

Liquid Soap from Waste Cooking Oil Containing Betel and Aloe Vera Creates Low Risk to Allergic Contact Dermatitis in Rat Skin

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Abstract

The quality of used cooking oil can be improved by purifying it using bagasse activated charcoal. Purified used cooking oil can be used as raw material for soap. Use of soap can cause allergic contact dermatitis (ACD). Skin suffering from ACD is characterized by inflammatory cell infiltration of the skin, the inflammatory cells involved are mast cells, macrophages and polymorphonuclear cells (PMN), namely basophils, eosinophils and neutrophils. Therefore, inflammatory cells were chosen as a parameter for the ACD response to the use of used cooking oil liquid soap containing betel and aloe vera. Liquid soap formed was use an extract concentration of 4.5, 9 and 15 %. Skin sensitivity testing was tested on male Wistar rats aged 2 - 3 months for 14 days. Quantitative data was obtained through histopathological examination of the number of inflammatory cells, which were analyzed statistically. The results showed that the use of liquid soap with an extract concentration of 9 % (4.24 ± 0.74) and 15 % (3.96 ± 0.25) had no effect on basophil infiltration. The use of liquid soap with an extract concentration of 4.5 % (2.96 ± 1.17 , 6.42 ± 0.86 , 5.96 ± 0.38), 9 % (3.12 ± 0.47 , 4.62 ± 0.68 , 6.42 ± 1.06) and 15 % (3.36 ± 0.41 , 5.08 ± 0.30 , 6.44 ± 0.55) had no effect on infiltration of neutrophils, eosinophils and macrophages. The use of liquid soap with an extract concentration of 9 % (4.60 ± 0.86) had no effect on mast cell infiltration. Therefore, it can be concluded that the best concentration of 9 % extract betel and aloe vera showed no infiltration of inflammatory cells in rats' skin given soap from waste cooking oil. Thus, that the soap formed from waste cooking oil treated with bagasse activated charcoal and contain betel and aloe vera is predicted safe to used.

Keywords: Liquid soap, Betel leaf, Aloe vera, Allergic contact dermatitis, Polymorphonuclear cells, Mast cells, Macrophages

Introduction

High use of cooking oil results in high waste of cooking oil. It is estimated that used cooking oil produced in 2019 will be around 3 million kiloliters [1]. The quality of used cooking oil can be improved through a purification process using adsorbents [2]. Rahayu *et al.* [3] stated that adsorbents derived from bagasse are effective in reducing free fatty acid levels in used cooking oil so that they can be used to improve the quality of used cooking oil. Used cooking oil that has gone through a refining process can be used as raw material for non-food products such as biodiesel, candles and soap [4]. Soap is a product that

is necessary in everyday life. Thus, through the saponification process, used cooking oil can be reused as raw material for making soap [5].

Soap is used as a skin cleansing product, besides that it should be able to inhibit and even kill pathogenic microorganisms. Thus, the addition of antiseptics to soap plays a role in protection against pathogenic microorganisms [6]. Apart from chemical preparations, antiseptics can also be found in natural ingredients derived from secondary metabolites. Utilizing plant extracts as antiseptic agents has fewer side effects [7]. Betel (*Piper betle* L.) and aloe vera (*Aloe vera* L.) have been proven to have antiseptic benefits [8,9]. Aloe vera ethanol extract has antibacterial activity against *Escherichia coli* (*E. coli*), *Shigella*, *Salmonella* Spp. and *Staphylococcus aureus* (*S. aureus*) [10]. Apart from that, the ethanol extract of green betel leaves also has antibacterial capabilities which can inhibit *E. coli*, *S. aureus* and *S. epidermidis* [11-13].

The addition of betel leaf extract and aloe vera acts as a natural antiseptic compound in soap. Even though the ingredients in soap come from natural ingredients, it does not rule out the possibility that it can cause allergic reactions [14]. Goossens [15] stated that several ingredients contained in skin care products including soap can cause contact dermatitis reactions. Kumar and Paulose [16] stated that as many as 12 % of patients were confirmed to have ACD caused by soap. Skin suffering from ACD is characterized by infiltration of inflammatory cells in the skin tissue. Basophils, eosinophils, neutrophils, mast cells and macrophages are inflammatory cells involved in ACD [17-21].

Inflammatory cell infiltration is an inflammatory response caused by microorganisms or non-microorganisms, such as chemicals [22,23]. When tissue is traumatized, the tissue will activate leukocyte chemotaxis from the circulation to the trauma site. Extravasated leukocyte cells are also called inflammatory cells [24,25]. These inflammatory cells will produce cytokines thereby inducing an inflammatory response [26]. For this reason, evaluating the effect of soap on skin tissue requires *in vivo* testing.

Relatively many *in vivo* tests use rodents as animal models. Animal models used in *in vivo* tests must be appropriate to the testing requirements. Based on the structure of their skin, rat have more similarities to human skin than other rodents [27]. Thus, in this study, rats were used as an animal model in evaluating the safety of soap.

