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The Effect of the Extracts of Betel (*Piper betel)* Leaves on the Content of IL-1β as the Inflammatory Cytokine in Experimental Mice Using an Acute Wound Healing Modeling

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Abstract

This study aims to determine the effect of ethyl acetate derived from the leaves extraction of betel *(Piper betel)* on the content of Interleukin-1β (IL-1β) in experimental mice using an acute wound healing modeling. The experimental design used randomized post test only group with 72 experimental mice which divided into 4 groups and each group consisted of 6 mice with three replications. The negative control group (I) was given vaseline after incision. The positive control group (II) was given bioplasenton. The third group (III) was topically given the extracts of betel leaves at 2% concentration and the fourth group (IV) was given the extracts of betel leaves at 3% concentration after incision respectively. The determination of IL-1β applied Immunoassay Quantikine Elisa (IQE).

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The quantitative analysis (statistical test) used complete randomized design using one-way anova with Spearman's test and expressed as means±standard deviation, whereas, the qualitative analysis of the data was presented with the figure of time period of the concentration of IL-1β for the negative control and control groups as well as both the extracts of betel leaves at 3rd day, 7th day and 14th day respectively. Results revealed that the content of IL-1β was not statistically correlated with both the negative and positive control groups for both the treatment of vaseline (ρ < 0,217, r = 0,218) and bioplasenton (ρ < 0,249, r = 0,190) respectively. In addition, the content of IL-1β was also not statistically correlated with the treatment of the extracts of betel leaves at 2% concentration (ρ < 0,060, r = 0,420). In contrast, the content of IL-1β was statistically correlated with the treatment group of the extracts of betel leaves at 3% concentration (ρ < 0,002, r = 0,701). Results of the study concluded that the treatment of extracts of betel leaves at 3% concentration affected wound healing process in experimental mice and increased in line with the ascending time in wound healing process, whereas, both the negative and positive control groups as well as the treatment group of the extracts of betel leaves at 2% concentration did not affect the wound healing process among experimental mice.

Keywords: Piper betel; interleukin-1; wound healing.

1. Introduction

The study of the effect of betel *(Piper betel)* in wound healing process is feasible to know among nurse practitioners. It is considered important for the reason that the responsibility boundaries of nurse practitioners are changing, not only because of increased demands, but also they should demonstrate and develop their competence in varied extended and expanded practice roles [1].

Betel plant (*Piper betel)* is popularly regarded as a medicinal plant in Southeast Asia. Its leaves benefit for wound healing process and their extracts contain bioactive molecules like polyphenols, alkaloids, steroids, saponins and tannins [2]. The chief constituents of the leaves are volatile oils that contain phenol, betel-phenol, chavibetol and chavicol, cadinene, and hydroxychavicol which possess anti-oxidant and anti-carcinogenic activities [3,4,5,6,7]. The extracts of betel leaves also exhibits biological capabilities of detoxication, antioxidation, and antimutation against various ailments including liver fibrosis and carcinoma [8]. In addition, it is used for antidiabetic, antiulcer, antiplatelet aggregation, antifertility, cardiotonic, antitumour, antimutagenic, respiratory depressant and antihelminthic [9-15], antimicrobial [16], gastroprotective [17], hepatoprotective [18] and antimotility effects on washed human spermatozoa [19].

The health benefits of betel leaves in wound healing process can be identified by investigating the mechanism of wound healing process in the skin of the living species. Wound healing is a complex process involving several overlapping stages that include inflammation, formation of granulation tissue, reepithelialization, matrix formation and remodeling. Upon injury to the skin, the epidermal barrier is disrupted and keratinocytes release prestored interleukin-1 (IL-1). IL-1 is the first signal that alerts surrounding cells to barrier damage [20-30].

IL-1β is a pro-inflammatory cytokine. It is expressed by many cells including macrophage, NK cells, monocytes, and neutrophiles. It belongs to the IL-1 family that includes the IL-1a and IL1-RN genes. The

caspase 1 (CASP1/ICE) gene proteolitically activates IL-1β. This gene is involved in the proliferation, differentiation and apoptosis of cells that associates with wound healing [31].

