance Number 00848/KKEP/FKG.UGM/EC/2016.

The study of the average study number of macrophages can be seen in Table 1 and Figure 1. From Table 1, it is apparent that there are differences in mean number of macrophages in the control group and the treatment group. The highest

average number of macrophages is on the 3rd day in the control group as well as in the treatment group, but it decreased there after.

The normality test of Shapiro Wilk obtained the p value of >0.05, which indicated that the distributed data are normal. Analized homogenity by using Levene Test obtained p value of >0.05. There is no difference of variance between two groups or variant of homogeneous data. Data were qualified for normality and homogeneity and thus can be followed by two-way ANOVA test.

In the independent group, there is a significant difference between the number of macrophages in the control group and the treatment group. In the day variable, p=0.00 (p<0.05). It means that there is a significant difference between the number of macrophages between observation days, such

as day 0, 3rd, 7th, and 12th. Interaction of group variables with observation day obtained p value of 0.039, which means there is an interaction of group variable with day of observation to macrophage or there are differences of macrophage number in various treatment groups from time to time.

The results of the study on the average number of new blood cell is presented in Table 3 and Tigure 2. The average number of blood vessels between the control group and the treatment group over time has a difference. In the treatment group (Table 3), there was an increase of the mean number from day 0, 3rd, 7th, 12th and peak on the day 12th.

Normality test data in this research using Shapiro Wilk obtained p value of >0.05, which indicated normally distributed data. Homogenity test using Levene Test obtained p value of >0.05, meaning

Table 1. Mean and standard deviation number of macrophages in the treatment group and control group

Group	Day 0		Day 3rd		Day 7th		Day 12th	
	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation
Treatment	9.83	1.72	13.33	2.16	11.50	1.05	9.83	1.47
Control	9.67	1.97	18.83	3.92	15.50	3.45	12.83	1.72
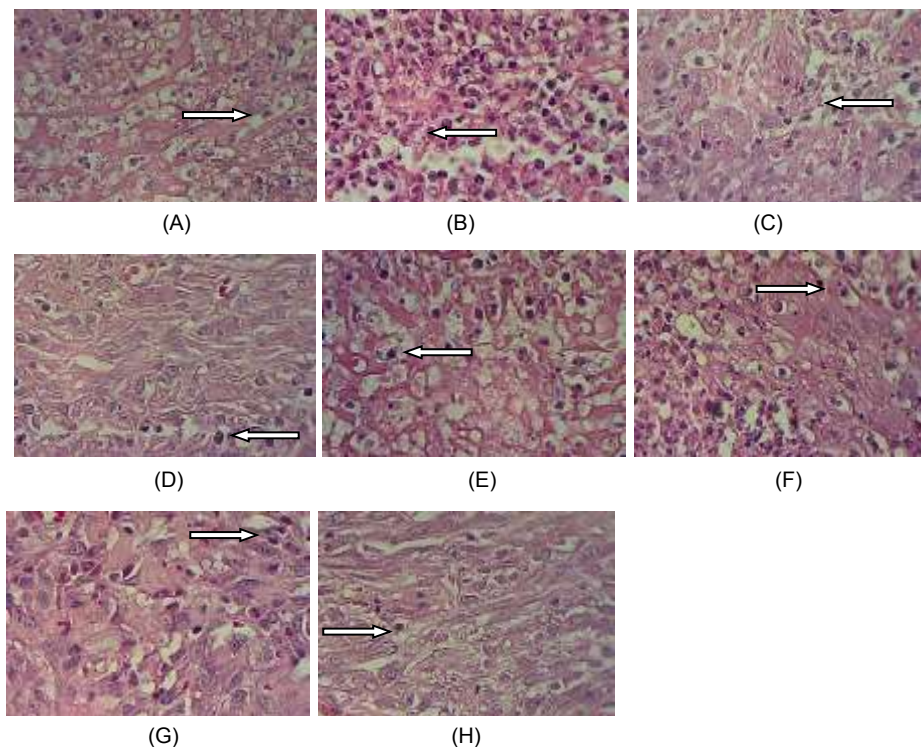
Figure 1. Macrophage histological picture (white arrowhead) on: (A) day 0 treatment group (± 9.83); (B) day 3rd treatment group (± 13.33); (C) day 7th treatment group (± 11.50); (D) day 12nd of the treatment group (± 9.83); (E) day 0 control group (± 9.67); (F) day 3rd of the control group (± 18.83); (G) day 7th of the control group (± 15.50); (H) day 12nd of the control group (± 12.83)

that there is no difference of variance between two groups or variant of homogeneous data. Data were qualified for normality and homogenity and thus can be followed by two-way ANOVA test.