Liquid soap containing 3 % aloe vera extract is effective in inhibiting *E. coli* and *S. aureus* [28]. Apart from that, liquid soap containing green betel leaf extract also has the same antibacterial ability as soap containing chlorhexidine and triclosan [29]. However, research by Takayama and Yoshioka [30] states that there is infiltration of inflammatory cells when using soap containing neem oil. Therefore, there has been no research regarding the effect of liquid soap with green betel leaf extract and aloe vera on the skin through parameters such as inflammatory cell infiltration. Thus, this research was conducted to test the safety of using used cooking oil liquid soap containing betel leaf and aloe vera extracts by observing the infiltration of polymorphonuclear inflammatory cells (PMN), namely basophils, neutrophils and eosinophils; macrophages; and mast cells.

Materials and methods

Preparation and extraction of green betel

Samples of green betel leaves (*Piper betle* L.) were obtained from Balai Penelitian Tanaman Rempah dan Obat (Balitro), Bogor. The green betel leaves chosen are young, fresh, thick, shiny leaves [31,32]. A total of 5 kg of betel leaves were washed with running water and then air-dried. After that, the betel leaves are dried in an oven at 50 °C until the water content of the betel leaves decreases to ≤ 10 %. The dried betel

leaves are then crushed and filtered using a sieve of $\pm 40 - 60$ mesh [33,34]. Betel leaf extraction uses the maceration method with a ratio of dried betel leaf and 96 % ethanol is 1:10. A total of 500 g of dried betel leaf was macerated using 5000 mL of 96 % ethanol and left for 72 h. Next, the sample is filtered using filter paper to separate the residue and filtrate. The filtrate obtained was evaporated using a rotary vacuum evaporator at a temperature of 55 °C to obtain a thick extract of betel leaves [35].

Preparation and extraction of aloe vera

Samples of aloe vera (*Aloe vera* L.) were obtained from the Aloe Vera Plantation, Kalisuren, Bogor. The aloe vera used was harvested aloe vera $\pm 10 - 12$ months old, the fronds were large and relatively old [36]. A total of 5 kg of aloe vera was peeled and washed with running water until the sap disappeared. The aloe vera leaf flesh is dried in an oven at 50 °C until the water content reduces to ≤ 94 % [34,37,38]. After that, 500 g of aloe vera leaf flesh was ground and then macerated with 1,000 mL of 96 % ethanol, for 72 h. The aloe vera filtrate was evaporated using a rotary vacuum evaporator at a temperature of 55 °C to obtain a thick extract of aloe vera [39,40].

Preparation of bagasse activated charcoal

Making activated charcoal bioadsorbent from bagasse refers to Rahayu *et al.* [3] which has been modified. The bagasse is cleaned and dried in the sun. After drying, the bagasse is burned for 15 min in a furnace at a temperature of 600 °C. The carbonized bagasse is ground and sieved with a 250 μm sieve. After that, 50 g of bagasse charcoal was activated with 250 mL of 10 % KMnO_4 for 24 h. The activated charcoal was washed with distilled water and dried in an oven at 115 °C for 1 h [3].

Refining used cooking oil

The used cooking oil used is oil after going through the tofu frying process 3 times [41]. Purification of used cooking oil uses bagasse activated charcoal that has been previously made. Purification of used cooking oil refers to Rahayu *et al.* [3]. A total of 10 g of bagasse activated charcoal is soaked in 150 g of used cooking oil for 72 h. After letting it sit, the used cooking oil is filtered first before being used to make liquid soap.

Preparation of used cooking oil liquid soap

The method for making liquid soap refers to Bidilah *et al.* [42] which has been modified. A total of 50 mL of purified used cooking oil was added with 30 mL of 35 % KOH, stirred until there was no unsaponified oil. After a thick soap mass is obtained, distilled water is added with a ratio of distilled water to soap, namely 2:1. After stirring for 60 min to obtain a liquid soap base, 10 mL of glycerin was added and stirred again for 5 min until a thick soap mass was obtained. The addition of a combination of aloe vera and betel extracts in liquid soap is 1:2, with a variation in the extract concentration used of 4.5, 9 and 15 %.

Animals

Based on calculations using the Federer formula, the number of repetitions for each group or treatment is 5 rats, so 25 rats are needed. The test animal used was a white rat (*Rattus norvegicus*), Wistar strain, male, approximately 2 - 3 months old, weighing 150 - 250 g, healthy with active movement, clean hair, clear eyes and had never received treatment [43]. Rats were obtained from Unit Pelaksanaan Fungsional (UPF) Badan Kebijakan Pembangunan Kesehatan, Bogor. Rat's acclimatization was carried out for 3 days [44]. Rats were placed in individual cages with cages measuring 50 \times 35 cm². The environmental conditions of the cage include a room temperature of around 27 °C, a lighting cycle of 12 h light and 12 h dark, with

air humidity around 50 - 60 %, the bedding used is wood shavings with a thickness of 2 cm, the cage and bedding are cleaned twice a week. The white rats are fed standard pellets at 10 % of their body weight every morning and drink ad libitum [43,45,46]. The rat's hair on the back was shaved 24 h before testing to an area of $\pm 2 \times 2$ cm² for the sensitivity test site, after which the skin was disinfected using 70 % alcohol [47]. Ethical approval for animal care and testing (No.015/KEH-BIO/UNJ/2023) was granted by the Animal Ethics Committee of Jakarta State University.