Early reports of the effect of the extracts of betel leaves as the inflammatory cytokine in experimental mice prompted the authors to evaluate its effect as the inflammatory cytokine in experimental mice. Therefore, the aim of this study is to analyze the effect of the extracts of betel leaves on IL-1β as the cytokine inflammation in experimental mice.

2. Materials and Methods

2.1 Plant materials

Piper betel leaves which popularly known as "sirih" in Indonesia was taken from a 300-1000 m elevation above sea level in Soppeng Regency of South Sulawesi Province in the view of the fact that such an elevation was favorable environmental condition for the growth of *Piper betel*.

2.2 Experimental animals

The healthy adult Wistar mice weighing approximately 250-300 grams with the 4-to-6 week interval of age were used. Wistar strain is one the mice strains originated from the American continent. The experimental mice were obtained from the Veterinary Laboratory A of Gadjah Mada University, and then they were transported to the veterinary laboratory of Medicine Faculty of Hasanuddin University.

2.3 Experimental design

The experimental Wistar mice were divided into 4 groups and they were kept in their cages (30 cm x 50 cm x 15 cm) for at least five days prior to commencement of the experiment to allow for acclimatization to the laboratory conditions [32] and they were handled daily during this period.

During the experiment period, the experimental mice were put in the cages at 22 ± 2^{o}C with 50%-60% air moisture and fed on a standard pellet diet (300 gr/mouse/day) and water *ad libitum* made at the veterinary laboratory of Medicine Faculty, Hasanuddin University. The cages were labeled and cleaned routinely for identification purposes and the light cycle was maintained in a 12 hour light/12 hour dark cycle in their respective groups for 15 days. There were 6 mice per group of treatment and the total experimental animals were 72 mice and they were grouped into 4 random groups with three replications which one mouse was used as demanding supply [33].The four groups of experimental mice were divided as follows: 1) the negative control group was treated with vaseline (K-), 2) the positive control group was treated with bioplasenton (K+), 3) the treatment group given by the extracts of betel leaves at the 2% concentration (P1), and 4) the treatment group given by the extracts of *Piper betel* leaves at the 3% concentration (P2). Results of the extracts of *Piper betel* leaves in the form of acetyl acetate were given at 3rd day, 7th day and 14th day after excision by examining the concentration of IL-1β respectively.

2.4 Wound creation

The hair on the back of each of the rats was removed with electrical clippers and bare areas were disinfected using a solution of 0.5% chlorhexidine in 70% alcohol, which was allowed to dry after application. The rats were anaesthetized with isoflurane (0.01 μg/kg-0.05 μg/kg) and the ficzation was conducted at pronation position. Evenly spaced wounds were made in the skin within the bare area of the back of mice using a punch to obtain biopsies with a diameter of 6 mm [34]. The hair on the back of each of the mice was removed with clippers and bare areas were cleaned using hair removal cream (Veet), which then disinfected using 0,5% chlorhexidine in 70% alcohol, which allowed to dry after application. Excision was made at the back area of experimental mice (0,8 x 0,8 cm) using a punch to obtain biopsies [35].

2.5 Extraction of Piper betel leaves

Betel leaves were chosen and chopped into small sizes, which then left to dry at room temperature in order to preserve the compounds in betel leaves for 7 days. Subsequently, 180 grams of dried betel leaves were put into 3 Erlenmeyer flasks; each flask contained 60 grams of dried betel leaves. After then, dried betel leaves were soaked into 96% ethanol where the volume of each ethanol solution was 600 ml, and the extracts were macerated for 48 hours. Later, the extracts in Erlenmeyer flask were filtered using muslin cloth and then evaporated until dry.

