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*Original Article*

# The Effects of Activated Carbon and Bentonite on Physicochemical Characterization of Red Fruit Oil (*Pandanus conoideus* Lam)

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**Abstract:** This research aims to see the quality of red fruit oil (RFO) before and after being treated with activated carbon and bentonite. The use of activated carbon and bentonite as adsorbents is because they are relatively cheap and widely available. The quality of red fruit oil from wet extraction can be seen from the analysis of physicochemical characterization determination, which includes the determination of peroxide value (PV), acid value (AV), saponification value (SV), p-anisidine value (PAV), and iodine value (IV). In this study, AV RFO ranged from 9.22 – 10.23 mg KOH/g, PV RFO ranged from 5.57 – 7.56 meq/kg, SV RFO ranged from 96.57 – 110.24 mgKOH/g, IV RFO ranged from 70.00 – 70.83 $gI_2$/100g, and PAV RFO ranged from 5.97 – 7.00 meq/kg. The purification of RFO using activated carbon and bentonite has a significant effect ($p < 0.05$) affected the physicochemical characteristics of the oil, where the process succeeded in improving the quality of RFO by increasing the iodine value, but reducing the peroxide value, acid value, saponification value, and p-anisidine value.

**Keywords:** Activated carbon, Adsorbent, Bentonite, Red fruit, Wet extraction

## 1. INTRODUCTION

Papua is one of the island that has red fruit (*Pandanus conoideus* Lam)[1][1]. Red fruit contains many active components such as tocopherols, carotene, cryptoxanthin, oleic acid, and palmitate acid[2], [3], [4], [5]. The Papuan people widely utilize red fruit as a source of edible oil and as an antioxidant[2], [3]. Traditionally, Papuans use a wet extraction method to produce red fruit oil. The wet extraction method requires adding a certain amount of water during the extraction process[6].

Free fatty acids (FFA) may be present in extracted red fruit oil (RFO), lowering its quality. The quality of RFO can be seen by looking at its characterization, namely by determining peroxide value (PV), acid value (AV), iodine value (IV), saponification value (SV), and p-anisidine value (PAV). The PV indicates the degree of oil deterioration. The amount of FFA in the oil is indicated by

the AV. The IV indicates the unsaturation of the fatty acids that make up the fat. The SV indicates the molecular weight of the oil. The anisidine value shows the number of secondary oxidation products in the oil[6], [7]. To improve the quality of RFO, RFO needs to be purified by adding adsorbents that aim to absorb impurities in oil such as pigments, components, and FFA. Bentonite, attalpugite, chitosan, and activated carbon are examples of adsorbent used in oil refining. [8]. In this study, RFO purification was carried out using bentonite and activated carbon as adsorbents because they are relatively cheap and easily found in everyday life. Previous studies reported that the use of the bentonite and activated carbon can improve the quality of hazelnut oil and used cooking oil [9], [10]. This study aims to see the quality of RFO before and after being given bentonite and activated carbon adsorbents. The addition of bentonite and activated carbon will likely improve the quality of RFO.

## 2. MATERIALS AND METHODS

### *2.1. Materials*

Reagents used were ethanol p.a, chloroform, Wijs reagent, glacial acetic acid (Supelco, Germany), sodium thiosulfate, hydrochloric acid (HCl), anhydrous sodium sulfate ($Na_2SO_4$), potassium hydroxide (KOH), potassium iodide (KI) (Merck, Germany), and distilled water. The adsorbents used were activated carbon and bentonite. The red fruit used is collected from Papua, Indonesia.

#### 2.1.1. Preparation of RFO

Oil from red fruits was prepared using the wet extraction method. Red fruits were peeled to separate the drupes from the pedicels using a knife and mixed with water (1:2 by weight/volume). The mixture was then heated for 20 minutes at 103°C and filtered. The paste obtained was then reheated for 60 minutes at 103°C. Next, centrifuge the paste for 10 minutes at 3000 rpm to separate the RFO from water and solid residue[11], [12]. Next, the oil was put into a centrifuge tube and anhydrous sodium sulfate ($Na_2SO_4$) was added as much as 10% of the sample weight, then homogenized with a vortex for 5 minutes and continued with centrifugation for 15 minutes at 5000 rpm. The precipitate and oil were separated and the oil was put into a new flask. After that, add the adsorbent previously activated by heating 250°C for 1 hour as much as 2% of the weight of the oil. Furthermore, the oil was stirred for 30 minutes at 500 rpm while heated at 40°C to improve adsorption kinetics. The oil was separated from the sediment and put into a dark-bottle[13].

### *2.2. Characterization of RFO*

#### 2.2.1. Determination of PV, AV, IV, and SV

Determination of PV, AV, IV, and SV in RFO according to the standard methods for the analysis of fats and oils in the official analytical methods of AOAC International[14].

#### 2.2.2. Determination of PAV

Determination of PAV in RFO samples using IUPAC standard method (1987) using UV-Vis spectrophotometer[15].

### *2.3. Statistical analysis*

One-way ANOVA from Minitab (version 21) software was used to examine the RFO characterization results, with a significance level of $p < 0.05$. The Tukey test is used to discover significant differences in the analysis if the data from the results show a significant difference.

## 3. RESULTS AND DISCUSSION

Red fruit extracted using the wet extraction method to produce oil is added with anhydrous sodium sulfate ($Na_2SO_4$) to reduce the water content in the oil extract because water can affect the quality and stability of oil[16]. RFO is then purified by adding adsorbents. The utilized adsorbents consists of activated carbon and bentonite. Bentonite is a clay containing the main mineral montmorillonite and is used as a vegetable oil bleach[17]. Activated carbon is charcoal that is activated to have a high absorption of odor, color, toxic substances, and other chemical substances used for food oil purification[18]. Before being added, bentonite and activated carbon are activated to enlarge the pores so that the surface increases size and affects adsorption[19]. The appearance of red fruit oil before and after the addition of bentonite and activated carbon is visually not much different (Figure 1).


**Figure 1.** Visual appearance of crude RFO (a), RFO applied to activated carbon treatment (b), and RFO applied to bentonite treatment (c).

All RFOs were characterized to determine the peroxide value (PV), acid value (AV), iodine value (IV), saponification value (SV), and p-anisidine value (PAV). The RFO characterization results can be seen in Table 1. These charaterization parameters provide an overview ogf the quality and grade of the red fruit oil. Unfortunately, there is no Indonesia National Standard (SNI) for red fruit oil to date.

**Table 1.** Peroxide value, acid value, iodine value, saponification value and p-anisidine value of crude RFO and RFO purified with activated carbon and bentonite

| Parameters | Crude RFO | RFO treated with activated carbon | RFO treated with bentonite | p-Value |
|---|---|---|---|---|
| Acid value (mgKOH/g) | $10.23 \pm 0.03^{a}$ | $9.73 \pm 0.03^{b}$ | $9.22 \pm 0.03^{c}$ | 0.000 |
| Peroxide value (meq/kg) | $7.56 \pm 0{,}00^{a}$ | $6.57 \pm 0.00^{b}$ | $5.57 \pm 0.00^{c}$ | 0.000 |
| Saponification value (mgKOH/g) | $110.24 \pm 0{,}78^{a}$ | $103.89 \pm 0.79^{b}$ | $96.57 \pm 0.83^{c}$ | 0.000 |
| Iodine value (g $I_2$/100g) | $70.00 \pm 0.22^{b}$ | $70.40 \pm 0.25^{ab}$ | $70.83 \pm 0.22^{a}$ | 0.013 |
| p-anisidine value (meq/kg) | $7.00 \pm 0.04^{a}$ | $6.56 \pm 0.01^{b}$ | $5.97 \pm 0.01^{c}$ | 0.000 |

* Significant differences ($p < 0.05$) are indicated by the presence of different letters in the same row.

