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The Effectiveness of Ethanolic Extract of *Centella asiatica* (L.) on Healing Minor Recurrent Aphthous Stomatitis in Wistar Male Rats (*Rattus norvegicus*)

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ABSTRACT
One of the most common oral diseases is Recurrent Aphthous Stomatitis (RAS) and the most common form is Minor RAS. Gotu kola leaves (*Centella asiatica* (L.) Urban) contain active components that can help the healing process that widely used as traditional medicine. The aim of this study was to determine the effectiveness of the 70% ethanolic extract of *C. asiatica* on the healing of Minor RAS. This study is a *true experimental* with a *Pretest-Posttest Randomized Control Group* design. The *purposive sampling* technique is used. The sample of this study are 24 male wistar rats divided into 4 sample groups, a negative control group and 3 experimental groups (treatment with ethanolic extract of *C. asiatica* at a concentration of 25%, 50%, and 75%). The formation of the lesion minor RAS was by making a lesion on the lower labial oral mucosa of the rats by using a 10% H_2O_2 solution, then giving the treatment in each group for 7 days twice a day. Observations were made by measuring the diameter of minor RAS lesions in all samples using a *sliding caliper* every morning for 7 days. This research conducted in August 2021. The results of the measurement of lesion diameter were tested by the *Shapiro-Wilk* normality test. *One Way ANOVA* test was performed, the results showed that there was no significant difference in the diameter of the lesion between experimental groups and negative control group ($p>0.05$). Ethanolic extract of *C. asiatica* did not significantly heal minor RAS lesions.

Keywords: *Gotu kola; Centella asiatica (L.); Ethanolic extract of C. asiatica; Minor recurrent aphthous stomatitis; Rattus norvegicus.*

1. INTRODUCTION

One of the most common oral diseases is Recurrent Aphthous Stomatitis (RAS), affecting up to 25% of the total population with recurrence rates reaching 50%. This disease includes chronic inflammation of the oral mucosa characterized by ovoid ulceration, small, sometimes more than one ulceration, recurrent, has a yellow base, and is surrounded by an erythematous halo [1]. The most characteristic symptom of this disease is recurrent onset with one or several sore erosions and ulcerations that appear usually on the oral mucosa of the lips, tongue, and cheeks [2].

There are three forms of RAS clinical appearance, namely minor, major, and herpetiform. Minor recurrent aphthous stomatitis is the most common form of RAS and approximately 85% of patients have this type of lesion. RAS minor usually occurs mostly in mobile mucosa located above the minor salivary glands [3]. Recurrent aphthous stomatitis may involve non-keratinizing mucosa in the oral cavity. The ulceration is superficial, usually <1 cm in diameter, and does not cause scarring even after years of recurrent ulceration, and tends to heal in 7-14 days [1].

The etiopathogenesis of RAS is still not fully understood. Potential trigger factors include: genetic predisposition, hormonal defects, increased oxidative stress, microelement and vitamin deficiency, systemic diseases (Crohn's disease, AIDS, ulcerative colitis), anxiety, food allergies, mechanical injury and viral and bacterial infections [2].

Recurrent aphthous stomatitis (RAS) is a disease of the oral cavity characterized by recurrent disorders of the oral mucosa in the form of painful ulcers [4]. Oral mucosal lesions that occur such as minor RAS are diseases that are known to be very uncomfortable so that they can reduce the patient's quality of life, due to a stinging sensation that gets worse when performing daily activities, such as talking, eating, or even drinking.

Drug treatment is considered for patients who have repeated RAS of the mouth and/or present with symptoms such as directional pain and difficulty eating [1]. Treatment for RAS is symptomatic; aims to reduce number and size of ulcers, pain, healing time, and to increase the disease-free period because the main etiology of this disease is still not clearly understood. Currently treatment options include topical agents, topical and systemic steroids, corticosteroids, mouthwashes containing active enzymes, antibiotics, laser treatments, cauterization, and combination therapy [5]. Topical treatment is aimed at preventing superinfection, protecting existing ulcers, analgesia, reducing inflammation, and treating active ulcers [4].

