Effectiveness of cocoa beans (Theobroma cacao L.) extract gel 8% and 16% in the healing of wistar rats (Rattus norvegicus) gingiva incision wound

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Abstract

Objective: To determine the effectiveness of 8% and 16% cocoa bean extract gel in the healing of Wistar rats gingival incision wound. Material and Methods: Cocoa beans were extracted using the maceration method, diluted with a concentration of 8% and 16%, and the addition of CMC-Na as gel basis. The 24-tailed mice are divided into 6 mouse-tails of treatment 1 given the 8% cocoa bean extract gel, 6-tails of treatment 2 given the 16% cocoa bean extract gel, 6-tailed mice positive groups given the drug Oxoferin solution 0.001%, and 6-tails of negative group given aquades. The treatment and wound length assessment of rats were carried out on the 1st day, 3rd day, 5th day, 7th and 10th days, then euthanasia on day 10 using ether anesthesia and carried out the cutting of the mandibular bone using the gingival tissue to wound re-epithelialization observation with a microscope (Olympus BX-41) 200X magnification. Results: One-way ANOVA and Post-HOC LSD tests presented a significant difference in the length of Wistar rats gingival wound closure between the treatment group that applied 16% cocoa bean gel extract, the negative and positive control groups, while between the 8% gel extract, the negative and positive control groups showed insignificant differences. Mean gingival incision wound re-epithelialization of each group on day 10 was 24.57 µm, 28.93 µm, 50.63 µm, 48.03 µm respectively. Conclusion: The 16% cocoa bean extract gel treatment is effective in healing the gingival incision wound of Wistar rats after applied for 10 days.

Keywords: Cocoa bean extract gel, Gingiva, Incision wound, Oxoferin, Wound healing DOI: 10.15562/jdmfs.v7i2.1286

Introduction

Wounds are injury that is usually characterized by discontinuity of normal tissue structures due to exposed by sharp tools, objects, or something that can injure body tissues. Wounds can occur anywhere on the surface of the body, one of which is in the oral cavity. The cause of the wound can be due to trauma, surgery, or extraction.

The wound healing process consists of three complex and overlapping stages. The inflammatory phase is divided into the initial inflammatory phase (hemostasis) and late inflammation, the proliferative phase, and the remodeling phase. After the injury, the body’s response to wound healing, which consists of the third phase, allows wound closure to prevent microbial invasion or infection of other agents into the tissue and prevention of chronic infection formation.

Clinically, wound healing usually occurs within two weeks of wound closure, but if the wound is deep and large, the wound closure process can take more than two weeks. Wound healing can be assessed using a variety of assessment methods, one of them are quantitative and objective measurements, which are better used to compare the effects of different wound treatments, including the ruler-based assessment method. Ruler-based assessment means an assessment by measuring the size of the wound or measurement based assessment.

Clinical wound assessment is supported by histological assessment, one of which is reepithelialization that plays a role in restoring tissue integrity when a wound occurs. A wound completely healed when the wound area has undergone complete epithelialization, so that epithelial cells migrate and join in the center of the wound until the wound edge is completely closed and free from external contamination.

Methods to accelerate wound healing are very diverse and currently developing, both by bringing the edges of the wound together, with chemicals, or alternatives with natural ingredients. Therapy with natural ingredients was developed because it is considered relatively safer and has minimal side effects with proper and correct use compared to synthetic or chemical drugs, and many plants have diverse contents, so they have synergistic effects and benefits of more than one pharmacological effect. Compared to chemical or synthetic drugs that are considered to have side effects that are not good for the body in long term. Chemical drugs for wound healing that available in drugstore or...
pharmacy mostly have relatively expensive prices, one of which is Oxoferin.5

Oxoferin is a topical patent drug with one of its active ingredients, namely tetrachlorodecaoxide (TCDO) which is used to treat infectious wounds, slow healing of surgical or traumatic wounds, and as a post-labioplasty therapeutic agent in the field of Oral Surgery.7 It can cause side effects, such as burning, itching, allergic reactions in some people, and pain, although this cases are very rare.10

One of the alternative natural ingredients that can be used for wound healing is cocoa beans.11 Cocoa beans are known in health because they have good sources of antioxidants and anti-inflammatory.11,12 Research on the effectiveness of cocoa bean extract gel in wound healing has been carried out by Kurniawati, et al (2019) who stated that 8% cocoa bean extract gel was effective in accelerating the wound healing process of male Wistar rats tooth extraction by reducing the number of macrophage cells on day 3.13

Based on the considerations described above, the researcher is interested to examine the effectiveness of 8% and 16% cocoa bean extract gel on the healing of gingival incision wounds in Wistar rats.

