
Phytochemicals potential of *Cyperus rotundus* Linn. root extract Kalimantan for treatment of Oral mucosa traumatic ulcer : "Healing process enhancement with *Cyperus rotundus* L. root"

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Abstract: *Cyperus*(C.) *rotundus* Linn(L.) root extract Kalimantan have been shown empirically for decades as a medicine for the treatment of toothache, swollen gums and thrush in the Island of Borneo (Kalimantan). This study was aimed to analyze the phytoconstituents of *C. rotundus* L. root extract and the effect of its concentration in inducing the expression of TGF- β 1 and the fibroblasts proliferation in the healing process of oral mucosa traumatic ulcer in Wistar rats. Materials and Methods: This was an experimental study with randomized post test only control group design. The gel extract of *C. rotundus* L. based 3% Natrium carboxymethyl cellulose (CMC-Na) made by maceration method was used as treatment group while gel-based 3% CMC-Na was used for control group. Forty-eight rats were divided equally into two groups. Twenty-four rats were observed on day 5 and 24 others on day 7. Histological evaluation was performed by Hematoxylin Eosin (HE) staining for fibroblast and Immunohistochemistry(IHC) staining for TGF- β 1. Statistical analysis was performed using One Way Anova and Post Hoc Tukey's Honestly Significant Difference (HSD) test. Results:*C. rotundus* L. gel extract showed significant difference between the groups as a whole and between each group. The 4% was the best concentration compared to 2% and 8% of *C. rotundus* L. based 3% CMC-Na. There were strong correlation between TGF- β 1 expression and the number of fibroblasts. Conclusion: The concentration of four percent *C. rotundus* L. root extract Kalimantan, effectively increases the expression of TGF- β 1, triggers migration and increases the proliferation of fibroblasts, which ultimately increases the amount of fibroblasts in the wound area.

Keywords: Wound healing; Expression of TGF- β 1; Fibroblast; Root *Cyperus rotundus* L; Central Kalimantan Isolate; Tannin and Steroid.

1. Introduction

Oral mucosa serves as a protective barrier against trauma, pathogens, and carcinogenic agents. It can be affected by a wide variety of lesions and conditions, some of which are harmless, while others may have serious complications¹. Identification and treatment of these pathologies are an important part of total oral health care. Hence, oral soft tissue examination is crucial, and it should be done in a systematic manner to include all parts of the oral cavity.

Traumatic ulcer, according to Greenberg², is an ulcerative lesion of oral mucosa caused by trauma. Almost everyone has had a traumatic ulcer in oral mucosa (83.6%), and no significant differences are present between male and female. In males, the percentage is 81.4% and in females 85%, the majority were due to poor dentures, fractured restorations and sharp edges of the teeth^{3,4}. In a large group of Saudi dental patients over 15 years old there was a prevalence of oral lesion as much as 1.9%⁵.

Traumatic ulcers in oral mucosa occur on the lips, buccal folds, gingiva, labial mucosa and floor of the mouth, but they are most common in labial and buccal mucosa. Damage resulting from physical trauma, if occurs in a long time, may cause the patient to suffer from malfunctioned speech, chewing, and swallowing, and will even result in decreased body condition. The treatment provided until today is still symptomatic, by reducing existing pain, eliminating the causal factors, and accelerating ulcer healing. Severe traumatic ulcers may be managed with topical treatments, such

as topical corticosteroids. Antibiotics may be given to avoid sustained infection.

The use of natural ingredients as a medicine tends to increase. Traditional medicine has several advantages, one of which is a relatively low side effect. Currently the utilization of medicinal plants as herbal medicine has not been optimal. *C. rotundus* L. root extract Kalimantan is one of the plants that can be efficacious as a medicine, grew wild in the open space or slightly protected from sunlight, on vacant land, roadside or on farmland. It is a weed plant that difficult to eradicate, and thrives in peat soil⁶. The plant is a multipurpose plant that is believed to have many benefits and widely used in traditional medicine, not only in Indonesia, but throughout the world⁷. The people of Kalimantan have been using *C. rotundus* L. for generations for a variety of treatments, including toothache, swollen gums and mouth ulcers, by taking the root plant then boiled it in the water and used as a mouthwash.