Skin sensitivity test

Treatments were grouped into 5 groups with each group consisting of 5 rats. The 5 groups consisted of liquid soap with an extract concentration of 4.5 % (P1), 9 % (P2) and 15 % (P3), positive control (Asepto liquid soap, KP) and negative control (liquid soap without extract, KN). Skin sensitivity test refers to Putri *et al.* [47] which has been modified. The test scheme is presented in **Figure 1**. The dosage of the soap preparation used is 0.5 mL [48]. The backs of rat that had been shaved were previously smeared with soap 10 times and then left for 4 h [49]. Soap rinsing follows the method of Iwashita *et al.* [50] which has been modified, the back skin is rinsed using 2 mL of warm distilled water at a temperature of 37 ± 1 °C, 5 times [50,51]. The sensitivity test was carried out for 14 days and was carried out once a day.

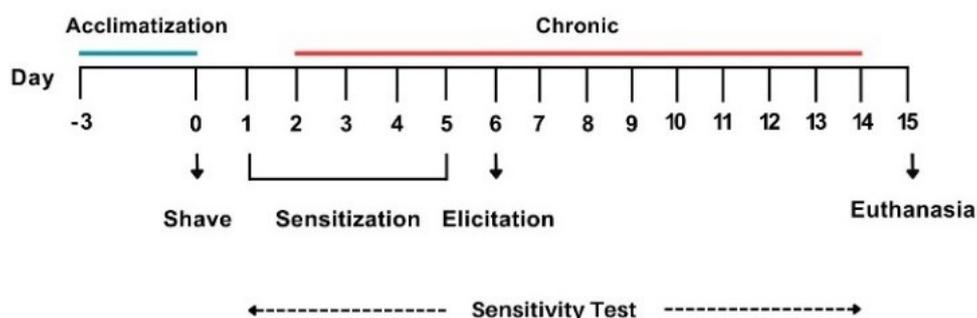


Figure 1 Schematic of skin sensitivity test timing.

Histopathological examination of inflammatory cell infiltration

On the 15th day, rats were euthanized by intraperitoneal injection with 10 % ketamine at a dose of 150 mg/kgBW and 2 % xylazine at a dose of 30 mg/kgBW. After the rats were euthanized, a skin biopsy was performed on the back skin which had been tested for sensitivity [52]. The skin samples obtained were then fixed with 10 % Neutral Buffered Formaldehyde (NBF) solution. Next, the dehydration, clearing, infiltration, embedding, sectioning processes are carried out sequentially. Then the staining process was carried out with hematoxylin and eosin (H&E) [53,54]. Observation of the number of inflammatory cells using a light microscope with 400 \times magnification in 10 fields of view, the number of counts was averaged.

Statistical analysis

Quantitative data obtained included PMN inflammatory cells (basophils, neutrophils and eosinophils), macrophages and mast cells. Data analysis was carried out using the Statistical Program for Social Science (SPSS) version 25. Data was analyzed using 1-way Analysis of Variance (ANOVA) with a significance level of 5 %. If there is significance < 5 % then proceed with the Duncan Multiple Range Test (DMRT) with a confidence level of 95 % [55].

Results and discussion

Inflammatory cell infiltration is one of the responses that marks the occurrence of ACD [56]. Histopathological examination of rat skin was carried out to calculate the infiltration of inflammatory cells in the skin tissue after testing the skin’s sensitivity to liquid soap. The results of the study showed that there was inflammatory cell infiltration in the 5 groups (**Figure 2**).