Indonesia has a variety of natural resources that are useful for human life [6]. Sources of natural wealth include plants that can be used for health and beauty. Along with increasing public awareness of the importance of maintaining health, natural ingredients are widely used as traditional medicine, so that gotu kola leaf (*Centella asiatica* (L.) Urban) is the choice of simplicia for traditional medicine in the future [7].

The tropical Asia region, especially in various countries such as the Philippines, China, India, Sri Lanka, Madagascar, Africa, and Indonesia is the origin of the area and the growing area of the gotu kola plant [8]. Gotu kola can be found in plantation areas, fields, roadsides, rice fields, or in slightly wet fields [6]. Gotu kola (*Centella asiatica* (L.) Urban) is a plant found throughout Indonesia that grows on slightly moist soil and gets enough sunlight, such as in meadows, rice fields, the edges of ditches, and so on [9].

The health benefits of gotu kola include antioxidant, anti-inflammatory, antibacterial, antifungal, wound healing and anti-ulcer [10]. For the human body, gotu kola has benefits in improving the immune system because all of its active substances are antioxidants [7]. Gotu kola leaf also has a very low level of toxicity so that it can be a potential herbal plant that can be applied to many treatments, as evidenced by its long use as a natural product [10].

Gotu kola leaves (*Centella asiatica* (L.) Urban) are known for their high pentacyclic triterpenoid content, so they can be called *"centelloids*" [11]. Triterpenes are the most abundant and most important active substance in gotu kola leaves. The most commonly found triterpenes in gotu kola leaves are pentacyclic triterpenoid acids (sapogenins), such as madecassic acid and asiatic acid, and triterpenoid glycosides (saponins), such as asiaticoside and madecassoside [12]. Asiatic acid, madecassic acid, asiaticoside, and madecassoside are the most important active compounds from gotu kola leaves, so that they become components of biomarkers for assessing the quality of gotu kola leaves. Asiatic acid and madecasic acid have anti-inflammatory properties by regulating inflammatory mediators (prostaglandins), and asiaticoside inhibits the synthesis of oxidative stress that facilitates healing of ulcers [10]. The content of asiaticoside which is a saponin can stimulate the formation of collagen which will accelerate wound healing [13]. In previous studies, it was found that extract of *C. asiatica* at 25%, 50%, and 75% concentrations was as good as *povidone iodine* in wound healing, and extract of *C. asiatica* at 25% concentration was better than *povidone iodine* in wound closure indicators [14].

Gotu kola leaf is also known to have high antioxidant activity and is a good resource of antioxidants such as total phenolics, beta carotene, and ascorbic acid [9]. The phytochemical content of the phenolic group of gotu kola leaves is flavonoids and tannins [12]. The flavonoids found in gotu kola leaves include catechins, epicatechins, kaempferol, quercetin, and glycosides [11].

Preclinical testing in pharmacology is a test process carried out on experimental animals and or on other biological materials to prove the scientific truth of the efficacy and safety of a material. Experimental animals are widely used in experimental studies of the medical and scientific branches [15]. Rats have a physiological system similar to humans, available in large numbers, economical prices and varied strains [16]. White rats (*Rattus norvegicus*) are widely used as experimental animals because these animals are easily obtained in large quantities, have a fast response, provide a scientific picture that may occur in humans, and the price is relatively cheap [17].

There are so many active substances with healing properties contained in *Centella,* but no one has investigated the effect of ethanolic extract of *Centella* on RAS. Therefore, aim of this study was to determine the effectiveness of the 70% ethanolic extract of *C. asiatica* on the healing of Minor Recurrent Apththous Stomatitis that will be seen from the reduction in the diameter of the Minor RAS lesion on *Rattus norvegicus*.

2. METHODS

This type of research is a *true experimental* laboratory research with a *pretest-posttest randomized control group* research design. This research groups the experimental and control group members randomly. This study began with measurements in the experimental and control groups, then continued with treatment in the experimental group, after which the second measurement

was carried out [18]. This research conducted on August 2021, in Pharmacology Laboratory of the Faculty of Medicine UMS, Surakarta, Indonesia.