The data in Table 1 shows that RFO refining using activated carbon and bentonite has a significant effect ($p < 0.05$) on AV, PV, IV, SV, and PAV. The refining process using bentonite and

activated carbon can improve the physicochemical characteristics of oil by adsorbing oxidation products including peroxides, ketones, FFA, aldehydes, and phosphatides in oil [20].

The application of activated carbon and bentonite to RFO has successfully reduced AV, PV, SV, PAV, and increased IV. The AV is a measure of the quantify of FFA in the oil that are the result of the hydrolysis process of the oil during the extraction and storage process[6], [7], [21]. In this study, the AV of crude RFO was 1023 ± 0.03 mgKOH/g, RFO treated with activated carbon was 9.73 ± 0.03 mgKOH/g, and RFO treated with bentonite was 9.22 ± 0.03 mgKOH/g. The high acid value of RFO is due to the hydrolysis reaction in the extraction process, which uses water and high temperature heating, thus increasing the oil's free fatty acid content [22]. The AV of RFO with the addition of bentonite has better quality. This is because the ability of bentonite to absorb free fatty acids is more remarkable due to its small particle size. The smaller the particle size, the greater the diffusion rate of solute molecules into the pores of the adsorbent[8]. In addition, the ability of bentonite to absorb free fatty acid components in oil is due to the silanol group (Si-OH) formed in the $SiO_2$ compound in bentonit during activation. Silanol groups are polar and able to bind to other polar compounds, such as free fatty acids. Hydrogen atoms from silanol groups on bentonite bind to oxygen-carbonyl groups (-C=O) on free fatty acids through hydrogen bonds so that free fatty acids are bound to the bentonite surface and can reduce the acid value in oil. This bonding occurs because hydrogen atoms tend to bond with oxygen atoms that are more electronegative [10], [23]. Peroxide value indicates the degree of oil damage[6], [7]. In this study, the PV of crude RFO was 7.56 ± 0.00 meq/kg, RFO treated with activated carbon was 6.57 ± 0.00 meq/kg, and RFO treated with bentonite was 5.57 ± 0.00 meq/kg. RFO PV with the addition of bentonite has better quality. This is because bentonite formed silanol groups (Si-OH) contained in the $SiO_2$ compound during activation. Silanol groups have polar properties and can bind to other compounds with specific functional groups, such as peroxide groups in peroxide compounds formed in oxidized oil. The silanol group on bentonite binds to the peroxide group in the peroxide compound through hydrogen bonds, which causes the peroxide molecule to bond to the bentonite surface so that the oxydative stability of the oil increases and the PV of the oil is reduced as the amount of peroxide in the oil decreases. This hydrogen bond occurs between the silanol group's hydrogen atom and the peroxide group's oxygen atom[10], [23].

The SV indicates the molecular weight of the oil[6], [7]. In this study, the SV of crude RFO was 110.24 ± 0.78 mgKOH/g, RFO treated with activated carbon 103.89 ± 0.79 mgKOH/g, and RFO treated with bentonite 96.57 ± 0.83 mgKOH/g. The saponification value of RFO in this study is in accordance with the report of Murtiningrum (2005), which ranged from 88.83 – 212.59 mgKOH/g. The molecular weight of fatty acids in the oil decreases as the SV increases. The addition of bentonite and activated carbon to RFO can reduce saponification value because bentonite and activated carbon adsorb impurities such as FFA and short-chain triglycerides in oil[24], [25], [26]. The unsaturation of the fatty acids that comprise up the fat is indicated by the IV[6], [7]. In this study, the IV of crude RFO was 70.00 ± 0.22 $gI_2$/100g, RFO treated with activated carbon 70.40 ± 0.25 $gI^2$/100g, and RFO treated with bentonite 70.83 ± 0.22 $gI_2$/100g. The provision of activated carbon and bentonite can increase the IV in RFO by absorbing unwanted components, such as saturated compounds so that the concentration of double bonds in the oil increases[27]. As the IV increases, the oil's degree of unsaturation increases, and the quality improves[13]. The PAV indicates the number of secondary oxidation products in the oil[6], [7]. In this study, the PAV of crude RFO was

7.00 ± 0.04 meq/kg, RFO treated with activated carbon was 6.56 ± 0.01 meq/kg, and RFO treated with bentonite was 5.97 ± 0.01 meq/kg. Activated carbon and bentonite can inhibit the formation of secondary oxidation products to reduce the PAV [28].

## 4. CONCLUSION

The purification of red fruit oil using activated carbon and bentonite significantly ($p < 0.05$) affected the physicochemical characteristics of the oil, such as PV, AV, SV, IV, and PAV. The process successfully improved the quality of RFO by decreasing acid, peroxide, saponification, and p-anisidine and increasing iodine value. Providing activated carbon and bentonite can be an alternative to improve RFO quality effectively and economically.

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**Conflicts of interest:** The authors declare no conflict of interest.

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*Original Article*

# Optimization of Hydroxypropyl Methylcellulose (HPMC) and Glycerine in Essence of Sheet Mask Containing *Centella asiatica* (L.) Urb. Extract and Snail Mucus (*Achatina fulica*) as A Moisturizer

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**Abstract:** Various natural ingredients have been formulated as moisturizers in cosmetics, both from natural and animal raw, especially *Centella asiatica* (L.) Urb. Extract and snail mucus (*Achatina fulica*). However, further research is needed to determine the synergistic effect of these two ingredients in one cosmetic preparation, namely sheet masks. The mask sleeve, which is a sheet mask applicator, will dry longer than other masks, therefore the penetration shown as a moisturizer is better. Gelling agents (such as *hydroxypropyl methyl cellulose* (HPMC)) and humectants (such as glycerine) are important ingredients in sheet masks. This research aims to optimization HPMC and glycerine in a sheet masks *Centella asiatica* and snail mucus. Optimization of HPMC and glycerine with a ratio of 0.3–3% was carried out using the Software Design Expert Stat-Ease® version 10 with *Simplex Lattice Design* method. The responses from optimization of the preparation were pH, viscosity, absorption ability and skin moisture. The optimum formula was obtained at a concentration of 0.589% HPMC and 2.711% glycerine with pH of 5.64 ±0.02; viscosity of 4581.8± 59.80 cPs, absorption power in scale of 4.6±0,55 , and skin moisture of 32.09%±3.21. The irritation test has a score of 0 or does not cause irritation.

**Keywords:** *Centella_asiatica*, snail_mucus, glycerine, HPMC, sheet_mask

## 1. INTRODUCTION

While Indonesia boasts abundant natural resources, the country suffers from significant environmental damage, contributing to climate change. Climate change leads to a rise in air temperature, which can result in quicker skin dryness and paleness [1]. One of the innovations to overcome this is to use a moisturizer in a sheet masks. Sheet masks are a form of moisturizing cosmetics that are currently popular because they are hygienic and practical to use. Sheet mask preparations are considered more profitable because they can be used more easily than other masks such as peel-off, organic, or paste masks. In addition, the mask sleeve, which is a sheet mask applicator, will dry longer than other masks so that the penetration shown as a moisturizer is better [2] [3].