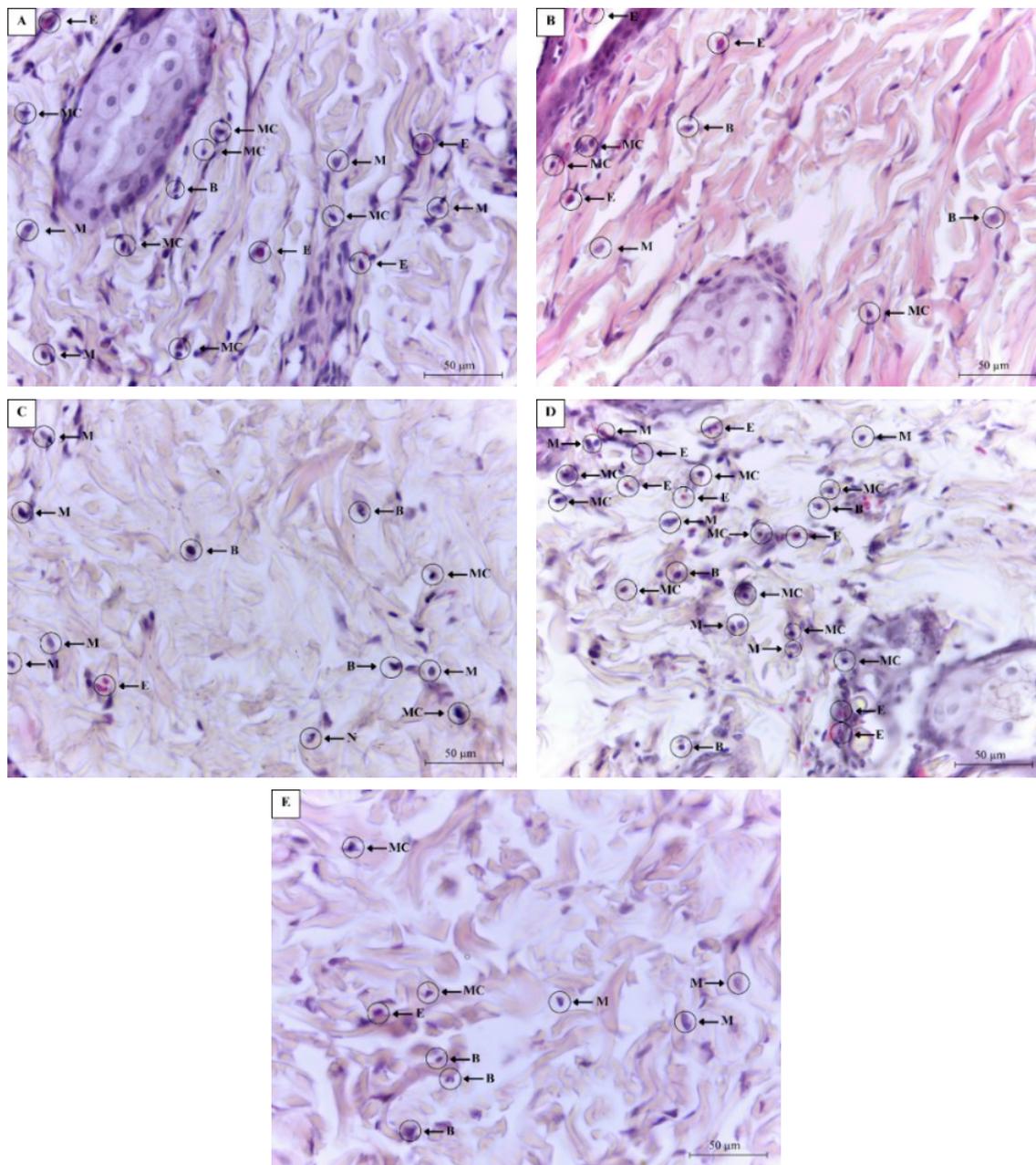


Figure 2 Histopathological images of inflammatory cell infiltration. H&E staining at 400× magnification. (A) group P1, (B) group P2, (C) group P3, (D) negative control, KN, and (E) positive control, KP. B = Basophils, N = Neutrophils, E = Eosinophils, MC = Mast cells and M = Macrophages.

Histopathological examination of the number of inflammatory cells from rat skin after the soap sensitivity test is shown in **Tables 1 - 3**. One-way ANOVA analysis of basophil, neutrophil and eosinophil infiltration showed that the treatment had a significant effect with a significance value of $p < 0.05$. Thus, to find out which group had a significant effect, DMRT test was carried out on the infiltration of basophils, neutrophils and eosinophils. The average number of PMN inflammatory cells is presented in **Table 1**.

Table 1 Average number of PMN inflammatory cells on histopathological examination of rat skin.

| Group | Basophil | Neutrophil | Eosinophil |
|-------|---------------------------|--------------------------|---------------------------|
| KP | 3.58 ± 0.21 ^a | 1.50 ± 0.15 ^a | 9.04 ± 0.82 ^a |
| KN | 7.04 ± 0.96 ^b | 6.52 ± 1.16 ^b | 15.44 ± 2.87 ^b |
| P1 | 5.40 ± 0.60 ^{ab} | 2.96 ± 1.17 ^a | 6.42 ± 0.86 ^a |
| P2 | 4.24 ± 0.74 ^a | 3.12 ± 0.47 ^a | 4.62 ± 0.68 ^a |
| P3 | 3.96 ± 0.25 ^a | 3.36 ± 0.41 ^a | 5.08 ± 0.30 ^a |
| Sig. | 0.004 | 0.005 | 0.001 |

Note: Data is the mean ± SE from the results of the 1-way ANOVA test with $\alpha < 0.05$ and the superscript letters are the results of the DMRT test at a 95 % confidence interval. Different letter notations in the same column indicate statistically significant differences ($p < 0.05$) between groups. KP is a positive control using Asepso liquid soap and KN is a negative control using liquid soap without extract. P1, P2 and P3, respectively, are liquid soap groups with 4.5 % extract, 9 and 15 %.

Based on **Table 1**, it is shown that basophil infiltration in groups P1 (5.40 ± 0.60), P2 (4.24 ± 0.74) and P3 (3.96 ± 0.25) was not statistically different from KP (3.58 ± 0.21). Groups P2 and P3 had statistically lower basophil infiltration numbers compared to KN (7.04 ± 0.96). Based on this, it shows that groups P2 and P3 have no effect on basophil infiltration because this group has a number of basophils that is not statistically different from KP and is statistically lower than KN so groups P2 and P3 are considered to have no effect on basophil infiltration of the rat skin

Based on the results of neutrophil infiltration presented in **Table 1**, it shows that the number of neutrophils in groups P1 (2.96 ± 1.17), P2 (3.12 ± 0.47) and P3 (3.36 ± 0.41) not statistically different from KP (1.50 ± 0.15). In addition, groups P1, P2 and P3 had statistically lower neutrophil counts compared with group KN (6.52 ± 1.16). Thus, it can be concluded that groups P1, P2 and P3 are groups that have no effect on neutrophil infiltration.