Material And Methods
This experimental laboratory study using posttest-only with control group design was conducted in November-December 2020. Cocoa bean extract gel was made in the Chemistry Laboratory of the Faculty of Teacher Training and Education, Syiah Kuala University, Banda Aceh and incision, treatments on animals and histologic preparation were performed at the Faculty of Veterinary Medicine, Syiah Kuala University, Banda Aceh. This study passed the ethical check by Commission of the Faculty of Dentistry, Syiah Kuala University, Banda Aceh as stated in the letter No. 230/ KE/ FKG/ 2020.

The cocoa beans (Theobroma cacao) as much as 2 kg obtained from Tiro/Truseb, Pidie District, Aceh, with the criteria of good cocoa beans from ripe cocoa pods. Furthermore, the experimental animal samples used were 24 Wistar rats using the Federer formula. They were chosen as population and samples were taken with these requirements: male rats, aged 8-12 weeks, weight of 200-250 grams, and healthy. The distribution of 24 samples was divided into 2 treatment groups with the application of 8% and 16% cocoa bean extract, Oxoferin as positive control group, and aquadest as a negative control group. Each group consisted of 6-tails.

The process of making the extract, 2 kg of cocoa beans, is left for approximately 5 days. Then, the bean pulp is peeled from the cocoa beans, then dried by aerating for ± 24 hours. Next, the cocoa beans are coarsely ground, aerated again to dry for ± 48 hours and grinded using a blender until a powder-like result of 0.2 kg is obtained. Cocoa beans that have been powdered are extracted by maceration method until a thick extract is obtained. Then, the cocoa bean extract was diluted using aquadest with a concentration of 8% and 16% and the addition of CMC-Na so that it became a cocoa bean extract gel.

Furthermore, the Wistar rats are acclimatized for 7 days in individual cages. Preparation for the Wistar rats includes cages, drinking bottles, and rat feed. During the study, animal feed was given ad libitum method. The location of incision wound is the labial gingival surface in the mandibular anterior teeth of mice. Prior to the incision, intramuscular anesthesia was performed using xylazine hydrochloride 2 mg/kgBW and ketamine hydrochloride 20 mg/kgBW as much as 0.5 ml. The wound was made using a scalpel and blade no. 11 with a length of 5 mm and depth of ± 1-2 mm.

Each group was applied 8% and 16% cocoa bean extract gel, Oxoferin, and aquadest twice a day at 8 A.M and at 4 P.M, starting on the first day of injury until the 10th day. Clinical observations were assessed by measuring the length of wound closure on days 1,3,5,7,10 using a periodontal probe (UNC-15) and histologically observing wound reepithelialization on day 10. Wistar rats that had been treated for 10 days were then euthanized using ether by inhalation and decapitated. The next step was cutting the mandible of Wistar rats, then cutting the tissue on the treated gingiva of Wistar rats with a size of 5x5 mm and a thickness of 2-3 mm, then the gingival tissue was taken and fixed in 10% Neutral Formalin Buffer (BNF) solution. Tissue staining using Hematoxylin-Eosin and observation of wound re-epithelialization were carried out using an Olympus BX 41 electron microscope with 200x magnification to observe an increase in epithelial thickness.

Data from the measurement of wound closure on days 1,3,5,7,10 were analyzed using the One Way ANOVA test with a significance level of 0.05. Then, Post-Hoc LSD (Least Significance Different) test was carried out. The data was processed using SPSS version 23 program.

Results
Extraction of 2 kg of cocoa beans was carried out by maceration method to obtain 8% and 16% cocoa
The results of the application of 8% and 16% cocoa bean extract gel, Oxoferin as a positive control, and aquadest as negative respectively showed wound healing which was marked by a decrease in wound size on days 1, 3, 5, 7, 10, figure 2. Histologically, the epithelialization was seen with an increase epithelial thickness in each group.

The results of measuring length of the wound for each group can be observed in the following figure and table, table 1.