Various natural ingredients have been formulated as moisturizes, both from plants and animals. *Centella asiatica* (CA) is a plant material that contains asiaticoside. This group will stimulate the formation of the extracellular matrix so that it will increase the percentage of collagen and

strengthen the skin [4]. Other sources are also obtained from animals, namely snails which have mucus. The mucus in snails contains allantoin, collagen, elastin, and glycolic acid. Allantoin is a secure and efficient substance for safeguarding the skin, aiding in the prevention of skin damage by promoting cell growth and facilitating the healing of wounds [5]. Moreover, it enhances the moisture level in the extracellular matrix and creates compounds with substances that can cause irritation and sensitivity [6].

Based on the description, researchers are interested in combining CA and snail mucus as an essence in sheet mask. The important excipients require additional ingredients to become preparations with good physical characteristics so as to support the function of these preparations. Gelling agent is an important material because it will affect in physical characteristics such as pH, viscosity, absorption of the preparation [7]. Besides HPMC, humectants are another component that is influential because they can retain water in the skin so that moisture activity is obtained in the skin. In this study, HPMC K4M was used as a gelling agent and glycerine as a humectant. The optimization of HPMC and glycerine used in this study used a range of 0.3%-3%. This was referred to from the concentration of glycerine as a humectant in the range of 0.2-65.7% [8] and the concentration of HPMC used for gelling agent was 0.25%-5% [9].

The purpose of this study was to determine the effect of combination of HPMC and glycerine on the chemical and physical characteristics of the essence sheet mask containing CA and snail mucus. Furthermore, this study found out the optimum ratio of HPMC and glycerine on the physical characteristics and moisture activity of the essence sheet mask containing CA and snail mucus.

## 2. MATERIALS AND METHODS

### *2.1. Material and Tools*

Materials used in this study were *Centella asiatica* (L.) Urb. (B2P2TOOT, Tawangmangu, Indonesia), snail mucus (CV. Keong Sumber Makmur, Indonesia), allantoin (Sigma Aldrich, catalague no 102505490), compressed paper masked (Fresca, Indonesia), HPMC K4M (Dow Chemical), glycerine (Brataco Chemical), propylene glycol (Brataco Chemical), DMDM-hydantoine (Clariant), perfume and aqua destillata.

Instruments used in this study were rotary evaporator (Heidolph), glass funnel, hotplate, magnetic stirrer, digital balance (Ohaus), Brookfield viscometer (DV-1 Prime), pH meter (Trans Instruments Walklab Series), infra red spectrophotometer (Agylent), and Skin Analyzer Moisture (BIA Skin Moisture Analyzer series ZL201430286553.9).

### *2.2. Methods*

#### 2.2.1. Identification and Determination

Determination to obtain certainty about the plants used in this research. Identification and determination of *Centella asiatica* was carried out at the Laboratory of the Research and Development Center for Medicinal Plants and Traditional Medicine Tawamangu, Karanganyar, Central Java. Snail determination was carried out in the Ecology and Biosystematics laboratory, Department of Biology, Faculty of Science and Mathematics, Dipenogoro University, Semarang.

#### 2.2.2. Extraction *Centella asiatica* (CA) and identification active substance

Extraction was carried out using the remaceration method by soaking dried CA herb powder in 70% ethanol in a ratio of (1:7) for 4 days, with solvent replacement every 24 hours. The extract

obtained was followed by a qualitative analysis test using thin layer chromatography (TLC) to determine the active substance content in the extract. The Rf obtained in the TLC test is compared with the Rf in the reference [10].

2.2.3. Analysis of allantoin compound in snail mucus sample

Identification of allantoin compound in snail mucus obtained from CV. Keong Sumber Makmur was carried out with FTIR instrument and compared with allantoin standard spectrum [11].

2.2.4. Optimization HPMC and glycerine in sheet mask containing *Centella asiatica*

Manufacturing begins by developing HPMC into a gelling agent in aqua distillate at a temperature of 70°C then leaving it overnight. In the subsequent stage, glycerine and propylene glycol were added and mixed on a hot plate at 70°C at a speed of 200-400 rpm until homogeneous, then mixed with HPMC gel. DMDM-hydantoin as a preservative was added until homogeneous. The active ingredients (CA and snail mucus) are weighed and added gradually. Finally, fragrance was added and the remaining solvent was added up to 60 grams. A sheet mask sleeve was prepared and then 15 grams of the preparation was placed in the sleeve, which was then placed in a tightly closed foil bag. The optimization used in this research was used range of 0.3%-3%, this is referred to from the concentration of glycerin as a humectant in the range of 0.2-65.7% [12] and the concentration of HPMC used for gelling agent is 0.25%-5% [9]. As for the optimization composition from Software Design Expert Stat-Ease® version 10 for each run can be seen in table 1.

**Table 1.** Formulation Essence Sheet Mask with Different Concentrations of HPMC and Glycerin

| **Formula** | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Ingredients (%w/w)** | **F1** | **F2** | **F3** | **F4** | **F5** | **F6** | **F7** | **F8** | **F9** | **F10** |
| **CA Extracts** | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| **Snail mucus** | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 |
| **Propilenglycol** | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| **HPMC** | **0.3** | **3** | **0.975** | **0.3** | **2.325** | **1.65** | **1.65** | **3** | **0.3** | **3** |
| **Glycerinee** | **3** | **0.3** | **2.325** | **3** | **0.975** | **1.65** | **1.65** | **0.3** | **3** | **0.3** |
| **DMDM-*hydantoine*** | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| **Perfume** | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| **Aquadest (ad)** | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |

2.2.5. Chemical and physical characteristic test of essence sheet mask [13] [14]

a. Organoleptic test

The organoleptic test is a physical test of the essence sheet mask that has been made which includes observing the smell, color and shape.

*b. pH test*

The pH test is a constant pH check by a pH meter after calibration with pH 4 and pH 7 buffer solutions. The electrode was immersed in the preparation, and the recorded pH value was noted.

c. Viscosity test

Viscosity test is a viscosity test that is carried out using a Brookfield viscometer. This test uses a spindle diameter of 62 mm and speed of 4 rpm.

d. Absorption ability test

The absorption ability test was carried out by weighing 15 grams and then placing it in a porcelain cup, then inserting the mask sleeve. The time required for the preparation to absorb into the mask sleeve is given the appropriate score. The researcher has formulated their own range for the data to facilitate assessment. This custom range was designed to simplify evaluation (table 2).