The sample of this study was white rat wistar strain (*Rattus norvegicus*) which had minor RAS lesions on the labial mucosa. The sampling technique used purposive sampling technique by selecting white male Wistar (*Rattus norvegicus*) rats (so that there is no hormonal influence if choosing female sex) aged 4-5 months with a body weight of 300-400 grams and healthy.

The size of the sample is determined by calculation using the Federer formula. The total number of samples of male wistar strain rats needed in this study was 24 rats and contained 6 white wistar rats in each sample group. There were 4 groups of research samples, including the control group that was not treated (group I) and 3 experimental groups that were given the treatment of gotu kola leaf ethanol extract at a concentration of 25% (group II), 50% (group III), and 75% (group IV).

Making extracts in this study using the maceration method. Grind the dried simplicia, then sieved with a mesh size 44. The extraction stage of the maceration method begins by mixing one part (grams) of simplicia powder with ten parts of 70% ethanol solvent (liters) in a macerator pan. Mixing results are stirred for 10 minutes, once a day. Soaking or maceration is done for 2 x 24 hours. The maceration results were filtered using a glass funnel and filter paper. The filtering results are stored in a place that is not exposed to sunlight, then remaceration.

The result of maceration (maserate) was evaporated with a *rotary evaporator*, at a temperature of 78˚C until the extract thickens but can still be poured. *Waterbath* with a temperature of 70˚C until the extract thickens and there is no alcohol smell. The thickened extract was stored in a desiccator. Calculate the yield of the extract, by calculating the percentage between the weight of the extract obtained and the weight of the simplicia powder of *Centella* leaf used when making the extract (w/w) [19]. Dilution of the extract to form concentrations of 25%, 50%, and 75% using sterile distilled water. Mix until homogeneous.

Adaptation of the test animals for 10 days, the treatment was fed with BR I pellets and drinking water. All test animals were anesthetized with inhalation of diethyl ether on cotton wool, then waited until the test animals lost consciousness marked by the absence of a kicking reflex, then applied *lidocaine prilocaine* (EMLA 5%) cream on the labial mucosa. The formation of the lesion was conditioned like a minor RAS patient, namely by making a lesion on the lower labial mucosa of the oral cavity of the test animal by using a 10% H2O2 solution on a cotton bud for 2 seconds. The lesion that formed the next day was confirmed to be 1 lesion and not more than 1 cm in diameter.

The treatment on test animals was carried out by giving ethanolic extract of *C. asiatica* according to the concentration in each treatment group II, III, and IV (25%, 50%, and 75%). The extract was administered 2 times in the morning and evening every day for 7 days with a 0.2 ml injection syringe dripped onto minor RAS lesions that had formed in the test animals and maintained for 10 seconds.

Observations were made by measuring the size of minor RAS lesions in all samples of test animals using a *sliding caliper* every morning at 08.00 WIB for 7 days before giving the extract. The size of the diameter of the lesions in the experimental group of 25%, 50% and 75% concentration of experimental animals was compared with the group of negative control test animals that were not given any treatment.

The data that has been obtained is checked for accuracy first, then the data is entered into *Microsoft Excel*. Data analysis using *IBM SPSS Statistics 25* software. Analysis of distribution normality test data using the *Shapiro-Wilk* test, because the data is less than 50. Statistical analysis test uses unpaired comparative parametric hypothesis testing *One Way ANOVA*, if the results of the *Shapiro-Wilk* normality test obtained are normal data distribution.

3. RESULTS AND DISCUSSION

In this study, ethanolic extract of *C. asiatica* was obtained with a yield percentage of 62%. Making extracts in this study using the maceration method. This maceration method was chosen because it is more practical, uses less solvent, does not require heating [20]. The optimum time for maceration of gotu kola leaves is 24 hours. However, after passing the optimum time the yields produced were not significantly different and tended to remain [21].

Ethanol is a semi-polar solvent and almost all substances can be dissolved in it, so it is expected that the active compounds contained in the gotu kola herb can be extracted in ethanol solvent [22]. In this process, 70% ethanol solvent is used because ethanol can extract more active compounds than other types of organic solvents. Ethanol has a low boiling point of 79℃ so it requires less heat for the concentration process [20].