Observational data showed that the reduction of wound length in each group generally started to occur on the 5th day. The negative control group that was applied aquadest began to reduce wound length on day 5 in mice (No. I, III, V, VI) which was 4 mm. The reduction in wound length on day 7 was 3 mm in rats (No. III and V). On 10 day there was a reduction in wound length in rats (No. III and V) which was 2 mm.

The positive control group that was applied Oxoferin began to reduce wound length in rats (No. III and IV) by 4 mm on the 3rd day. The reduction in wound length on day 5 in rats (No. IV) was 3 mm. On the 7th day the reduction of wound length in rats (No. III) was 2 mm. The reduction in wound length on the 10th day was 1 mm in rats (No. III and VI).

The first treatment group which was applied 8% cocoa bean extract gel began to reduce wound length on the 3rd day by 4 mm in rats (No. VI), while the other rats on day 5. The reduction in wound length on day 7 was 3 mm in rats (No. III, IV, VI) and on day 10 was 2 mm.

The second treatment group which was applied 16% cocoa bean extract gel began to reduce wound length by 4 mm on day 3, except for rats (No. III, V, VI). The reduction in wound length on day 5 was 3 mm in rats (No. V and VI). On day 7 all rats reduce wound length, except rat (No. VI). The reduction in wound length on day 10 was 0 mm in rats (No. II, IV, V, VI).

Based on the table above, the highest average re-epithelialization of wound healing in wistar rats was in the 16% cocoa bean extract gel group at 50.63 µm, the lowest was in the negative control group with aquadest at 24.57 µm. The average re-epithelialization of the 8% cocoa bean extract gel group was 28.93 µm the Oxoferin positive control group was 48.03 µm. Histopathological feature reepithelization on day 10 (200x): A. Negative control (aquadest), B. 8% cocoa beans extract gel, C. 16% cocoa beans extract gel, D. Positive control (Oxoferin).

Based on the picture above, on day 10 the negative control group given aquadest showed the wound area with epithelial thickness was not completely covered, the treatment group using 8% cocoa bean extract gel showed the wound area was closed but with incomplete epithelial thickness, and the treatment group 2 using 16% cocoa bean extract
The results of the data normality test using shapiro-Wilk showed a normal distribution of data in the 8% cocoa bean extract gel, 16% cocoa bean extract gel, oxoferin, and aquadest with p-values of 0.820, 0.212, 0.110 and 0.093 respectively (p>0.05). Levene's variance homogeneity test showed a significance value of 0.732 (p>0.05). These results indicate that each group in assessment of wound length has a homogeneous variant.

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Table 1. Data on wound length measurement results (mm)

| Treatment group             | Day | Mean ± SD |
|-----------------------------|-----|-----------|
|                             | 1   | 3         | 5         | 7       | 10     |
| Negative Control (aquadest) | Mice 1 | 5 | 5 | 4 | 4 | 3 | 4.1±0.3 |
|                             | Mice 2 | 5 | 5 | 5 | 4 | 3 |   |
|                             | Mice 3 | 5 | 5 | 4 | 3 | 2 |   |
|                             | Mice 4 | 5 | 5 | 5 | 4 | 3 |   |
|                             | Mice 5 | 5 | 5 | 4 | 3 | 2 |   |
|                             | Mice 6 | 5 | 5 | 4 | 4 | 3 |   |
| Positive control (Oxoferin) | Mice 1 | 5 | 5 | 4 | 3 | 2 | 3.6±0.2 |
|                             | Mice 2 | 5 | 5 | 4 | 3 | 2 |   |
|                             | Mice 3 | 5 | 4 | 4 | 2 | 1 |   |
|                             | Mice 4 | 5 | 4 | 3 | 3 | 2 |   |
|                             | Mice 5 | 5 | 5 | 4 | 3 | 2 |   |
|                             | Mice 6 | 5 | 5 | 4 | 3 | 1 |   |
| 8% cacao Bean extract gel   | Mice 1 | 5 | 5 | 4 | 4 | 3 | 3.9±0.2 |
|                             | Mice 2 | 5 | 5 | 4 | 4 | 2 |   |
|                             | Mice 3 | 5 | 5 | 4 | 3 | 2 |   |
|                             | Mice 4 | 5 | 5 | 4 | 3 | 2 |   |
|                             | Mice 5 | 5 | 5 | 4 | 4 | 2 |   |
|                             | Mice 6 | 5 | 5 | 4 | 3 | 2 |   |
| 16% Cacao Bean extract gel  | Mice 1 | 5 | 5 | 4 | 3 | 1 | 3.3±0.3 |
|                             | Mice 2 | 5 | 5 | 4 | 3 | 0 |   |
|                             | Mice 3 | 5 | 4 | 4 | 3 | 1 |   |
|                             | Mice 4 | 5 | 5 | 4 | 3 | 0 |   |
|                             | Mice 5 | 5 | 4 | 3 | 2 | 0 |   |
|                             | Mice 6 | 5 | 4 | 3 | 3 | 0 |   |