**Table 2.** Absorption Ability Test Scores

| Score | Time |
|---|---|
| 5 | 0 second – 1 minutes 59 seconds |
| 4 | 2 minutes – 3 minutes 59 seconds |
| 3 | 4 minutes – 5 minutes 59 seconds |
| 2 | 6 minutes – 7 minutes 59 seconds |
| 1 | 8 minutes – 10 minutes |

e. Skin moisture test

The moisture test was carried out on 12 volunteers and it was confirmed that they did not have skin diseases. This test already has an Ethical approval from Stifar Yayasan Pharmasi Semarang, Indonesia with number permit 427/YP/NA/KEPK/STIFAR/EC/XI/2023. The test used a skin moisture analyzer with the pre-post-test group design method. The test preparation was applied to the panelists on the upper arm, measuring 3x5 cm in the morning and evening. Determination of the percentage of skin moisture was carried out for 2 hours, 3 days and 7 days after using the preparation [15] using BIA Skin Moisture Analyzer series ZL201430286553.9. The results are then calculated:

$$\%Mositure\ activity = \frac{(volunteers'\ skin\ moisture\ scores{-}n){-}(volunteers'\ skin\ moisture\ scores\ day{-}0)}{volunteers'\ skin\ moisture\ scores\ day{-}0}\ x\ 100\% \text{.....(1)}$$

When the moisture score is in the range of 0-45%, then the skin is categorized as dry skin; a score of 46-55% is categorized as moist skin; and 56-100% is categorized as very moist skin (BIA Skin Moisture Analyzer).

f. Skin irritation test

This test has already received ethical approval from Stifar Yayasan Pharmasi Semarang, Indonesia, under permit number 427/YP/NA/KEPK/STIFAR/EC/II/2023. The irritation test employed a specific method, which involved conducting an open patch test on the inner forearm of ten panelists. In this procedure, 1 mL of the prepared solution was applied to a designated area (measuring 2.5 x 2.5 cm), typically on the inner left arm. The arm was left exposed for approximately 24 hours, and any skin reactions that occurred were carefully observed. The presence of redness, itching, or swelling on the treated inner forearm skin indicated a positive irritation reaction [16].

**Table 3.** Grading of Erythema and Oedema [17]

| Erythema and Eschar Formation | Score | Oedema formation | Score |
|---|---|---|---|
| No erythema | 0 | No oedema | 0 |
| Very slight erythema (barely perceptible) | 1 | Very slight oedema (barely perceptible) | 1 |
| Well-defined erythema | 2 | Slight oedema (edges pf area well defined bu definte raising) | 2 |
| Moderate to severe erythema | 3 | Moderate oedema (raised approximately 1 mm) | 3 |
| Severe erythema (beef redness) to eschar formation preventing grading of erythema | 4 | Severe oedema (raised more than 1 mm and extending beyond area of exposure) | 4 |

## 3. RESULTS AND DISCUSSION

### *3.1. Extract Centella asiatica*

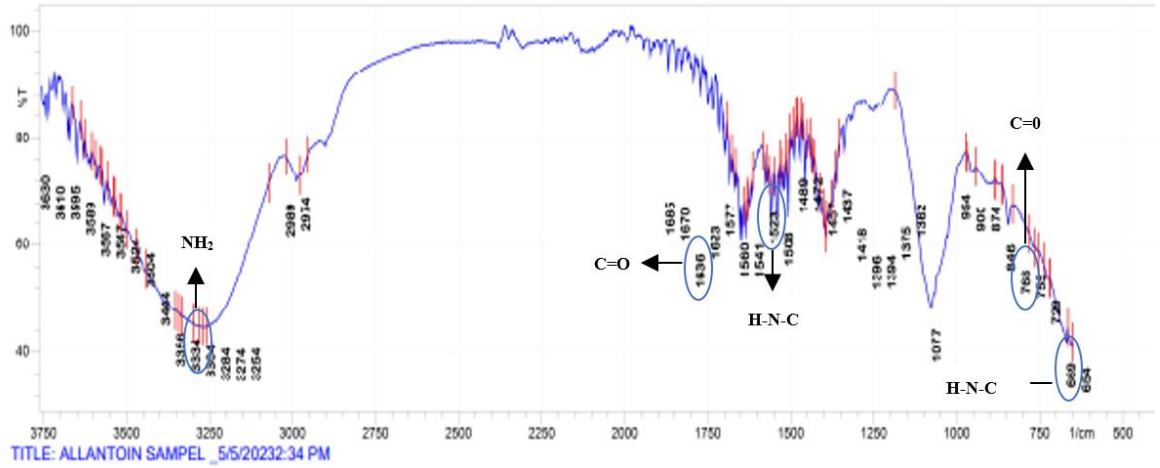
The extract obtained from 200 grams of *Centella asiatica* herba is 38.5300 grams (the yield percentage produced was 19.26%). The choice of the remaceration method was based on previous research of Rahimah et al., 2021 [18] using the remaceration method to extract CA and obtained a yield of 30.1%, while compared the research of Hapsari et al., 2017 [10] which used the maceration method to obtain a yield of 5.9%. The solvent used in the extraction this time was 70% ethanol because research stated that asiaticoside was mostly found in 70% ethanol extractor compared to 30% and 50% ethanol. An ethanol free test was also carried out on the extract to ensure that the extract was free from ethanol solvent. A positive ethanol-free result is indicated by the absence of ester odor from the ethanol extract which has been reacted with concentrated sulfuric acid and 1% acetic acid [19]. The results of a qualitative analysis with TLC showed that the extract contained asiaticoside, flavonoids, tannins, saponins and steroids (Table 5).

**Table 5.** Qualitative Analysis with TLC Result of *Centella asiatica* [10, 20]

| Compounds | Research Results | Reference Results | Conclusion |
|---|---|---|---|
| Asiaticoside | Purple Staining at UV 254 nm; Rf: 0.25 | Blue or purple staining; Rf: 0.24 | Positive |
| Flavonoids | Brownish yellow stain; Rf : 0.66 | Yellow or yellow-brown stain; Rf: 0.5-0.7 | Positive |
| Alkaloids | No stains are formed | Brown or orange stains; Rf: 0.2-0.8 | Negative |
| Saponin | Yellow stain; Rf: 0.46 | Blue to blue violet stains, sometimes in the form of red, yellow, dark blue, purple, green or yellow-brown spots in visible light; Rf: 0.11-0.40 | Positive |
| Tannin | Blackish purple stain; Rf: 0.78 | Blackish purple stain; Rf: 0.66-0.79 (Fatih, 2016) | Positive |
| Steroids/terpenoids | Purple stain; Rf: 0.30 | Red, violet, green or purple stains; Rf: 0.06-0.70 | Positive |

*3.2. Snail Mucus Analysis*

Allantoin in snail mucus sample was identified by using FTIR. The spectra analysis in Figure 1 reveals that the allantoin present in snail mucus closely resembles the spectra profile of the allantoin standard used as a reference. However, the allantoin in snail mucus exhibits a reduced intensity. This disparity in intensity can be attributed to the impurities present in the snail mucus sample, implying that the purity level of the sample is inferior to that of the standard allantoin [21].




_____ : Snail Mucus Spectra (sample)

_____ : Allantoin Spectra (sigma)

**Figure 1.** Comparison of Spectra Results between Snail Mucus Sampel and Allantoin Standard (Sigma no 102505490)

Based on previous research, bands within the range of 3500–3350 $cm^{-1}$ and 3450–3150 $cm^{-1}$, which correspond to the asymmetric and symmetric stretching vibrations of amino groups, were discernible. Additionally, bands associated with the vibrations of H-N-C groups were detected in the range of 1550–1500 $cm^{-1}$. Absorption bands around 1636–1640 $cm^{-1}$ were likely attributed to stretching vibrations of C = O and C = C groups, particularly associated with amidic groups. A stretching vibration of low intensity in the range of 1120–910 $cm^{-1}$ was observed, likely originating from the C-$NH_2$ aliphatic amine group. Bands spanning from 860–510 $cm^{-1}$ and a separate band at 450 $cm^{-1}$ were indicative of N-H out-of-plane bending vibrations. Bands at 1840–1640 $cm^{-1}$ and 780–760 $cm^{-1}$ could be linked to C = O vibrations. Notably, the bands identified in each sample exhibited variations from the characteristic functional group bands of the allantoin standard, suggesting the presence of allantoin compounds in the analyzed samples of snail mucus.