A study conducted by Nuryana⁸ on the effect of root extract of *C. rotundus* L. on the epithelialization process of wound healing, reported that the grass root extract gel concentration of 1%, 2%, and 4% could effectively accelerate the epithelial process of wound healing in wistar rats. The aim of this study was analyze the phytochemical essential of *C. rotundus* L. root extract⁹ and to identify the effect of *C. rotundus* L. from Central Kalimantan, in concentrations of 2%, 4% and 8% on fibroblast count and TGF- β 1 expression in wound healing process. This study found that 4% *C. rotundus* L. root extract Kalimantan effectively accelerated

epithelial process, thereby enhanced the wound healing in Wistar rats.

2. Materials and Methods

This was an experimental study using randomized post test only control group design. Healthy male Wistar rats, 48 in number with body weight of 150-250 g and aged 2-3 months were used according to Institutional Ethics Committee guidelines. Subjects were divided into treatment group (24 rats) and control group (24 rats). Each of them was divided further into 2 subgroups based on the decapitation period. Traumatic ulcers with a diameter of 2 mm and a depth of 2 mm were treated with burnisher no. 4, heated with burner spray for 1 minute and touched for 1 second in the labial mucosa below the mouse. The ulcer was characterized by a pale white color, a more concave area than the surrounding mucosa¹⁰.

2.1 Phytochemical analysis

Qualitative phytochemical analysis:

Air dried powders of *C. rotundus* L. were subjected to phytochemical screening according to standard published methods^{25,14,19}

Quantitative phytochemical analysis:

Three hundred gram powder of air-dried aerial parts of each plant was extracted by percolation in 1 L of ethanol (95%) (4 times x 4 days) until complete exhaustion¹⁰. The total ethanol extract was concentrated under reduced pressure at 350C. The percentage yield of each plant extract was calculated according to the dry weight. The determination of moisture, total ash, acid insoluble ash and water soluble ash were carried out according to published method¹⁵. Quantitative analysis of primary and secondary metabolites percentage were carried out according published methods for carbohydrates,

proteins, lipids, phenols, flavonoids, alkaloids and tannins on day 5 and 7.

2.2 Treatment for the experimental animals

On day 3 the control group was treated with gel-based 3% Natrium Carboxymethyl Cellulose (CMC-Na) and three treatment groups with topical *C. rotundus* L. root extract gel-based 3% CMC-Na. Concentrations of 2%, 4%, and 8% were applied to the ulcer. The gel-based material used was 3% CMC-Na, a mixture of 100 ml distilled water and CMC-Na gel carrier materials.¹¹. Three grams of CMC was dissolved in 100 ml of hot water while being stirred constantly, chilled and mixed with 2 grams of *C. rotundus* L. extract to make 2% *C. Rotundus* root extract gel. Then, 4 grams and 8 grams of *C. rotundus* extract was mixed with 3% CMC-Na. Each of them was performed to make 4% and 8% *C. rotundus* L. root extract gel respectively. Zero percent concentration was made only from gel-based 3% CMC Na. This concentration was then used in the control group (placebo)¹¹.

Gel application was done by using micropipette as much as 0.05 ml for 1 minute, 2 times a day (morning and afternoon) on the third day after traumatic ulcer until the day fifth and seventh.

2.3 Experimental animals euthanasia

On day 5 and 7, all rats in treatment and control groups were sacrificed by inhalation. The rats were inserted into bottles without air and were given with 10% ether. Dead rats were subjected to lower mandibular cutting by including the ulcer portion and the normal parts were then inserted into the fixation solution (10% formalin buffer (pH 7.4)), and, subsequently the dead rats were buried¹².

2.4 Tissue-processing techniques using paraffin method

The first paraffin method is fixation. It was done to stop the autolysis process in the cells caused by subsequent treatment. Thereafter, the tissue fixation was rinsed with running water for 6-9 hours. Then followed by dehydration I, clearing I, infiltration I, dehydration II and clearing II, infiltration II, embedding, and the last stage was the cutting, which was done by rotary microtome serially with a thickness of 5 μ . From these selected pieces, the fine ones were put into water bath. Tissue incisions were attached to the glass object and then ready to be stained. HE staining was performed to see the fibroblasts count and immunohistochemistry test was performed for TGF- β 1 expression examination.