Based on **Table 1**, the results of eosinophil infiltration show that groups P1, (6.42 ± 0.86), P2 (4.62 ± 0.68) and P3 (5.08 ± 0.30) have eosinophil numbers that are not statistically different from KP (9.04 ± 0.82). Groups P1, P2 and P3 had statistically lower eosinophil counts compared with KN (15.44 ± 2.87). Therefore, groups P1, P2 and P3 had no effect on eosinophil infiltration.

Table 2 Average number of mast cells on histopathological examination of rat skin.

| Group | Mast Cell |
|-------|---------------------------|
| KP | 5.12 ± 0.50 ^{ab} |
| KN | 8.04 ± 1.14 ^{bc} |
| P1 | 9.10 ± 1.17 ^c |
| P2 | 4.60 ± 0.86 ^a |
| P3 | 5.30 ± 0.73 ^{ab} |
| Sig. | 0.020 |

Note: Data is the mean ± SE from the results of the one-way ANOVA test with $\alpha < 0.05$ and the superscript letters are the results of the DMRT test at a 95 % confidence interval. Different letter notations in the same column indicate statistically significant differences ($p < 0.05$) between groups. KP is a positive control using Asepso liquid soap and KN is a negative control using liquid soap without extract. P1, P2 and P3, respectively, are liquid soap groups with 4.5 % extract, 9 and 15 %.

One-way ANOVA analysis of mast cell infiltration showed that treatment had a significant effect with a significance value of $p < 0.05$. Thus, a DMRT test was carried out on mast cell infiltration in each group. Results on mast cell infiltration are presented in **Table 2**.

Based on the results of the analysis of mast cell infiltration presented in **Table 2**, it shows that group P1 (9.10 ± 1.17) had a statistically high number of mast cell infiltration compared to KP (5.12 ± 0.50), but group P2 (4.60 ± 0.86) and P3 (5.30 ± 0.73) had mast cell numbers that were not statistically different from KP. Groups P1 and P3 had mast cell counts that were not statistically different from KN (8.04 ± 1.14) and only group P2 was statistically low compared to KN. Thus, only the P2 group had no effect on mast cell infiltration.

The results of 1-way ANOVA analysis of macrophage infiltration showed that the treatment had a significant effect ($p < 0.05$). Based on this, a DMRT test was carried out to determine which groups had a significant influence. The results of the average number of macrophage infiltrations are presented in **Table 3**.

Table 3 Average number of macrophages on histopathological examination of rat skin.

| Group | Macrophage |
|-------|---------------------------|
| KP | 8.10 ± 0.36 ^a |
| KN | 16.00 ± 1.52 ^b |
| P1 | 5.96 ± 0.38 ^a |
| P2 | 6.42 ± 1.06 ^a |
| P3 | 6.44 ± 0.55 ^a |
| Sig. | 0.001 |

Note: Data is the mean ± SE from the results of the 1-way ANOVA test with $\alpha < 0.05$ and the superscript letters are the results of the DMRT test at a 95 % confidence interval. Different letter notations in the same column indicate statistically significant differences ($p < 0.05$) between groups. KP is a positive control using Asepso liquid soap and KN is a negative control using liquid soap without extract. P1, P2 and P3 respectively are liquid soap groups with 4.5 % extract, 9 and 15 %.

Based on the analysis of macrophage infiltration shown in **Table 3**, groups P1 (5.96 ± 0.38), P2 (6.42 ± 1.06) and P3 (6.44 ± 0.55) had a number of macrophages that were not statistically different from KP (8.10 ± 0.36). In addition, groups P1, P2 and P3 had a statistically low number of macrophages compared with KN (16.00 ± 1.52). Based on this, it can be concluded that groups P1, P2 and P3 have no effect on macrophage infiltration.

Soap functions in cleaning the skin because soap is a type of surfactant made from natural oils or fats. Surfactants have a bipolar structure, namely the tail group is hydrophobic and the head group is hydrophilic. The tail group will stick to the dirt and the head group which is associated with water helps spread and rinse the dirt [57,58]. Even though soap can clean the skin, the ingredients in soap can cause contact dermatitis [15]. In addition, products containing plants are believed to be better and have relatively few side effects, but they can still be involved in adverse skin reactions such as causing ACD [59].

The ACD reaction is mediated by T cells, this reaction can be triggered by irritants and allergens [19,60]. Triggers that can cause ACD reactions are called haptens. Haptens are chemicals with low molecular weight, one of which comes from natural substances such as plant extracts and natural fragrances [61]. A substance, including haptens, can penetrate the skin through several pathways, namely through the intercellular pathway into the lipid matrix, the intracellular pathway, or the sweat gland and hair follicles pathway. The penetration route through hair follicles can enhance penetration and can be a reservoir for penetration because substances deposited there can diffuse into the surrounding space, across capillary walls, and even reach the circulatory system. The time required for penetration is generally high because it takes hours to get through the skin or into the dermis [62].