Two ways ANOVA in Table 4 show. The group variables obtained p value of 0.00 (p<0.05), which means that there is a significant difference between the number of blood vessels in the control group and the treatment group. In the day variable, p value was 0.000 (p<0.05), meaning that there was a significant difference between the number of blood vessels between observation days, namely day 0, 3rd, 7th, 12th. Interaction of group variable with observation day obtained p value of 0.10 (p>0.05), meaning that there was no interaction of group variable with observation day to blood vessel or there was no significant difference in the number of blood vessels in various treatment groups from time to time.

The results of the research on the average number of epithelium are presented in Table 5 and Figure 3. There is an increase in the average number of epithelium on the 7th and 12th days both in the control group and the treatment group (Table 5). The standard deviations from each group on the 7th and 12th days show a high value. It is possible that the highest mean value has a very large difference to the lowest average value of each day with the possibility of abnormally distributed data.

The Shapiro Wilk data normality test shows p>0.05, which means the data have abnormal distribution. Because the data distribution is not normal, the next data analysis using non-parametric test is Kruskal-Wallis test. The Kruskal-Wallis test showed significance value of 0.00 (p<0.05) indicating that there was a significant difference between the two control groups and the treatment on the calculation of epithelial thickness.

Table 2. Two-way analysis of variance (ANOVA) of macrophages number

Variable	F	p
Group	20.281	0.000
Days	16.109	0.000
Group *days	3.067	0.039

Table 3. Mean and standard deviation number of blood vessel in the treatment group and control group

Group	Day 0		Day 3rd		Day 7th		Day 12th	
	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation
Treatment	1.67	0.81	4.33	1.21	5.83	1.47	7.67	1.03
Control	1.67	0.81	2.16	0.75	4.16	1.32	5.50	1.76
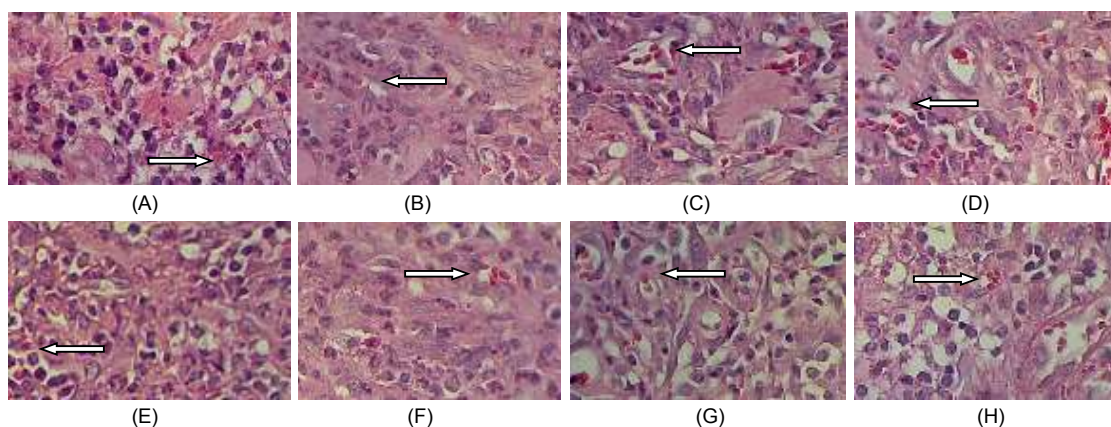
Figure 2. Histological description number of blood vessel (white arrowhead) on: (A) day 0 of the treatment group (± 1.67); (B) day 3rd of the treatment group (± 4.33); (C) day 7th of the treatment group (± 5.83); (D) day 12nd of treatment groups (± 7.67); (E) day 0 of the control group (± 1.67); (F) day 3rd of the control group (± 2.16); (G) day 7th of the control group (± 4.16); (H) day 12nd of the control group (± 5.50)

Table 4. Two Way ANOVA test of blood vessel

| Variable	F	p
Group	18.837	0.00
Days	38.004	0.00
Group * Days	2.209	0.10

Table 5. Mean and SD number of re-epithelisation in the control group and treatment group

Group	Day 7th		Day 12th	
	Mean	Standard deviation	Mean	Standard deviation
Treatment	46.83	10.88	174.11	29.35
Control	26.83	9.94	102.73	28.93