2.5. Fibroblast count and TGF- β 1 expression

Fibroblast count and TGF- β 1 expression was determined based on the observation of the paraffin blocks and histochemical preparations under a light microscope with 400 x magnification at 5 visual fields in order to facilitate reading and preventing cell duplication. The data were obtained as an average in each visual field.

2.6 Data Analysis

Levine test was done to test the homogeneity of the data. Normally distributed data ($p > 0.05$), followed by parametric test using One Way Anova was done to identify mean difference in group as a whole with 95% confidence level ($\alpha = 0.05$) and Post Hoc Tukey HSD was done test to determine significant difference between groups.

3. Results

Phytochemical analysis of *C. rotundus* L. root extract Central Kalimantan

indicated the presence of relative amounts of constituent bioactive substances presented in Figure 1.

Figure 1. Percentage of each content in root extract of *C. rotundus* L. of Central Kalimantan.

Figure 2. (Upper) Fibroblast count between studied groups on day 5; Control group (A); *C. rotundus* L gel extract. 2% (B); *C. rotundus* L gel extract 4% (C); *C. rotundus* L gel extract. 8% (D). (Lower) Fibroblast count between studied groups on day 7; Control Group (E) *C. rotundus* L gel extract 2% (F), *C. rotundus* L gel extract 4% (G); *C. rotundus* L gel extract 8% (H).

Table 1. Fibroblast count on day 5 and 7 in healing process of traumatic ulcer in oral cavity mucosa between groups.

Table 1 shows fibroblasts on day 5 where the mean \pm SD of the treatment group was lower than that on day 7, and the control group had lower mean \pm SD on day 5 compared to that on day 7. *C. rotundus* L. gel extract showed significant difference between the groups as a whole and between each group. The 4% treatment group showed the highest on the day 5 and 7, respectively.

Figure 3. (Upper) TGF- β 1 expressions between studied groups on day 5; Control Group (K) (A), *C. rotundus* L. gel extract 2% (B) *C. rotundus* gel extract 4% (C) *C. rotundus* L. 8% (D). (Lower) TGF- β 1 expressions between studied groups on day 7; Control Group (E), *Cyperus Rotundus* L. root 2% (F) *Cyperus Rotundus* L. root 4% (G) *Cyperus Rotundus* L. root 8% (H).

Table 2. Mean \pm SD of TGF- β 1 expression on day 5 and 7 healing process of traumatic ulcer in oral cavity mucosa between groups.

Table 2 shows that TGF- β 1 expression in treatment group is higher on day 5

compared to that on day 7. Control group had lower mean \pm SD. Stastical analysis showed significant difference between the groups as a whole and between each group. The 4% treatment group showed the highest on day 7.

Figure 4. TGF- β 1 expression against fibroblasts count on day 5 and 7

Table 3. Correlation test of TGF- β 1 expression against fibroblasts count on day 5 and 7.

TGF- β 1 expression had strong correlation with fibroblasts count on day 5 and 7 with correlation coefficient of 0.941 and 0.980, respectively. This indicated that the two variables had a close correlation in the form of linear positive correlation.

4. Discussion

Wound healing, as a normal biological process in human body, is achieved through four precise and highly programmed phases: hemostasis, inflammation, proliferation, and remodeling. For a wound to heal succesfully, all four phases must occur in the proper sequence and time frame. Many factors can interfere with one or more phases of this process, thus causing improper or impaired wound healing. The factors include oxygenation, infection, age and sex hormones, stress, diabetes, obesity, medications, alcoholism, smoking, and nutrition 22.

C. rotundus L. extract Kalimantan was considered as a source of bioactive compounds, as it was able to produce a great variety of secondary metabolites, characterised by a broad spectrum of biological activities. The wound healing potential of the plant extract was reinforced by the phytochemical analysis of the root extract which revealed the presence of several bioactive and therapeutic metabolites.