Hapten penetration into the stratum corneum can trigger ACD [61]. However, haptens are not immunogenic but can be recognized by the immune system after the hapten binds to a non-specific skin protein, or what is known as a carrier protein. After the hapten is able to penetrate the stratum corneum, it will bind to the carrier protein to form a complex called the hapten-carrier-complex. Hapten binding to carrier proteins is formed from strong covalent bonds or weaker non-covalent bonds. In addition, lipophilicity, chemical structure and protein binding affinity are factors that influence the hapten sensitization ability. These factors are very important in influencing the penetration process into the stratum corneum and through the deeper layers of the epidermis or dermis to reach the antigen presenting cells (APC) [61]. Increased penetration and risk of sensitization from haptens can also be caused by impaired skin barrier function, such as inflamed skin [61].

The hapten-carrier complex will be captured by APCs, one of which is dendritic cells (DC), to form hapten-specific T cells in skin-draining lymph nodes (LN). This phase is called the sensitization phase and is the initial phase of ACD. Apart from that, in this phase alarmins and cytokines will also be produced by keratinocytes which create a pro-inflammatory environment in the skin. Thus, under the influence of mediators secreted by keratinocytes, DC will migrate from the skin tissue to the LN. During the migration process, the DC undergoes a maturation process and then the DC will mature. Mature DC will present antigen/hapten to naive T cells, or what is known as immunological priming. This results in clonal expansion of hapten-specific T cells and the formation of memory and effector T cells that will circulate throughout the body. Next, the hapten-specific T cells will be in the LN, blood and skin, this stage is the final stage of the sensitization phase [61].

When re-contact with the same hapten, hapten-specific T cells will infiltrate the skin in contact with the hapten and mediate a skin inflammatory response dominated by T helper (Th)-1 cells and CD8⁺ T cells or cytotoxic T cells (Tc cells), this phase is referred to as the elicitation phase [60]. The elicitation phase begins with re-contact with the hapten which causes the hapten to penetrate the stratum corneum. Similar to the sensitization phase, the hapten will bind to the carrier protein to form a hapten-carrier complex, which

will be captured by APC. Next, APCs will present them to hapten-specific T cells so that hapten-specific T cells are recruited en masse to the skin [61]. CD8⁺ T cells are localized in the epidermis and dermis, and are responsible for the infiltration of inflammatory cells, especially neutrophils. Meanwhile, CD4⁺ T cells or T helper cells are responsible for initiating extravasation of leukocyte cells into tissues [63]. Thus, leukocyte cells will extravasate into inflamed skin [18].

When leukocyte cells extravasate, interactions between leukocytes and endothelial cells (EC) are involved in the extravasation process. Leukocyte cells can migrate to inflamed tissue involving several stages. The initial stage of extravasation is chemoattraction. This stage results in activation of leukocyte rolling and adhesion by chemokines. Next, selectin (on the endothelium) will bind to its ligand (which is expressed by leukocytes). This will cause regulation of leukocyte rolling on the endothelium. After the rolling stage, leukocytes will adhere to blood vessels by expressing integrins on the leukocyte surface to bind to their ligands expressed on ECs. Next, leukocytes will cross the EC that lines the blood vessels, this stage is called trans-endothelial migration, and is the final stage in the extravasation process so that leukocytes can infiltrate the tissue [18]. These leukocyte cells will infiltrate the epidermis and dermis [64].

Leukocyte cells that infiltrate tissue, also known as inflammatory cells, will play their role in ACD inflammation. Neutrophils are the 1st immune cells to infiltrate sites of inflammation, especially in acute inflammation during hypersensitivity [65]. Neutrophils are also involved in the sensitization and elicitation phases of ACD. During the sensitization phase, mast cells trigger neutrophil infiltration which in turn causes neutrophils to trigger positive feedback by releasing mediators resulting in additional neutrophil infiltration into the inflammatory site [66]. Neutrophils also trigger a strong proinflammatory reaction by secreting large amounts of reactive oxygen species (ROS), exocytosis of granule proteins (including proteases) and the release of various cytokines (one of which is interleukin (IL)-13) [66,67]. In addition, neutrophils are capable of producing and releasing various pruritogens [68]. Pruritogens are one of the itch mediators that cause an itchy sensation on the skin [69].

Infiltration of potent inflammatory cells such as neutrophils, eosinophils and macrophages are also driven by chemoattractant (including IL-8 and C-C motif chemokine ligand (CCL)-5) secreted by basophils [70]. In addition, basophils will crosstalk with other inflammatory cells and play an important immunoregulatory role in the secretion of cytokines such as IL-4 and IL-13 in inflammatory tissue. Basophils secrete IL-4 which induces upregulation of adhesion molecules such as vascular cell adhesion molecule-1 on EC thereby facilitating eosinophil infiltration. IL-4 secreted by basophils also cooperates with tumor necrosis factor (TNF)- α in stimulating fibroblasts and EC to produce eotaxins, including CCL11 and CCL24, which can attract eosinophils. The development and maturation of basophils and eosinophils is strongly influenced by the granulocyte macrophage colony-stimulating factor (GM-CSF) family of cytokines, such as IL-3, IL-5 and GM-CSF [18,70].