The preparation procedure of topical cream was conducted by suspended its cream base. The suspension of cream base was performed for all kinds of cream bases, viz. absorption base, hydrocarbon and percolating water in hot mortars at ± 60^{o}C while stirring them until achieved homogeneous extracts and left to dry. The ethanol extracts of *Piper betel* leaves were made through the re-suspension of the prepared cream bases and then adding the extracts a little bit until achieved homogeneous extracts and formed cream.

2.6 Preparation of tissues for determination of the content of IL-1β

The extracts of betel were given orally for 15 days; the mice were fasted overnight and sacrificed by cervical decapitation. Afterward, the hairs on the back of each of the rats were removed with electrical clippers and bare areas were disinfected using a solution of 0.5% chlorhexidine in 70% alcohol, which were allowed to dry after application. The excision of biopsy was made approximately 1.5 x 2.5 cm^2. Samples for the examination of tissues of wound were analyzed using ELISA. The determination of the content of IL-1β used Rat IL-1β Immunoassay Quantikine Elisa.

2.7 Statistical analysis

All values were expressed as the mean±standard error. The quantitative analysis was performed using SPSS statistical package for WINDOWS (SPSS version 17). Results related to the content of IL-1β were statistically analyzed using Spearman's test. Data were presented with figures and tables and expressed as means±standard deviation (SD). Statistical test was repeated one-way ANOVA, *p*-values ≤0.05 were considered significant. The qualitative analysis was the time period of the content of IL-1β for four groups at 3rd day, 7th day and 14th day

respectively.

3. Results

Based on the experiment with three replications conducted among 72 experimental mice, mean values for 4 groups of experimental mice treated by vaseline (the negative control group), bioplasenton (the positive control group), the extracts of *Piper betel* leaves at both 2% and 3% concentrations were illustrated in Figure 1.

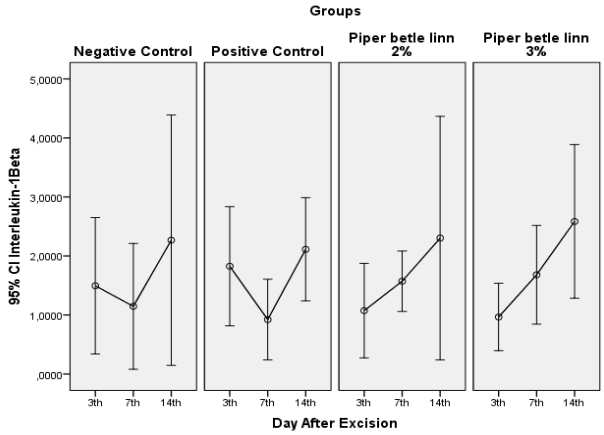

Figure 1: Comparison of mean values of the concentration of IL-1β among four groups of experimental mice (n=6) consisted of the negative control group (vaseline), the positive control (bioplasenton), the extracts of *Piper betel* leaves (2% and 3% concentrations) consecutively. Each concentration of IL-1β for each group of experiment was determined for 3rd day, 7th day and 14th day respectively. Data were presented as mean±standard deviation ($p \leq 0.05$).

The data in Figure 1 were then transformed into Table 1, where the lowest concentration of IL-1β at 3rd day was the extracts of betel leaves at 3% concentration (0.97±0.46), whereas, the highest concentration of of IL-1β at the 3rd day was the positive control group (1.83±0.81).

At 7th day after the treatment, the lowest concentration of IL-1β at the 3rd day was the positive control or bioplasenton (0.92±0.55), whereas, the highest concentration of IL-1β was the extracts of betel leaves at 3% concentration (2.67±1.71).

As illustrated in Table 2, the mean differences of the concentration of IL-1β for the negative control were -0.35 (3rd day and 7th day), -0.112 (7th day and 14th day), and -0.770 (3rd day and 14th day) respectively, whereas, the mean values of the concentration of IL-1β for the positive control was 0.90 (7th day and 14th day), -1.19 (3rd day and 14th day), and -0.290 (3rd day and 14th day) respectively. On the other hand, the mean differences of the concentration of IL-1β for the extracts of *Piper betel* leaves at 2% concentration were -0.49 (3rd day and 14th day), -0.73 (7th day and 14th day), and -1.23 (3rd day and 14th day) respectively, whereas, the mean differences of the concentration of IL-1β for the extracts of *Piper betel* leaves at 3% concentration were -0.72 (3rd day and 7th day), 0.90 (7th day and 14th day), and -1.26 (3rd day and 14th day) respectively.