The optimization design used in the Design Expert version 10 software program is a design using the Simplex lattice design method. This method was chosen because SLD (simple lattice design) is an optimization method that can determine the physical properties between two or more mixtures, so it can be used on materials that can be mixed physically. Apart from that, the optimized material has the same concentration range, namely 0.3-3%.

In making an essence sheet mask containing Centella asiatica extract and snail mucus, HPMC and glycerin were optimized, these two ingredients were optimized because they can affect the physical characteristics of the preparation, especially the viscosity of the preparation.

### 3.3. *Physical Characteristic of Essence Sheet Mask*

**Table 4.** The Physical Characteristic of Essence Sheet Mask

| Formula | pH | Viscosity (cPs) | Absorption Ability (minute) | Skin Moisture (%) |
|---|---|---|---|---|
| **F1** | 5.18 | 4244 | 5 | 35.61 |
| **F2** | 5.42 | 5601 | 2 | 22.45 |
| **F3** | 5.34 | 4739 | 4 | 28.21 |
| **F4** | 5.22 | 4544 | 5 | 3636 |
| **F5** | 5.41 | 4911 | 2 | 23.61 |
| **F6** | 5.40 | 4784 | 3 | 24.39 |
| **F7** | 5.39 | 4851 | 3 | 24.44 |
| **F8** | 5.40 | 5661 | 1 | 20.83 |
| **F9** | 5.20 | 4664 | 5 | 36.36 |
| **F10** | 5.41 | 5946 | 2 | 20.83 |

**Table 5.** Anava Results

| Response | p-value prob > F (Modl) | Description | p-value Prob > F *Lack of Fit* | Description |
|---|---|---|---|---|
| pH | <0.0001 | *Significant* | 0.3365 | *Not significant* |
| Viscosity | 0.0003 | *Significant* | 0.3022 | *Not significant* |
| Absorption | <0.0001 | *Significant* | 0.8060 | *Not significant* |
| Skin Moisture | <0.0001 | *Significant* | 0.0785 | *Not significant* |

**Table 6.** Equation Simplex Lattice Design

| Response | Equation |
|---|---|
| pH | Y = 1.63188 (A) + 1.55610 (B) + 0.049960 (A)(B) |
| Viscosity | Y = 1814.26154 (A)+ 1376.42678 (B) – 175.65519 (A)(B) |
| Absorption | Y = 0.43712 (A) + 1.69068(B) – 0.20215 (A)(B) |
| Skin Moisture | Y = 6.68612 (A) + 11.98755 (B) – 2.27753 (A)(B) |

The HPMC coefficients (+1.63188) and glycerine (+1.55610) showed almost the same effect of increasing pH, but the HPMC coefficient values were higher than those of glycerine (table 4). This means that HPMC has a greater effect than glycerine in increasing the pH value. The resulting pH is caused by the difference between the two ingredients, HPMC 10% used in this study has a pH of 5.07 and glycerine has a pH of 4.62. The pH value of HPMC is more alkaline than the pH value of glycerine.

The HPMC coefficient value (+)1814.26154 also indicates that the effect is greater in increasing the viscosity of the preparation compared to glycerine, which has a coefficient (+1376.42678). In this study, 10% HPMC had a viscosity of 4439 cPs and glycerine had a much lower viscosity value of 442.4 cPs. This causes a difference in viscosity resulting from the effect of adding each ingredient to each formula. HPMC is a polymer derived from cellulose. This polymer molecule enters the cavity formed

by water molecules, resulting in hydrogen bonds between the hydroxyl groups (-OH) of the polymer and water molecules. Therefore, the higher the concentration of HPMC, the more hydroxyl groups are bound, so the viscosity is higher [22] [23]. This means increasing amount of HPMC, the more liquid will be retained and bound by HPMC, finally the viscosity will increase.

On the other hand, the coefficient value of HPMC (0.43712) is smaller than that of glycerine (+1.69068), this indicates that the effect of glycerine in increasing the absorption ability of the preparation is greater. this is because the high HPMC content will increase the viscosity of the preparation, the preparation becomes thicker and difficult to absorb in the sheet mask sleeve.

The HPMC coefficient value is also smaller with a value of (+6.68612) while glycerine is (+11.98755) in increasing skin moisture. Glycerine can form a hygroscopic layer, therefore it can absorb water from the air and be able to retain it. This process can also prevent dehydration of the stratum corneum layer. The advantage of glycerine as a humectant compared to other humectant ingredients is that glycerine can maintain moisture in the skin because of the large number of hydroxyl groups, making it stronger in binding and holding water in the skin [24] [25].

Determination of the optimum formula obtained from Software Design Expert Stat-Ease® version 10 Simplex Lattice Design method with the optimum parameters of pH, viscosity, absorption ability and skin moisture. The importance of each parameter can be determined from unimportant to important, with a weight of 1 (not important, +) to most important (+++++) with a weight of 5. The importance, in this case, is determined by the parameter's influence on the preparation of the essence sheet mask.

The pH parameter was assigned a maximum target with a weight of 3 (+++). This decision was made because all pH values produced in the running formula fall within the safe range accepted by the skin. A pH that is too low can cause irritation, while a pH that is too high can cause scaly skin. The desired pH in an optimum formula must fall within the required range of 4.5-6.5.

The viscosity parameter was designated as a minimum target with a weight of 5 (+++++). This is due to viscosity being a crucial parameter in this preparation. Viscosity directly affects the dosage form produced. In this research, the aim is to obtain a liquid result to ensure that the preparation can still be absorbed by the mask sleeve. Low viscosity results in a liquid preparation that can be easily absorbed into the mask sleeve, serving as an applicator. Viscosity is a parameter directly linked to the absorption ability parameter.

The absorption ability parameter was assigned a maximum target with a weight of 5 (+++++). This choice was made because proper absorption of the essence sheet mask into the sleeve is crucial. If the preparation cannot be absorbed properly into the sleeve, it will not be evenly distributed when used. Absorption also aids in determining the effectiveness of the preparation upon application. If the preparation cannot be absorbed properly into the sleeve, it cannot be classified as a sheet mask, resulting in similar application methods to other types of masks. The moisture parameter was assigned a maximum target weight of 5 (+++++). A higher humidity level yields better moisture activity, facilitating the desired therapeutic effect.

The optimum formula obtained is a formula with a concentration of 0.589% HPMC and 2.711% glycerine with a desirability value of 0.711. The optimum formula was composed of a predetermined ratio of HPMC and glycerin. The manufacturing steps were conducted in the same manner as during the formula's preparation, and testing was performed using identical procedures.

The physical appearance of the essence sheet mask was depicted in Figure 2. The test results of the essence sheet mask were presented in Table 9.

**Table 9.** Optimum Formula Test Results

| Response parameters | Test Result |
|---|---|
| pH | 5.64 ± 0.02 |
| Viscosity (cPs) | 4581.80 ± 59.80 |
| Absorption Ability (minute) | 4.60 ± 0.55 |
| Skin Moisture (%) | 32.09 ± 3.21 |


**(a)** **(b)**

**Figure 2.** Optimum formula of (a) essence of sheet mask containing CA and snail mucus (b) sheet mask containing CA and snail mucus after absorption ability test

Irritation is an inflammation of the skin that occurs due to the presence of foreign compounds. Symptoms include a burning feeling that occurs due to dilation of blood vessels characterized by redness (erythema) and swelling (edema). The irritation test was carried out covered with gauze. The resulting preparations meet the cosmetic requirements that are safe for human skin, proven by a score of 0 on the irritation test for all volunteers [11]. The results of the irritation test show that the irritation score caused by the preparations is 0 or not irritating.