The results of the study in the form of data on the average diameter measurement of minor RAS lesions of all test animals in each sample group in the healing process of Minor RAS male white rats wistar strain (*Rattus norvegicus*) for seven days are shown in Table 1.

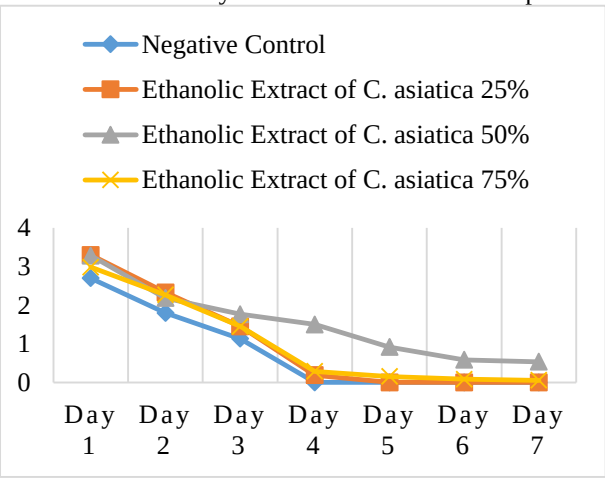
Table 1. Results of the mean diameter of minor RAS lesions per day

Group	Mean Lesion Diameter (mm)						
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
I	2.7	1.8	1.13	0	0	0	0
II	3.3	2.33	1.45	0.18	0	0	0
III	3.3	2.18	1.76	1.5	0.916	0.583	0.533
IV	2.9	2.26	1.45	0.28	0.15	0.083	0.05

Based on Table 1, the data obtained from the average daily diameter of minor RAS lesions were processed using the *IBM SPSS Statistics 25* software. The data processing began with the normality test of these data to determine the distribution of the data using the *Shapiro-Wilk* normality test and obtained the result of the significance value is less than 0.05 ($p < 0.05$), then the data distribution is not normal. Data transformation is performed to normalize the data distribution using the log function. The data for measuring the diameter of minor RAS lesions per day for groups I, II, III, and IV that have been transformed, were subjected to the *Shapiro-Wilk* normality test and the significance value was greater than 0.05 ($p > 0.05$), then the data distribution was normal, so the statistical analysis test used the *One Way ANOVA* parametric hypothesis.

In the *One Way ANOVA* parametric test, the significance value ($p = 0.773$) or $p > 0.05$, so it can be concluded that there was no significant difference in the reduction in lesion diameter from day 1 to day 7 between ethanolic extract of *C. asiatica* 25%, 50%, 75 % and without treatment.

The results of the average diameter of minor RAS lesions in this study can also be seen in Graphic 1.



Graphic 1. Results of the mean diameter of minor RAS lesions

Based on Graphic 1, the curve that decreases from the first to the seventh day shows a decrease in the mean diameter of minor RAS lesions from day to day in the negative control sample group and in the treatment group. The reduction in the diameter of the lesions in the treatment group showed a healing effect on minor RAS lesions from the ethanolic extract of *C. asiatica* descriptively, although the results of the hypothesis test showed no significant differences between the sample groups.

The healing activity of minor RAS lesions in the treatment group given the ethanolic extract of *C. asiatica* can be influenced by the immunity and nutritional intake of the test animals, as well as the action of the active substances contained in the *C. asiatica* extract. The action of the active substance of the extract depends on the level of absorption of the extract in minor RAS lesions. The active substances from gotu kola (*Centella asiatica*) leaves, one of which is a triterpenoid group, have anti-inflammatory properties, namely asiatic acid and madecasic acid which regulate prostaglandins, and asiaticoside substances inhibit the synthesis of oxidative stress which facilitates healing of ulcers [10]. Recurrent aphthous stomatitis is an idiopathic oral mucosal lesion, where the etiopathogenesis of RAS is still not fully understood, there are several predisposing factors associated with the formation and development of this disease. These factors include family history of RAS, food hypersensitivity, smoking, psychological stress, disorders of the immune system, as well as local factors such as trauma that occurs in individuals who are genetically susceptible to RAS, and microbial factors, as well as nutritional factors such as folate deficiency and B-complex vitamins [23], [24].