Table 1: The values of mean ± SD of the concentration of IL-1β for experimental mice using an acute wound healing modeling based on the division of groups and time period.

Groups	**n**	**Concentration of IL-1β (Mean ± SD)**		
		3rd day	**7th day**	**14th day**
Negative control (vaseline)	6	1,49±0.93	1,15±0.86	2.67±1.71
Positive control (bioplasenton)	6	1.83±0.81	0.92±0.55	2.11±0.70
Extracts of *Piper betel* leaves (2%)	6	1.07±0.65	1,57±0,41	2,30±1.66
Extracts of *Piper betel* leaves (3%)	6	0.97±0.46	1.68±0.67	2.58±1.05

Table 2: Mean differences of the concentration of IL-1β for experimental mice using an acute wound healing modeling based on the division of groups and time period.

Groups	**Mean Differences**		
	3rd-7th	**7th-14th**	**3rd-14th**
Control			
Negative	-0,35	-1,12	-0,77
Positive	-0,90	-1,19	-0,29
Exp.			
Piper betel (2%)	-0,49	-0,73	-1,23
Piper betel (3%)	-0,72	-0,90	-1,62

As shown in Table 3, *p*-value for the negative control group (vaseline) was 0.217. Hence, the correlation between the overall time period (3rd day, 7th day and 14th day) was not statistically significant with the concentration of IL-1β for the negative control group (r = 0.218). Therefore, there was probability the regular

increase of the concentration of IL-1β in wound healing process. On the other hand, *p*-value for the positive control group (bioplasenton) was 0.249. Hence, the correlation between the overall time period (3rd day, 7th day and 14th day) was not statistically significant with the concentration of IL-1β for the positive control group (r = 0.190). Accordingly, there was probability the regular increase of the concentration of IL-1β in wound healing process.

Table 3: The correlation between the overall time period and the concentration of IL-1β for experimental mice based on an acute wound healing modeling.

Groups	**Correlations**	
	p*	***r****
Negative	0,217	0,218
Positive	0,249	0,190
Piper betel (2%)	0,060	0,420
Piper betel (3%)	0,002	0,701

*p** One-Way Anova Statistical Test.

*r** Spearman's correlation

p-value for the treatment group for the extracts of *Piper betel* leaves (2% concentration) was 0.060. Hence, the correlation between the overall time period (3rd day, 7th day and 14th day) was not statistically significant with the concentration of IL-1β for this treatment group (r = 0.420). Therefore, there was probability the regular increase of the concentration of IL-1β in wound healing process. On the contrary, *p*-value for the treatment group for the extracts of *Piper betel* leaves (3% concentration) was 0.002. Hence, the correlation between the overall time period (3rd day, 7th day and 14th day) was statistically significant with the concentration of IL-1β for the treatment group for the extracts of *Piper betel* leaves at 3% concentration (r = 0.701). Therefore, there was probability the regular increase of the concentration of IL-1β in wound healing process.

4. Discussion

Both *p*-value and *r*-value for the negative control group based on statistical test using Spearman's correlation were 0.217 and 0.218 respectively. Hence, there was not any correlation between the overall time period (3rd, 7th and 14th) and the content of IL-1β for the negative control group. Therefore, there was probability the decrease of IL-1β content at the overall time period (3rd, 7th and 14th) in wound healing process. On the other hand, both *p*-value and *r*-value for the positive control group based on statistical test using Spearman's correlation were 0.249 and 0.190 respectively. Hence, there was not any correlation between the overall time period (3rd, 7th and 14th) and the content of IL-1β for the positive control group. Therefore, there was probability the decrease of IL-1β content at the overall time period (3rd, 7th and 14th) in wound healing process.