## 4. CONCLUSION

HPMC and glycerine have effect on the physical characteristics and moisture activity test of the essence sheet mask combination of CA extract and snail mucus. HPMC can increase the pH and viscosity in the preparation. Glycerine can increase the absorption ability of the essence to the mask sleeve and skin moisture.

Both HPMC and glycerine are compatible in essence with sheet masks containing CA and snail mucus with the optimum composition in the proportion of 0.589% HPMC and 2.7111% glycerine. The response results for the optimum formula were test results of pH 5.64 ± 0.02, viscosity 4581.8 ± 59.80 cPs, absorption ability 4.6 ± 0.55, and humidity 32.09 ± 3.21%.

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*Original Article*

# Formulation and Physical Evaluation of Hand Sanitizer Gel with Various Concentration Ambon Banana Peel Extract (*Musa paradisiaca Var. sapientum (L)*)

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**Abstract**: Hand washing is an activity that can reduce microorganism contamination, but some people whose work environment is far from hand washing facilities will experience difficulties. This gave rise to the innovation of practical hand washing without water, commonly called a hand sanitizer. Currently, the innovation of materials for making hand sanitizers uses natural ingredients, one of which can be Ambon banana peels, where the use of banana peels can also increase their economic value. The purpose of this study was to evaluate the physical preparation of hand sanitizer gel from Ambon banana peel extract at various concentrations. This study used Ambon banana peels extracted by the UAE method. Next, three hand sanitizer gel formulations were made with the active ingredient, a dry extract of Ambon banana peel. Formulation 1 (F1) uses 7% extract, Formulation 2 (F2) uses 10% extract, and Formulation 3 (F3) uses 13% extract. Each formulation was subjected to physical evaluation, including organoleptic tests, homogeneity, pH, spreadability, and adhesion, for three replications. The results showed that the three formulations had almost the same organoleptic visuals, namely a brown color, a soft texture, and the typical smell of Ambon bananas. Each formulation also fulfills the requirement of being homogeneous, and the pH is within the pH range of the skin. Spreadability and adhesion to the three formulas also meet the requirements for topical preparations. It can be concluded that the three formulations of Ambon banana peel hand sanitizer gel fulfill the requirements as topical preparations based on their physical evaluation. The three formulas did not show significant differences, so the concentration of the active ingredients in this study had no significant effect on the physical state of the preparations.

**Keywords:** Peel of Ambon banana; Gel hand sanitizer; Physical test; Formulation

## 1. INTRODUCTION

Humans have organs that are interconnected with one another to support the whole body. The skin is one of the largest organs of the body and is located on the entire surface of the human body, covering bones, flesh, and the blood stream [1,2]. The skin functions as a shock barrier from outside the body so that the organs in the body do not become inflamed or damaged. Hands are parts of the body that often make physical contact, so they are easily exposed to microorganisms, both normal and pathogenic flora, and actions such as hand washing are needed to clean microorganisms attached to the palms of the hands [2].

Along with the rise in activity among Indonesians, particularly in metropolitan areas, and the proliferation of instant product. Then a innovative hand wash solution without the use of water but with an antiseptic was created and called a hand sanitizer [3]. Hand sanitizer is a product that contains an antiseptic substance in which there is alcohol with a concentration of 60–95% [4]. The

alcohol in hand sanitizers has effective bactericidal and antibacterial activity against gram-negative and gram-positive bacteria that can develop on the palms of the hands, including Escherichia coli and Staphylococcus aureus. However, alcohol has the weakness that its effectiveness for removing bacteria is only short-term because it is volatile and dries quickly. Besides that, excessive use of alcohol will cause skin irritation and even a burning effect [5]. It is necessary to have an alternative to replace the use of alcohol with natural ingredients that are relatively safer for the skin.

Bananas are one of the commodities that come from the fruit group, which is quite well known among Indonesians. There are more than 200 types of bananas in Indonesia that have the opportunity to be used [6]. Ambon banana is a type of banana that is widely consumed by Indonesians. These bananas are widely sold in traditional and modern markets and are spread across almost all of the Indonesian islands [7]. Banana (Musa paradisiaca) of the Ambon variety has many health benefits. One of them is useful for digestion because it has a high fiber content. Apart from that, it is not only useful in its fruit, but the sap of the Ambon banana plant is also useful as an antiseptic because it has a chemical content in the form of tannins [8]. Banana sap is usually found in the midrib and banana skin. Banana peels, often discarded as waste, actually offer numerous benefits that most people may not realize. Therefore, we should optimize the use of banana peels to enhance their selling value. Ambon banana peel has compounds that act as antimicrobial agents, including flavonoids, saponins, steroids, glycosides, and tannins [9,10].

Several studies using banana peels as wound antiseptics have shown effective results [10]. Other research also shows that banana peel is useful as an antibacterial and produces a good inhibition zone (> 5mm) against Escherichia coli and Staphylococcus aureus [9,11]. The use of banana peels as an antiseptic or antibacterial needs to be developed with formulations that are more diverse and functional according to the needs of the community so that the value of the benefits will be more optimal. Based on the description above, the researcher is interested in conducting research related to the formulation of hand sanitizer gel preparations from various concentrations of thick extract of Ambon banana peel by paying attention to the physical properties of the preparations.

## 2. MATERIALS AND METHODS

### *2.1. Materials*

The materials used are Ambon banana skin from the dampit plantation in Malang, distilled water, hydroxypropyl methyl cellulose (HPMC) (Merck), carpobopol 940 (Merck), Triethanolamine (TEA) (Merck), glycerin (Merck), methyl paraben (Merck), Ambon banana essences (Red bell), and 96% ethanol (Merck).

The tools used include analytical scales, a mortar, chamber, stamper, glassware (Iwaki), a waterbath, an oven (Menmert), a rotary evaporator (RE100-Pro), a set of power spreaders, a set of adhesion tools, a glass plate, and a pH meter.

### *2.2. Ambon Banana Peel Extraction*

Extraction using the ultrasound-assisted Extraction (UAE) method with 96% ethanol (1:10). 545 g of Ambon banana peel powder were dissolved and extracted for 3 x 5 minutes. The ethanol extract obtained was then concentrated using a rotary evaporator until thick. Then dried using an oven to remove any residual ethanol that is still left behind. The yield of dry banana peel extract was calculated.

*2.3. Manufacture of Ambon banana peel hand sanitizer gel*

Weigh each material to be used. Mixed carbopol 940 gel base and HPMC into the mortar, then stirred until a swelled base formed, and TEA was added to the base, then homogenized. Dissolve methyl paraben in hot distilled water and then add it to the base mixture. Furthermore, dry banana peel extract was dissolved in glycerin, sprinkled into the base mixture little by little, and stirred until homogeneous. The last step is adding one drop of Ambon banana essence as a fragrance and homogenizing. In this study, three hand sanitizer gel formulas were prepared with different concentrations dry banana peel extract. The formula's gel hand sanitizer is shown in Table 1.