Table 6. Kruskal wallis test of re-epithelisation

	Epitel
Chi-square	46.300
df	7
Asymp. sig.	.000
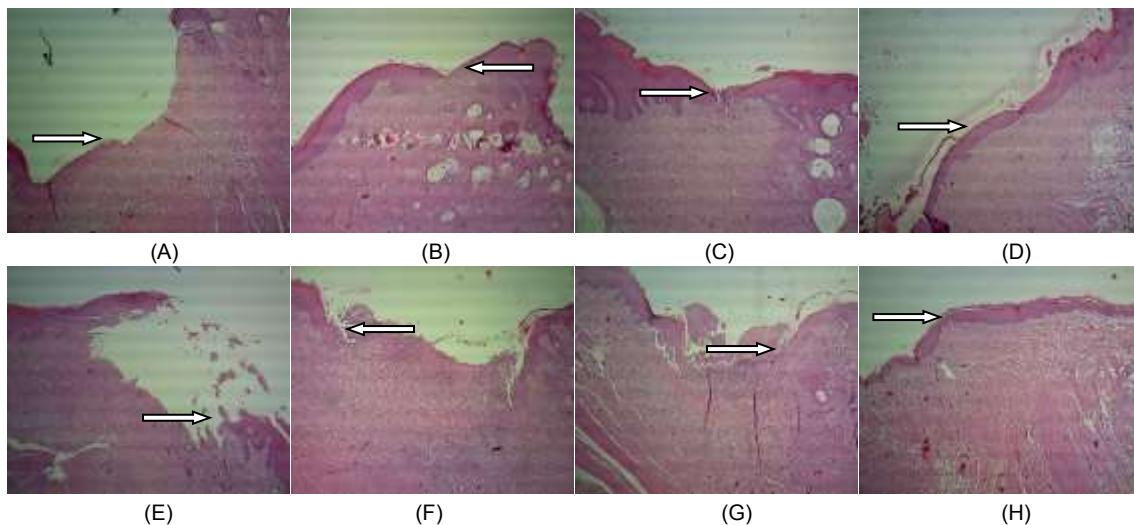
Figure 3. Histology of re-epithelialization (white arrowhead) at: (A) day 0 of the treatment group; (B) day 3 of the treatment group; (C) The 7th day of the treatment group (± 46.83 µm); (D) day 12nd of the treatment group (± 174.11 µm); (E) day 0 of the control group; (F) day 3rd of the control group; (G) day 7th of the control group (± 26.83 µm); (H) day 3rd of the control group (± 102.73 µm)

DISCUSSION

The results correspond to the theory that after wounding, neutrophils will migrate to the wound area during the first 24 hours of functioning to phagocytosis foreign bodies and bacteria, and it will be replaced by macrophage cells on day two or three that have a greater role in wound healing. Macrophages also act as antigen presenting cells during the wound healing process by synthesizing various growth factors. Macrophages that are affected by various mediators are found in the microenvironment around the wound, causing macrophage cells to undergo a change in properties according to need at the wound site. The highest peak of macrophage increase occurred on the day 5th.[8]

Angiogenesis is stimulated by angiogenic growth factors such as TGF-β and VEGF. This growth factor binds to the receptor on the surface of the endothelium. The activated endothelial cells

then proliferate and grow outward through the basal membrane to form the capillary buds that will become new blood vessels. Growth factors and chemical mediators are released by platelets and epidermal cells. Re-epithelization occurring in the proliferative and cytokine phases involved are EGF and TGF-α produced by platelets and keratinocytes. Because this process has a high metabolic activity, there will be an increase in the need for oxygen and nutrients. The need for increased oxygen and nutrients results in an increase in the formation of new blood vessels or angiogenesis. Papaya leaf is one of the plants that have antibacterial and anti-inflammatory effect. These effects are resulted from the active ingredients, namely flavonoids and papain enzymes. Papain enzyme is one of the most potent anti-inflammatory agents. This papain enzyme will work with vitamins A, C and E to prevent inflammation.[9] In addition, this papain enzyme is a proteolytic enzyme that has the ability to break protein bonds into arginine. Arginine will increase the activity of macrophage phagocytosis by producing nitric oxide (NO), which will act as a toxic mediator and is in charge of eliminating bacteria.