Both *p*-value and *r*-value for the treatment group of the extracts of *Piper betel* leaves at 2% concentration based on statistical test using Spearman's correlation were 0.060 and 0.420 respectively. Hence, there was not any correlation between the overall time period (3rd, 7th and 14th) and the content of IL-1β for this group. Therefore, there was probability the decrease of IL-1β content at the overall time period (3rd, 7th and 14th) in wound healing process. On the other hand, both *p*-value and *r*-value for the treatment group of the extracts of *Piper betel* leaves at 3% concentration based on statistical test using Spearman's correlation were 0.002 and 0.701 respectively. Hence, there was correlation between the overall time period (3rd, 7th and 14th) and the content of IL-1β for this control group. Therefore, there was probability the increase of IL-1β content at the overall time period (3rd, 7th and 14th) in wound healing process.

IL-1β is a member of the interleukin 1 family of cytokines. This cytokine is produced by activated macrophages, leukocytes, keratinocytes, and fibroblasts. Its target cells are endothelial cells, macrophages, leukocytes, keratinocytes and fibroblasts. Its signaling/receptors are ICAM-1, VCAM-1, IL-1 in which such receptors have pivotal roles in inflammation, angiogenesis, re-ephitelization, and remodeling of tissues [36].

Piper betle is used in the traditional medicine as diuretic as the antioxidant activity of ethanol, chloroform and ethyl acetate extract of leaves and stem of *Piper betle* where the ethanol extracts of leaves and stems of betel have antioxidant activity [37].

Collagen is the key component in the phases of wound healing. As soon as after wounding, the exposure of fibroblastic collagens in blood streams leads to the aggregation and activation of trombosite and releasing chemotactic factors beginning the process of wound healing. Fragments of collagens release leukocyte collagenase to pull fibroblasts into the injury areas. After then collagens are used to form new extracellular matrix [38].

A previous study [39] that investigates the inflammatory cytokines such as IL-1α and IL-1β, IL-6 and TNF (tumor necrosis factor-alpha) during wound inhaling process in experimental rats using enzyme-linked immunosorbent assay and immunostaining for an observation interval of 0-240 hours (three days) provides facts that the concentrations of TNF-α and IL-1β begin to rise after the onset of wounding and achieve the peak period for 12 hours from the onset of wounding. Infiltration of various leukocytes shows acute inflammation at three hours and six hours, and the main source of this cytokine is immunohistochemical substance identified as neutrophil. Such a study provides fact that both TNF and IL-1β have pivotal roles in triggering the onset of inflammation. Rebound of the concentration of cytokines is 72 hours from the onset of wounding. Histological examination of the wound at 72 hours shows the migration of fibroblasts and the formation of new granulation as the evidence of the proliferative phase in wound healing process. This is positively correlated with the result of our study where the treatment of *Piper betel* leaves at 3% concentration increased the wound inhaling process at third day (72 hours). This is also correlates with the study conducted by another study [40] that investigates the effect of the extraction of *Piper betel* leaves on the erythrocytes of experimental mice showing that there is significant reduction in TBARS and significant increase in ascorbic acid, vitamin E, super oxide dismutase, catalase and membrane-bound ATPases among experimental mice fed with 0.2 gm of *Piper betel* leaves per day, the lower concentration than the concentration of *Piper betel* leaves used in our study (3% concentration).

5. Conclusion

The evidence from this study revealed that the extracts of *Piper betel* leaves at 3% concentration could increased the wound healing process among experimental mice than the treatment of vaseline and bioplasenton as well as the treatment of the extracts of Piper betel at 2% concentration where the wound healing process increased based on the ascending time at 3rd day, 7th day and 14th day. Results of this study showed the potency of *Piper betel* leaves in improving wound healing process. Further studies should be conducted in investigating the role of *Piper betel* leaves on the physiological and immunological mechanisms.

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