**Table 1.** Formula of Ambon's banana peel hand sanitizer gel

| Material | Function | Concentration | | |
|---|---|---|---|---|
| | | F1 | F2 | F3 |
| Ambon Banana's Peel Dry Extract | active substance | 7% w/v | 10% w/v | 13% w/v |
| HPMC | Base | 0.5% | 0.5% | 0.5% |
| Carbopol 940 | Base | 0.25% | 0.25% | 0.25% |
| TEA | *alkalizing agent* | 2% | 2% | 2% |
| Gliceryn | Humectant | 15% | 15% | 15% |
| Methyl Paraben | Preservative | 0.075% | 0.075% | 0.075% |
| Escence of Banana's Ambon | Fragrance | 1 drop | 1 drop | 1 drop |
| Aquades | Solvent | Ad 30 ml | Ad 30 ml | Ad 30 ml |

*2.4. Physical Evaluation*

2.4.1. Organoleptic Test

Visually observe the hand sanitizer gel preparations, including color, smell, and texture.

2.4.2. Homogeneity Test

The hand sanitizer gel is applied on a piece of transparent glass, sealed up, and checked for a harmonious arrangement and the absence of coarse grains.

2.4.3. pH Test

The pH value was determined by dissolving up to 1 g of Ambon banana peel hand sanitizer gel formulation in 10 ml of water, stirring until homogenous, and the value was measured using a pH meter.

2.4.4. Spreadability Test

On transparent glass that has been covered with mica paper, 1 g of the hand sanitizer gel preparation is applied, and after being loaded with 500 g, its dispersion is measured.

2.4.5. Adhesion Test

1g of hand sanitizer gel is applied to a set of adhesive tools that are equipped with a transparent glass plate, covered on the side of the glass plate with another glass plate, and then given a weight weighing 500 g. The weight is then allowed to stand for 2 minutes, the weight is transferred, the weight is removed, and the time is calculated required for the two glass plates to separate.

## 3. RESULTS AND DISCUSSION

Bananas are a plant that is in great demand by the public because every part of the plant can be used for their daily needs. The benefits of this banana plant can also be found in the skin of the

fruit. Not many people use banana peels, so this research is expected to help increase the value of the benefits of banana peels in the community.

This study used three formulations of ambon banana skin extract sanitizer gel preparations, each of which was replicated three times. Each formulation uses a different extract concentration. One of the reasons for choosing this concentration is based on research results showing that 10% banana peel extract can provide an optimal inhibition zone [9]. In this study, we selected three concentrations: below 10% (7%), 10%, and above 10% (13%). In addition, the formulation incorporates additional fragrance to enhance the banana aroma, signifying that the hand sanitizer originates from banana peel extract.

The first physical evaluation carried out on hand sanitizer gel is an organoleptic observation. Organoleptic testing, or sensory testing, is a test method using the human senses as the main tool for measuring product acceptance [12]. The organoleptic tests carried out included observing texture, smell, color, and taste. The observation results are shown in Figure 1 and Table 2.

**F1** **F2** **F3**

**Figure 1.** Visual organoleptic hand sanitizer gel Ambon banana peel extract

**Table 2**. The result of organoleptic test for Ambon banana peel extract hand sanitizer gel

| No | Formulation | Texture | Smell | Color | Teste |
|---|---|---|---|---|---|
| 1 | F1 Replicate 1 | Gentle | Typical smell of Ambon banana | Brown | Bitter, slightly sour |
| | F1 Replicate 2 | Gentle | Typical smell of Ambon banana | Brown | Bitter, slightly sour |
| | F1 Replicate 3 | Gentle | Typical smell of Ambon banana | Brown | Bitter, slightly sour |
| 2 | F2 Replicate 1 | Gentle | Typical smell of Ambon banana | Brown | Bitter, slightly sour |
| | F2 Replicate 2 | Gentle | Typical smell of Ambon banana | Brown | Bitter, slightly sour |
| | F2 Replicate 3 | Gentle | Typical smell of Ambon banana | Brown | Bitter, slightly sour |
| 3 | F3 Replicate 1 | Gentle | Typical smell of Ambon banana | Brown | Bitter, slightly sour |
| | F3 Replicate 2 | Gentle | Typical smell of Ambon banana | Brown | Bitter, slightly sour |
| | F3 Replicate 3 | Gentle | Typical smell of Ambon banana | Brown | Bitter, slightly sour |

The organoleptic test aims to determine the visual quality of the hand sanitizer gel preparation of Ambon banana peel extract. The dosage forms of the three formulas show that all the dosage forms are viscous. Ambon banana peel extract has a distinctive aroma of Ambon bananas that is not too strong, so it is given an Ambon banana fragrance. Based on observations, almost the same results were obtained, namely brown gel, soft texture, bitter taste, and typical Ambon banana smell. In terms of visual color, there is only a slight difference because the higher the concentration of the extract, the darker the color of the hand sanitizer gel preparation. This is in accordance with a study which states that the addition of active substances can affect the color and smell of the formulation [12].

The second physical evaluation is in the form of observing the pH of the preparation, where this pH has an important role in hand sanitizer gel preparations. The pH test aims to determine the safety of the hand sanitizer gel, which is formulated so as not to irritate the skin when applied. According to the Indonesian National Standard (SNI), the pH value of topical preparations is 4.5–8 [13]. The results of observing the pH of the preparation can be seen in Table 3.

**Table 3**. The result of pH test for Ambon banana peel extract hand sanitizer gel

| No | Formulation | pH | Mean | Standard Deviation |
|---|---|---|---|---|
| 1 | F1 Replicate 1 | 6.21 | 6.26 | 0.049329 |
| | F1 Replicate 2 | 6.30 | | |
| | F1 Replicate 3 | 6.29 | | |
| 2 | F2 Replicate 1 | 6.29 | 6.28 | 0.005774 |
| | F2 Replicate 2 | 6.28 | | |
| | F2 Replicate 3 | 6.29 | | |
| 3 | F3 Replicate 1 | 6.14 | 6.13 | 0.005774 |
| | F3 Replicate 2 | 6.13 | | |
| | F3 Replicate 3 | 6.13 | | |

The pH value is very important for topical preparations because it can affect the safety and comfort of using the product. Based on the observed data, it was determined that the gel preparations had a pH in the range of 6.1–6.3. These results met the requirements for topical preparations, namely 4.5–8 [13]. Other research sources also state that hand sanitizer gel preparations can be said to be safe if they match the skin's pH, which is 4.6–6.5 [14]. In this study, the concentration of banana peel extract did not have enough effect on changes in the preparation's pH because the results showed that the PH was not significantly different.

The third evaluation is the observation of the homogeneity of the preparation. This test was carried out with the aim of determining the homogeneity of the hand sanitizer gel preparation by looking at the uniformity of the particles in the preparation. A preparation is said to be homogeneous if it shows a homogeneous arrangement of particles and no coarse grains are visible [12,15]. The results of observing the homogeneity of hand sanitizer gel preparations are presented in Table 4.