Flavonoids are active ingredients that have anti-inflammatory effects.[10] Flavonoids can block the pathway of cyclooxygenase and lipooksigenase from arachidonic acid metabolism, leading to the synthesis of inflammatory mediators such as prostagladin, thromboxane inhibited to decrease inflammation.[11] The content of saponins in papaya leaf will stimulate the activity of TGF-β, which is capable of affecting the pericite of a kind of smooth muscle attached outside the capillary vessel that regulates proliferation, migration and cell differentiation. The presence of activation of endothelial cells, platelets, fibroblasts, and cytokine release locally will trigger endothelial cells to enter and migrate to the extracellular matrix, which then proliferates and forms new young tubules. Saponins can also trigger the formation of collagen that plays a role in the wound healing process. In addition to papain and flavonoid enzymes, papaya leaf also contain vitamin C. The role of vitamin C (ascorbic acid) is very important in collagen synthesis; the absence of vitamin C will cause interference in prokolagen and decrease collagen synthesis by connective tissue.[12]

Ulcer healing process consists of three phases of the inflammatory phase, proliferation phase and remodeling phase. Macrophages begin to appear in the inflammatory phase, peaking at day 5th and decreasing thereafter. Angiogenesis and re-epithelialization marked the occurrence of a proliferative phase that began on 3 days after the injury. Macrophages, angiogenesis, and re-epithelization are sequences of events that overlap and cannot be separated in the healing process. The healing process seen from the three indicators can be presented in graphical form as in Figure 4.

Macrophages act as antigen and phagocytic presenting cells during the wound healing process. They are also considered to have a role in the healing process through the synthesis of various growth factors. Macrophages that are affected by various
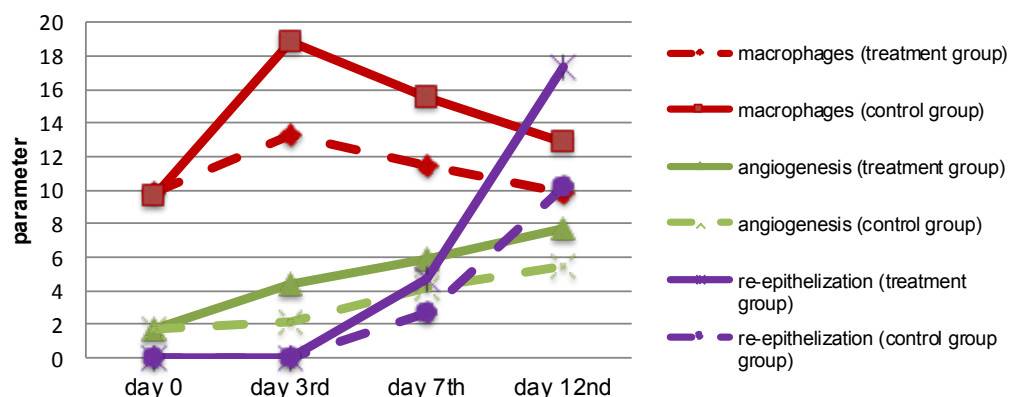

Figure 4. Graph of the healing process on three parameters

mediators are found in the microenvironment around the wound, causing macrophage cells to undergo character changes according to the need at the wound site. When the need for macrophages at the wound site decreases, the number of macrophages will also decrease.

Angiogenesis is stimulated by angiogenic growth factors such as TGF-β and VEGF. This growth factor binds to the receptor on the surface of the endothelium. The activated endothelial cells then proliferate and grow outward through the basal membrane to form the capillary buds that will become new blood vessels. Growth factors and chemical mediators are released by platelets and epidermal cells.

Re-epithelization occurring in the proliferative and cytokine phases involved are EGF and TGF-α produced by platelets and keratinocytes. Because this process has a high metabolic activity, there will be an increase in the need for oxygen and nutrients. The need for increased oxygen and nutrients results in an increase in the formation of new blood vessels or angiogenesis that is primarily affected by VEGF, bFGF and TGF-β.

Some obstacles found during the experiment was the researcher`s inability to control the rat`s habit to lick the ulcer area after application of extracts. Rat habits to lick the area of the ulcer can cause the extract no longer be attached to the ulcer, which needs to be anticipated. Another obstacle was the calculation of angiogenesis, only by blood vessels containing erythrocytes can be read and not all blood vessels contain erythrocytes. Staining preparations only use HE routine staining making it difficult to clearly observe endothelial cells. It is considerable to use immunohistochemical staining to provide clearer information about the content of various molecular elements in the cell.

CONCLUSION

The topical application of papaya leaf ethanolic extract can significantly accelerate the healing process of oral ulcers in Wistar rats. This conclusion is seen from three indicators of healing: macrophage cells, angiogenesis, and re-epithelization.

Subsequent studies should consider the addition of observation days to enable the observation of the entire healing process. It is also suggested that the papaya leaf ethanolic extract is made in gel in orabase preparations for easier attachment and insolubility in saliva. Further research is needed related to toxic effects of papaya leaf.

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