Phytochemical screening of various fractions of methanol extract reveals the presence of Tannin 57.88 %, followed by steroid (β -sitosterol) 39.25%, triterpenoids and polyphenols 1.2%, alkaloids 0.04%, saponins 0.83%, and flavonoids 0.6% (Figure 1).

Tannin is the main component of *C. rotundus* L. originating from Kalimantan, in contrast to *C. rotundus* originating from India which the main component is cyperene¹³. The high content of tannin is because most of the land in Kalimantan is peat land. Peat land is a soil formed from the accumulation of rotting plant debris that has high organic content. The benefit of organic nutrient content is essential. It plays a role in protein formation, in the regulation of catalytic mechanisms and catalysts such as photosynthesis, carbohydrate translocation, protein synthesis and others²³.

The astringent of tannin induces the formation of bonding compound complexes against microbial enzymes or substrates. The formation of tannin bonding complexes against metal ions increases the toxicity of tannin itself. Tannin is thought to be able to squeeze cell walls or bacterial cell membranes that disrupt the permeability of bacterial cells^{17;18}. Due to the disruption of the permeability, bacterial cells carry out life activities so that the growth is inhibited or even die. The antimicrobial and astringent properties of tannins and steroid are suggested to play critical role in wound healing process by increasing the rate of wound contraction, epithelialization and prevention of secondary bacterial infection, that would have complicated and delayed wound healing 19.

The content of *C. rotundus* originating from Kalimantan is able to shorten the

inflammatory phase to accelerate into proliferation phase. Inflammation promotes the ongoing pathogenesis of these diseases, causing major damage to various cells, tissues and organs throughout the body. If the cause of the inflammation persists or certain control mechanisms in charge of shutting down the process fail, the inflammatory responses become chronic, cell mutation and proliferation can result, often creating an environment that is conducive to the development of cancer. Uncovering and treating the cause of inflammation, rather than just treating the symptoms, is an important key to prevent any of these chronic diseases. Activated macrophages release cytokines and growth factors that play a role in inflammatory processes and tissue repair. Tannins alter the structure and/or function of some cells that may play an integral role in the production of inflammation such as vascular endothelial cells, platelets, T-lymphocytes, and alveolar macrophages. Tannin is also reported to exert other physiological effects, such as to accelerate blood clotting, reduce blood pressure, decrease the serum lipid level, and modulate immunoresponses.

This study showed that the number of fibroblast increased on day 5 and 7 in treatment group compared to control group. There was significant increase of fibroblasts on *C. rotundus* L. 4% concentration, on both day 5 and 7 (Table 1, Figure 2.), this is in accordance with the observations done by Tri Nuryana⁸, on the effect of root extract *C. rotundus* L. on the epithelial process in wound healing in Wistar rats. On day 7, *C. rotundus* L. with 4% concentration can effectively accelerate the epithelial process of wound healing in Wistar rats. An

increase in tissue epithelium leads to accelerated wound healing.

Tannin increases the number of capillary vessel formation and fibroblast cells. β -sitosterol, an estrogenic natural steroid, keep moisture in the wound area allowing cell growth. In the inflammatory phase, β -sitosterol limits the amount of prostacyclin, thus helping to accelerate the inflammatory phase. In addition, β -sitosterol also assists angiogenesis so as to speed up the proliferati-ve phase 16.

The amount of fibroblasts and TGF β -1 expression in the group receiving *C. rotundus* L. 8% concentration decreased both in day 5 and day 7 compared to the group receiving *C. rotundus* L. concentration of 4%. This is probably related to the recommended dosage be used in the healing of traumatic mucosal ulcer of the oral cavity of Wistar rats. The dosage is the dose of *C. rotundus* L that should be administered so as to give the desired pharmacological benefit. Optimal dosage is the right dose to give medical effect. The group of *C. rotundus* L. 4% concentration on day 5 and 7 showed the highest fibroblasts count and TGF β -1 expression, so that *C. rotundus* L. a concentration of 4% was the optimal dose, which was effective for the healing of traumatic mucosal ulcers of the oral cavity of wistar rats (Table 2, Figure 2.).