Table 2. Average re-epithelialization of gingival incision wound healing in group

| Group                        | Mean ± SD |
|------------------------------|-----------|
| Aquadest                     | 24.57 ± 1.23 |
| 8% cacao Bean extract gel    | 28.93 ± 0.74 |
| 16% Cacao Bean extract gel   | 50.63 ± 2.32 |
| Oxoferin                     | 48.03 ± 0.72 |

Statistical test using One-Way ANOVA showed a significance value of 0.000 (p<0.05), so there was a significant difference between groups. Next, the Post-Hoc LSD test was carried out [table 3](#).

Based on the table, Post-Hoc Least Significance Difference (LSD) test showed a significant difference between the 8% cocoa bean extract gel group and 16% cocoa bean extract gel group with p value = 0.000. The 16% cocoa bean extract gel group with positive control group showed p value = 0.039, while with negative control group showed a p value = 0.000. The positive control group and the negative control group showed a value of p=0.002 (p<0.05).

**Discussion**

This study was conducted to determine the effectiveness of 8% and 16% cocoa bean extract gel in the healing of Wistar rats gingival incision wound by assessing the length of wound closure on days 1, 3, 5, 7, 10 clinically using a periodontal approach.
using the Federer formula with a body weight of 150-200 grams and an incision was made on the labial gingiva with a length of 5 mm with a depth of ±1-2 mm.\textsuperscript{14,17} Wistar rats was chosen as the experimental animal for this study based on several aspects similar to humans including anatomy, physiology, genetics, biological characteristics and behavior. Wistar rats are also easy to breed, easy to treat, can represent mammalian biological systems.\textsuperscript{17,18} Male rats were chosen because they did not experience periodic physiological changes that usually occur in female rats so that the sample became easy to control, homogeneous, and the results were expected to be more accurate.\textsuperscript{18}

Then, the anesthesia used is intramuscular injection of xylazine and ketamine hydrochloride, because ketamine has the advantages of relatively fast onset of action, strong analgesic effect and relatively easy application, which can be injected intramuscularly, but the resulting muscle relaxation is not good.\textsuperscript{19} The side effects of ketamine can be minimized by combining it with premedication drugs that have a strong relaxing effect such as xylazine. The combination of ketamine and xylazine is very commonly used as induction of anesthesia used in small animals, it can produce better anesthesia, very strong analgesic effect, fast induction and recovery time, produce sedation and hypnosis, longer duration of anesthesia. longer, and rarely cause clinical complications.\textsuperscript{20}

This study used CMC-Na as a basis for the cocoa bean extract gel, because gel preparation functions to moisturize, cooling, and facilitate the penetration of extract on the skin or mucosa so that it has a healing effect, and can provide a comfortable state, so it reduce pain.\textsuperscript{13,21} Furthermore, the administration of the extract in a gel preparation on the wound was able to encourage the growth of epithelial cells to protect the wound from bacteria and fluid loss.\textsuperscript{16,22}

Cocoa beans are known to have anti-inflammatory activity against the number of macrophage cells.\textsuperscript{22} Active macrophages produce

The experimental animals used in this study were 24 male Wistar rats which were determined

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
Group & 8\% cacao Bean extract gel & 16\% Cacao Bean extract gel & Positive control & Negative control \\
\hline
8\% cacao Bean extract gel & & & S (0.000) & NS (0.061) \\
\hline
16\% Cacao Bean extract gel & S & & S (0.039) & S (0.000) \\
\hline
Positive control & NS & S & & S (0.002) \\
\hline
Negative control & NS & S & & S (0.002) \\
\hline
\end{tabular}
\caption{Post-hoc LSD test between group}
\end{table}

S=Significant; NS=Non-Significant

\textbf{Figure 3.} The “O” mark indicates the epithelium and epithelial thickness
chemotactic factors, growth factors, and cytokines that affect the proliferation, and migration of fibroblasts, endothelial cells, and epithelium so that clinically they can accelerate wound closure. Cocoa beans contain compounds such as polyphenols, flavonoids, tannins, saponins, and alkaloids. Cocoa bean extract gel on contains polyphenols, flavonoids, and alkaloids, so they have potential sources of antioxidants, they can supply a lot of antioxidants to ward off Reactive Oxygen Species (ROS) by increasing the activity of the enzyme Superoxide Dismutase (SOD) and glutathione transferase when damage to cells occurs due to injury, thus the wound will close quickly.