Extravasation of basophils into inflamed skin is mediated by T cell-secreted IL-3 [18]. In addition, IL-3 promotes differentiation, degranulation and synthesis of inflammatory mediators including type 2 cytokines in basophils. Degranulation of basophils results in histamine release. Furthermore, histamine mediates Th1 and Th2 responses. Basophils not only enhance the migration of innate and adaptive immune cells to inflammatory tissues but also support the maturation, differentiation and function of B and T cells [71]. Apart from that, basophil differentiation and development are also supported by IL-5, GM-CSF, transforming growth factor (TGF)- β and nerve growth factor (NGF). TGF- β promotes IL-3-induced basophil differentiation. Meanwhile, IL-5 and NGF act synergistically with GM-CSF to enhance basophil differentiation of myeloid progenitor cells. Although these factors contribute to basophil development, IL-3 is the main driver of basophil development. IL-3 is the most potent factor on basophils, when compared with IL-5, GM-CSF and NGF. Thymic stromal lymphopoietin (TSLP) is also a cytokine that plays a role

in the development of basophils. TSLP production is mainly by epithelial cells and plays a role in pathogenesis which has a Th2 response [71].

Secretion of GM-CSF, IL-8 and IL-10 by eosinophils, allows eosinophils to attract neutrophils, auto-stimulation and immunoregulation. Eosinophils can also exert cytotoxic effects, through the synthesis of TNF- α . In addition, eosinophils are a source of IL-31, which is a cytokine associated with inflammation and pruritus. Eosinophils can cause edema by releasing their granule proteins and by producing leukotrienes which have a direct vasodilation effect on blood vessels or indirectly through stimulation of mast cells and basophils [21].

The P1 treatment group had an average number of mast cells that was greater than the other treatment groups. In addition, the average number of mast cells in the P1 treatment group was not significantly different from the KN group. Mast cell infiltration is associated with both acute and chronic responses [19]. Mast cells also secrete proinflammatory mediators (such as IL-4, IL-5, IL-6, IL-13, TNF- α) and chemokines (such as CCL2, CCL3 and CCL4), proteases and growth factors [20,60]. Th2-specific cytokines promote mast cell degranulation which results in itching [19]. In addition, the degree of itching sensation correlates with the production of the cytokine IL-31 in ACD lesions [19].

Degranulation of mast cells to external stimuli can occur within seconds, allowing for a faster response than other immune cells in the tissue. Therefore, in many cases, mast cells act as initiators of the immune response [60]. In addition, mast cells also secrete bioactive amines (histamine and serotonin), triggering dilation and permeability of blood vessels, which ultimately leads to the formation of edema. The vascular response is further enhanced by TNF, proteases and eicosanoids which activate vascular EC. Then, mast cells will initiate neutrophil infiltration, such as through direct degranulation of TNF- α into the bloodstream which causes priming of circulating neutrophils and increases neutrophil extravasation, through the secretion of neutrophil attractants such as chemokine (C-X-C motif) ligand (CXCL)-1 and CXCL-2, and by IL-33 secretion. Mast cells also enhance neutrophil effector function [60,72]. In addition to controlling neutrophil numbers, mast cells also contribute to hapten sensitization in various ways. Meanwhile, in the elicitation phase, mast cells are one of the main factors that cause inflammation [20].

The KN treatment group was a group that had a higher average number of macrophages than other treatment groups. In addition, the average number of macrophages in all treatment groups was significantly different from the KN treatment group. When ACD occurs, monocytes migrate to the site of inflammation and differentiate into macrophages, in the elicitation phase [73]. Macrophages are stimulated by IL-6 to mature so that mature macrophages can carry out phagocytosis efficiently [74,75]. High macrophage activity correlates with high levels of cytokines or pro-inflammatory mediators released [74,75]. Macrophages are divided into M1 and M2 macrophages. M1 macrophages are activated classically, whereas M2 macrophages are activated alternatively. M1 macrophages are induced by toll-like receptor (TLR) and/or interferon gamma ligands, their cell surface markers and cytokines including IL-1, TNF- α , IL-12, IL-17 and IL-23. M1 macrophages express TLR-4, IL-1 β and nuclear factor kappa B (NF- κ B) and produce various inflammatory cytokines including TNF- α , IL-6 and IL-12. Meanwhile, M2 macrophages are induced by IL-4, IL-10, IL-13 and immune complexes [76]. M2 macrophages secrete large amounts of proinflammatory cytokines such as IL-1 β , IL-6 and TNF- α [73].

The results of this study show that the effect of liquid soap on inflammatory cell infiltration is greater for liquid soap without the addition of extracts. This can be caused by the alkyl structure of the soap which can affect the skin barrier because there is a correlation between the length of the alkyl surfactant chain and the effect of the surfactant on the skin [77]. The skin barrier is located mainly in the stratum corneum, consisting of corneocytes surrounded by intercellular lipid lamellae [78]. An intact skin barrier can prevent the penetration of dangerous molecules into the skin so that the skin barrier has an important role in the

occurrence of contact dermatitis. If the skin barrier is disrupted, irritants can penetrate the epidermis layer resulting in the release of cytokines which will cause inflammation. Additionally, a damaged skin barrier allows allergens to enter the skin. Thus, allergens can interact with Langerhans cells and T cells, triggering inflammation which results in ACD [79].