C. rotundus has properties to regulate cell function such as by stimulating the production of TGF- β 1, to increase chemotaxis and proliferation of fibroblasts in wound area. It is associated with granulation tissue formation, the growth factor for angiogenesis, as well as chemoattractant and mitogenic factor for fibroblasts in the inflammatory phase. It also has a dynamic role, in which the proliferative phase of TGF- β 1 plays a role

in the production of collagen by fibroblast and contributes to the extracellular matrix product and the remodeling phase of TGF- β 1 that inhibits substances that damage the extracellular matrix.

TGF- β 1 plays a role in the inflammatory phase by activating neutrophils and macrophages. In the proliferation and remodeling phase, TGF- β 1 expression falls, due to the inhibition of TGF- β 3 in the granulation tissue produced by leukocytes and macrophages. In the proliferative phase, fibroblasts exhibit angiogenesis stimulation, fibroblast proliferation, miofibroblast differentiation and extracellular matrix deposition.

The statistical analysis showed there was strong correlation between TGF- β 1 expression and fibroblast count on day 5 and 7 in the form of positive linear correlation (Figure 4, Table 3). This phenomenon showed that the induction of *C. rotundus* L. increased TGF- β 1 expression, and the production of TGF- β 1 increased proliferation of fibroblasts. Fibroblasts are major participants in

wound repair, which take part in granulation tissue's formation, collagen synthesis and interact with extracellular matrix, thereby promoting scar hyperplasia. Early in wound healing, fibroblasts synthesize and secrete a certain amount of collagen to accelerate wound healing.

5. Conclusion

Topical *C. rotundus* L root extract of Kalimantan tan increased the expression of TGF- β 1 as cell function regulator. The production of TGF- β 1 triggered migration and increase proliferation of fibroblasts which eventually increased fibroblast count in wound area. Four percent *C. rotundus* L. concentration effectively accelerated epithelial process of wound healing in wistar rats and increased tissue epithelium, leading to accelerated wound healing.

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TABLE

Table 1. Fibroblast count on day 5 and 7 in healing process of traumatic ulcer in oral cavity mucosa between groups.

Group	Day 5					Day 7				
	Mean \pm SD	2%	4%	8%	Control	Mean \pm SD	2%	4%	8%	Control
2%	14.83 \pm 0.753	-	0.001*	0.001*	0.001*	17.83 \pm 0.753	-	0.001*	0.001*	0.001*
4%	28.83 \pm 2.041	-	0.001*	0.001*	0.001*	35.33 \pm 2.733	-	0.001*	0.001*	0.001*
8%	19.50 \pm 1.049	-	0.001*	0.001*	0.001*	26.50 \pm 2.739	-	0.001*	0.001*	0.001*
Control	10.00 \pm 0.633	-	-	-	-	13.17 \pm 1.602	-	-	-	-

Note : *p<0.05 = significant differences

Table 2. Mean \pm SD of TGF- β 1 expression on day 5 and 7 healing process of traumatic ulcer in oral cavity mucosa between groups.

Group	Day 5					Day 7				
	Mean \pm SD	2%	4%	8%	Control	Mean \pm SD	2%	4%	8%	Control
2%	17.50 \pm 2.881	-	0.001*	0.001*	0.001*	14.67 \pm 1.211	-	0.001*	0.001*	0.001*
4%	34.00 \pm 2.898	-	0.001*	0.001*	0.001*	30.00 \pm 3.286	-	0.001*	0.001*	0.001*
8%	25.50 \pm 1.643	-	0.001*	0.001*	0.001*	23.00 \pm 3.464	-	0.001*	0.001*	0.001*
Control	9.17 \pm 0.753	-	-	-	-	12.17 \pm 1.169	-	-	-	-

Note : *p<0.05 = significant differences

Table 3. Correlation test of TGF- β 1 expression against fibroblasts count on day 5 and 7.