The concentration of cocoa bean extract selected in this study was 8% and 16%. Kurniawati et al. stated that 8% cocoa bean extract gel was effective in accelerating the healing process of tooth extraction wounds in male Wistar rats by reducing the number of macrophage cells on day 3. Prasetyo et al. stated that the addition of 15% cocoa bean extract gel to periodontal dressing was effective in healing gingival wounds by increasing the number of fibroblast cells. This showed that cocoa bean extract gel with concentration of 8% and 15% showed a good result for accelerating wound healing, especially in the oral cavity.

Next, the reason for choosing 16% cocoa bean extract gel is because it has twice concentration that has been proven to be effective so as to produce good wound healing. This is also in line with the research of Hasmila et al. which states that differences in the concentration of an extract affect wound healing activity, so the higher the concentration, the better the activity.

Wound healing in treatment group 2 given 16% cocoa bean extract gel was very good compared to the other three groups. The reason for faster and more effective wound healing with a higher concentration of extract compared to a lower one is due to an increase in the efficacious compounds in the extract thereby accelerating wound healing, so that content of bioactive substances in the extract increases, and optimal activity can be achieved.

Flavonoid in cocoa beans functions to increase Transforming growth factor-β (TGF-β), thus accelerating wound closure. Then, the tannins contain substances that bind to tissue, function to freeze proteins, so that the mucous membranes that are injured become dry and thick junctions are formed that are resistant to external inflammatory factors, increasing cell regeneration and proliferation, formation of granulation tissue, and epithelialization. Research by Pusparani et al. and Sari et al. stated that the content of saponins can accelerate the process of re-epithelialization in tissues, infiltration of inflammatory cells in the wound area, as a growth factor because of its mechanism of action that is able to stimulate the formation of new cells with the growth of vascular endothelial cells, muscle cells smooth blood vessels and fibroblasts, resulting in cellular growth that can eventually repair damaged blood vessel walls and accelerate wound closure.

Wound healing with oxoferin as a positive control was slower than the application of 16% cocoa bean extract gel. This is in line with the research of Reza et al. which state that natural plant extracts help accelerate wound healing, while the use of Oxoferin with intact and too strong concentrations causes the re-epithelialization process, only a slight increase in the epithelium caused by cells saturation.

Next, the control group given Aquadest experienced long wound healing, because it was the same as not giving other active substances or compounds that can accelerate the healing process, only cleaned to prevent foreign objects around wounds, so wounds heal normally. This study showed different and varied wound healing in each group due to differences in the body’s resistance of each rat, resulting in different responses.

The results of this study showed that treatment group 2 which was applied with 16% cocoa bean extract gel experienced a faster reduction in wound size compared to treatment group 1 which was applied to 8% cocoa bean extract gel, the positive control group was applied to Oxoferin, and the negative control group was applied to aquadest.

**Conclusion**

Based on the results of this study, it can be concluded that 16% cocoa bean extract gel has the best effectiveness compared to 8% cocoa bean extract gel in the healing of Wistar rats gingival incision wounds by accelerating the reduction of wound length and increase epithelial thickness in re-epithelialization observation. The 16% cocoa bean extract gel treatment is effective in healing the gingival incision wound of Wistar rats after applied for 10 days.

Statistical test using One-Way ANOVA showed a significance value of 0.000 (p<0.05), so there was a significant difference between groups. Post-Hoc LSD test showed a significant difference between the 8% cocoa bean extract gel group and 16% cocoa bean extract gel group with p value = 0.000. The
16% cocoa bean extract gel group with positive control group showed p value = 0.039, while with negative control group showed a p value = 0.000. The positive control group and the negative control group showed a value of p=0.002 (p<0.05).

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Conflict of Interest

The authors report no conflict of interest.

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