Apart from the alkyl structure of soap, the amount of free alkali in soap also affects the skin barrier. If a soap has a greater amount of alkali, it will result in a higher pH value in the soap [80]. Alkaline compounds in soap can neutralize or even damage (if it is too alkaline) the skin's acid mantle, which plays a role in blocking bacteria and viruses, and cause the skin to become dry due to loss of water, allowing irritation and allergies to occur [80]. Alkali that does not react with fatty acids during saponification affects the amount of alkali in soap [80]. The more alkali remaining from saponification causes more free alkali in the soap [81]. The lower the free alkali contained in the soap, the more it guarantees the perfection of the saponification reaction [42]. The saponification process in this study used KOH with a concentration of 35 %. When making soap with a KOH concentration of 35 %, the alkali content and pH of the soap are in accordance with the standards set by National Standardization Agency of Indonesia (SNI) [82]. Apart from that, this research used a volume of 50 mL oil and 30 mL KOH. This formulation is the best formulation in producing liquid soap volumes with soap characteristics that comply with SNI standards [42]. Based on this, the soap formulation used in this study produces an appropriate amount of free alkali so that the relatively greater effect of soap without the addition of extracts on inflammatory cell infiltration is not caused by the amount of free alkali in the soap. Meanwhile, liquid soap with the addition of betel and aloe vera extracts overall had a relatively smaller effect on inflammatory cell infiltration when compared to liquid soap without the addition of extracts. Thus, it shows that the addition of betel extract and aloe vera has a better effect on the amount of inflammatory cell infiltration.

The addition of betel leaf extract has an anti-inflammatory effect and minimizes irritation due to the bioactive compounds it contains. Betel leaves contain high levels of bioactive compounds such as polyphenols, flavonoids, alkaloids, eugenol, carvacrol and chavicol [83]. Essential oil from betel leaves has a high eugenol and linalool content and provides a strong, distinctive aroma [84]. The linalool compound has natural benefits such as calming the skin and minimizing irritating effects on the skin [84]. Meanwhile, the eugenol compound is the main component which has the highest concentration in betel leaf essential oil at 48.14 % and in betel leaf ethanol extract at 44.17 % [84,85]. Apart from having strong antibacterial properties, eugenol also has anti-inflammatory and anti-allergic properties [86,87]. However, on the other hand, it is stated that eugenol can irritate the skin and cause ACD [87]. Eugenol can cause ACD because it reacts directly with proteins to form hapten-proteins and is reactive. However, low concentrations of eugenol can act as an antioxidant and anti-inflammatory agent [87]. The anti-inflammatory effect of eugenol is in the form of inhibition of pro-inflammatory mediators such as cyclooxygenase-2, NF- κ B, IL-6 and leukotriene C4 [86]. Eugenol also shows an inhibitory effect on excessive leukocyte migration in the inflammatory process. This effect is not related to toxic effects because eugenol does not cause significant changes in cell viability [88]. In addition, eugenol is able to inhibit ROS production in human neutrophils [86].

Aloe vera also has anti-inflammatory effects, apart from having antimicrobial, antiviral and antibacterial effects [89,90]. This anti-inflammatory effect comes from the compounds contained in it such as aloe-emodin, aloin, aloesin, emodin and acemannan, which can relieve symptoms related to chronic inflammation [90]. *In vitro* research on the ability of aloe vera to protect the skin shows that aloe vera and its main compounds (aloesin, aloin and emodin) are able to provide protection, especially through antioxidant and anti-inflammatory mechanisms [91]. Aloin is able to reduce IL-8 production, lipid peroxidation and ROS formation [92]. Aloe vera is also able to weaken inflammation by inhibiting the pro-

inflammatory cytokines IL-6 and TNF- α . TNF- α plays an important role in the induction of inflammation activated by the immune response of macrophages and cytokines [90]. In addition, through inhibiting IL-6 and IL-8, reducing leukocyte adhesion, increasing IL-10 levels, and decreasing TNF- α levels, causing aloe vera to be effective in inhibiting inflammatory reactions [93].

Based on the results of this study, it was shown that liquid soap with extract concentrations of 9 and 15 % was not significantly different from the positive control in the infiltration of PMN cells, mast cells and macrophages. However, liquid soap with an extract concentration of 15 % was not statistically significantly different from the negative control on mast cell infiltration. Meanwhile, liquid soap with an extract concentration of 9 % was statistically significantly different from the negative control in the infiltration of PMN cells, mast cells and macrophages. Although the effect of liquid soap with an extract concentration of 9 % is not statistically significantly different from liquid soap with an extract concentration of 15 %. However, liquid soap with an extract concentration of 9 % is the liquid soap with the best extract concentration because liquid soap with this concentration is more efficient in the production process when compared to liquid soap with an extract concentration of 15 %. Therefore, it can be concluded that, liquid soap with an extract concentration of 9 % has the best effect because it causes relatively less inflammatory cell infiltration, its effect on the average number of inflammatory cells is close to the effect given by the positive control group with Asepso liquid soap, and has a more efficient concentration in the production process compared to liquid soap with an extract concentration of 15 %.

Conclusions

The use of used cooking oil liquid soap with betel and aloe vera extracts at an extract concentration of 9 % was considered to have no effect on the infiltration of PMN cells, mast cells and macrophages. Therefore, it can be concluded that using liquid soap made from used cooking oil containing betel and aloe vera extract with an extract concentration of 9 % is the soap with the best effect and is predicted to be safe to use.

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