Day	Coefficient correlation	Significance	p<0.05 = significant correlation
Day 5	0.941	0.000*	
Day 7	0.980	0.000*	

FIGURE

The Bioactive Substances in the Extract

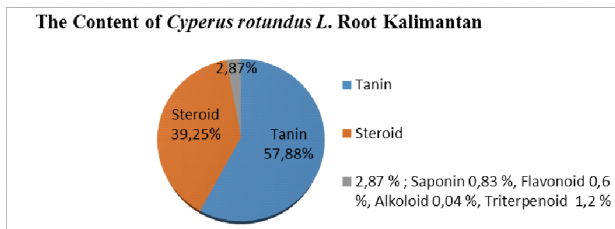
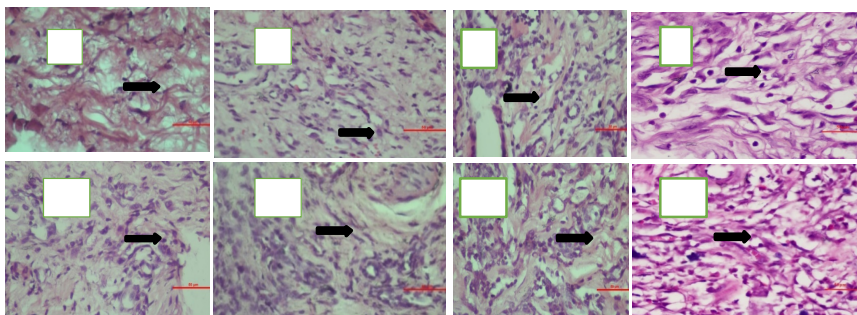


Figure 1. Percentage of each content in root extract of *C. rotundus L.* of Root Kalimantan.



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Figure 2. (Upper) Fibroblast count between studied groups on day 5; Control group (A); *C. rotundus* L gel extract. 2% (B); *C. rotundus* L gel extract 4% (C); *C. rotundus* L gel extract. 8% (D). (Lower) Fibroblast count between studied groups on day 7; Control Group (E) *C. rotundus* L gel extract 2% (F), *C. rotundus* L gel extract 4% (G); *C. rotundus* L gel extract 8% (H).

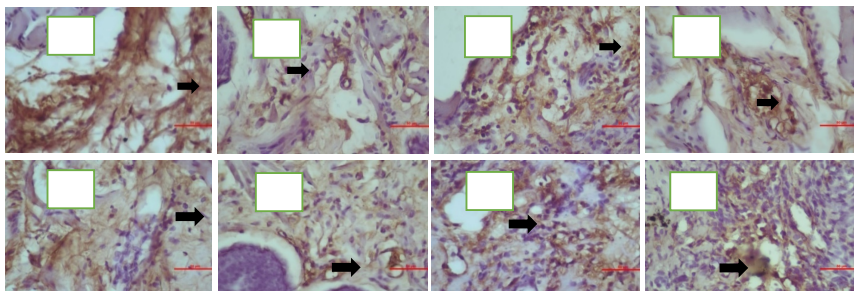


Figure 3. (Upper) TGF- β 1 expressions between studied groups on day 5; Control Group (K) (A), *C. rotundus* L. gel extract 2% (B) *C. rotundus* gel extract 4% (C) *C. rotundus* L. 8% (D). (Lower) TGF- β 1 expressions between studied groups on day 7; Control Group (E), *Cyperus Rotundus* L. root 2% (F) *Cyperus Rotundus* L. root 4% (G) *Cyperus Rotundus* L. root 8% (H).

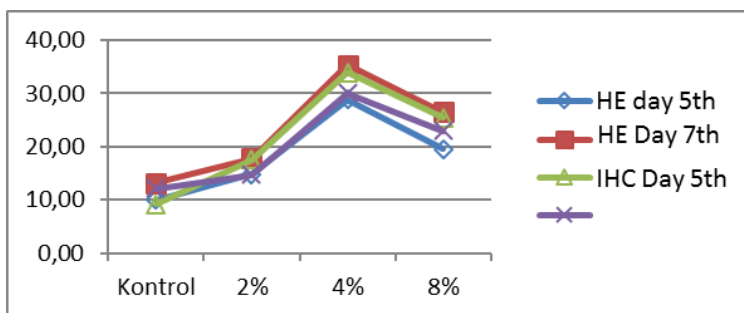


Figure 4. TGF- β 1 expression against fibroblasts count on day 5 and 7

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