The data on the mean diameter of minor RAS lesions are presented descriptively as shown in Table 2.

Table 2. Results of the mean (mean) diameter of minor RAS lesions

Group	Treatment	Mean ± Std. Deviation
I	Without Treatment	0.82 ± 1.12
II	Ethanolic Extract of *C. asiatica* 25%	1.04 ± 1.35
III	Ethanolic Extract of *C. asiatica* 50%	1.54 ± 0.98
IV	Ethanolic Extract of *C. asiatica* 75%	1.04 ± 1.21

Based on the data in Table 2, the results of the mean diameter of minor RAS lesions in the test animals of group II (25% ethanolic extract of *C. asiatica* treatment) and IV (75% ethanolic extract of *C. asiatica* treatment) had the same average and better than the group III average (ethanolic extract of *C. asiatica* treatment 50%). This correlates with previous research by Amaliya *et al* (2013) which stated that the *Centella* extract which had the best healing effect was a concentration of 25% [14].

4. CONCLUSIONS

There was no significant difference in the reduction in lesion diameter from day 1 to day 7 between ethanolic

extract of *C. asiatica* 25%, 50%, 75 % and without treatment ($p = 0.773$), it can be concluded that ethanolic extract of *C. asiatica* did not significantly heal minor RAS lesions, but the reduction in the diameter of the lesions in the treatment group showed a healing effect on minor RAS lesions from the ethanolic extract of *C. asiatica* descriptively.

The results of statistical tests which show that there is no significant difference between groups can be caused by several factors, including:

1. Differences in initial lesions and reduction in lesion size in each group of test animals can be influenced by differences in stress levels and nutritional intake of each test animal. In this study, researchers could not control the equivalence of nutritional intake in each test animal. Preferably, there is only one test animal in each cage, so that researchers can monitor nutrient intake and reduce stress levels in test animals. The cause of Recurrent Aphthous Stomatitis is still unknown, and therefore many factors are still implicated in the disease including trauma, food allergies, stress, hormonal changes, nutritional deficiencies, drugs, and tobacco [1].
2. The solubility of the extract preparation used during treatment can also affect. This can result in the active substance in ethanolic extract of *C. asiatica* not being able to provide a maximum healing effect on stomatitis. In this case, a drug can have an effect if it is completely dissolved in the carrier liquid and enters the target, and the absorption process occurs. It is recommended that the duration of administration of the extract to the lesion is maintained longer so that the active substances contained in the extract can be absorbed more optimally. Test animals that have a tendency to rub their mouth area can affect the absorption of the active substance compared to test animals that do not rub their mouth area.
3. The dosage form of the extract given can affect the effect of the active substance. The extract is still general, with many other secondary metabolites, which may result in a less than optimal healing effect. Further research is needed to further purge secondary metabolites from the gotu kola plant. There is a possibility that in the form of fractions or isolates, the active substance from gotu kola is more active, because in fractions or isolates, the compounds are more conical and focus on the single compound. The form of the gotu kola plant extract fraction has various names with the main active ingredients being triterpenoids (asiatic acid, madecassic acid, and asiaticoside), such as Titrated Extract of *Centella asiatica* (TECA), Total Triterpenoid Fraction of *Centella asiatica* (TTFCA), and Total Triterpenic Fraction (TTF). The isolates of active substances from the gotu kola plant which have been shown to have anti-inflammatory and wound healing effects include asiaticoside, madecassoside, asiatic acid, and madecassic acid. TECA has been shown to decrease the level of release of cytokines that stimulate inflammation [25].

AUTHORS' CONTRIBUTIONS

The authors confirm contributions to the paper as follows: study conception and design: S. D. Khofifah; data collection: S. D. Khofifah, M. R. Vernanda, N. M. I. T. Azmi; analysis and interpretation of results: S. D. Khofifah, N. R. Suparno, M. Sari; draft manuscript preparation: S. D. Khofifah, N. R. Suparno, M. Sari. All authors reviewed the results and approved the final version of the manuscript.

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