**Table 4**. The result of homogenity test for Ambon banana peel extract hand sanitizer gel

| No | Formula | Homogeneity | Particles/granules |
|---|---|---|---|
| 1 | F1 Replicate 1 | Homogeneous | No coarse particles/grains |
| | F1 Replicate 2 | Homogeneous | No coarse particles/grains |
| | F1 Replicate 3 | Homogeneous | No coarse particles/grains |
| 2 | F2 Replicate 1 | Homogeneous | No coarse particles/grains |
| | F2 Replicate 2 | Homogeneous | No coarse particles/grains |
| | F2 Replicate 3 | Homogeneous | No coarse particles/grains |
| 3 | F3 Replicate 1 | Homogeneous | No coarse particles/grains |
| | F3 Replicate 2 | Homogeneous | No coarse particles/grains |
| | F3 Replicate 3 | Homogeneous | No coarse particles/grains |

The results of the observations showed that the hand sanitizer gel preparation did not show any coarse grains and showed a harmonious arrangement. All formulations have fulfilled the homogeneity requirements of gel preparations.

The next evaluation is the measurement of the spreadability of the hand sanitizer gel. A spreadability test was carried out to determine the ability of the gel to spread on the skin surface [13]. The results of measuring the spreading power of hand sanitizer gel preparations are arranged in Table 5.

**Table 5.** The results of spreadability test for the Ambon banana peel extract hand sanitizer gel

| No | Formulation | Diameter of Scatter | Mean | Standard Deviation |
|---|---|---|---|---|
| 1. | F1 Replicate 1 | 5.2 cm | 5.266 | 0.208167 |
| | F1 Replicate 2 | 5.1 cm | | |
| | F1 Replicate 3 | 5.5 cm | | |
| 2. | F2 Replicate 1 | 5.4 cm | 5.4 | 0.2 |
| | F2 Replicate 2 | 5.2 cm | | |
| | F2 Replicate 3 | 5.6 cm | | |
| 3. | F3 Replicate 1 | 5.3 cm | 5.2 | 0.152753 |
| | F3 Replicate 2 | 5.4 cm | | |
| | F3 Replicate 3 | 5.1 cm | | |

Semi-solid products are expected to spread easily at distribution sites without significant pressure. The easier it is to apply to the skin, the greater the contact surface area of the nutrient substance with the skin and the optimal drug absorption. Semi-solid preparations that are comfortable to use have a spread of 5-7 cm [12].

Based on the results obtained, it was shown that all the formulas made met the requirements for a good distribution, namely in the range of 5. However, the results obtained did not show a significant effect related to the concentration of banana peel extract on the spreadability of the preparations. This is different from a study whose results stated that the concentration of the active component affected the spreadability of the preparation. The higher the concentration of the components used, the greater the spreadability of the preparation [12,13].

The last evaluation carried out in this study was the measurement of the adhesion of the hand sanitizer gel preparation. Measurement of adhesion aims to determine how long the contact between the hand sanitizer gel and the skin surface lasts [14]. The results of the adhesiveness test of the preparations are shown in Table 6.

**Table 6.** The results of adhesion test for the Ambon banana peel extract hand sanitizer gel

| No | Formulation | Sticking Duration | Mean | Standard Deviation |
|---|---|---|---|---|
| 1. | F1 Replicate 1 | 1.24 second | 1.1833 | 0.066583 |
| | F1 Replicate 2 | 1.20 second | | |
| | F1 Replicate 3 | 1.11 second | | |
| 2. | F2 Replicate 1 | 1.45 second | 1.653 | 0.415251 |
| | F2 Replicate 2 | 1.36 second | | |
| | F2 Replicate 3 | 2.12 second | | |
| 3. | F3 Replicate 1 | 1.20 second | 1.3833 | 0.141539 |
| | F3 Replicate 2 | 1.44 second | | |
| | F3 Replicate 3 | 1.54 second | | |

The results of measuring the adhesion of each formulation showed that the hand sanitizer gel from Ambon banana peel extract met the requirements because the adhesive duration was more than 1 second. A preparation can be said to be good if it has great adhesion because adhesion aims to determine how long the contact between the hand sanitizer gel and the skin surface is. The longer the contact time with the skin, the greater the working ability of the active substance, so that the effect is also greater [14,16].

The stickiness of hand sanitizer can be affected by the materials used in its manufacture. The use of materials such as carbopol and triethanolamine, which are gel developer agents, can increase adhesion. The more concentrated the gel is, the more sticky it will feel on the skin and last longer on the skin surface [17]. The results of the adhesion test for each formula in this study were not much different because the additives had the same concentration; it was just that the first formula (F1) had the smallest adhesive power compared to the other two formulas.

From the results of several physical evaluations, it was shown that the three hand sanitizer gel formulations of Ambon banana peel extract met the preparation requirements that could be accepted or applied to the skin. Physical evaluation of topical preparations is important to conclude that these preparations can be applied to the skin safely and comfortably. In addition, the concentration of the extract in the three formulations did not show a significant effect on each of the results of the physical evaluation of the preparations.

## 4. CONCLUSION

It can be concluded that all hand sanitizer gel formulations containing Ambon banana peel extract meet the physical evaluation criteria, so that their use can be said to be comfortable for the skin. The three formulas did not show significant differences, so the concentration of the active ingredients in this study did not have a major effect on the physical condition of the preparations. Making Ambon banana peel hand sanitizer gel can also be an interesting innovation because it increases the economic value of banana peels, which were previously considered trivial. Furthermore, further testing needs to be carried out regarding the irritation test to test the safety of the preparation and activity or potential test of this hand sanitizer gel preparation as an antiseptic.

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*Original Article*

# Profiling Rosmarinic Acid and Sinensetin Content of *Orthosiphon aristatus*. from Three Different Locations with Variety Ethanol Concentration

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**Abstract:** *Orthosiphon aristatus* is a well-known medicinal plant acknowledged for its therapeutic effect in treating urinary tract diseases, hypertension, diabetes mellitus, and arthritis. It is widely used as an ingredient in herbal medicine and distributed throughout the world, including China, Europe, and Indonesia. Sinensetin is normally used as a chemical marker to evaluate and control the quality of *O. aristatus*. However, in 2021, the European Medicines Agency changed the marker to rosmarinic acid. To determine the levels of rosmarinic acid and sinensetin in *O. aristatus* as well as the correlation between the two compounds, this study used high-performance liquid chromatography (HPLC) with a UV detector to analyze *O. aristatus* extract from three distinct locations with four different ethanol concentrations (p.a, 75%, 50%, and 25%). The results showed that the combination of solvent concentration and growing location had a significant effect on the levels of rosmarinic acid and sinensetin, with a p-value < 0.05. The Spearman test obtained a correlation coefficient (r) of - 0.423. It can be concluded that there is no correlation between the content of rosmarinic acid compounds in *O. aristatus* leaves and the content of sinensetin compounds.

**Keywords:** *Orthosiphon aristatus*; rosmarinic acid; sinensetin; ethanol concentration

## 1. INTRODUCTION

*Orthosiphon aristatus* (Blume) Miq., locally known "cat's whiskers" or as "kumis kucing", is a medicinal plant from the Lamiaceae family distributed widely throughout China, Europe and Southeast Asian countries, including Indonesia. The leaves of its plant have been used empirically to treat kidney stones and urinary tract diseases [1]. Furthermore, pharmacological studies have verified that this plant is therapeutically effective in treating diabetes mellitus [2], decreasing blood pressure [3], [4], [5], treating urinary tract diseases[6], and managing arthritis due to its anti-inflammatory properties [7]. Due to its potency, *O. aristatus* is widely used as an ingredient in herbal medicine product. According to the Indonesia Food and Drug Administration (BPOM), more than three hundreds herbal medicine products have been registered for containing *O. aristatus* [8]. In addition to being an economic opportunity, the growing interest in herbal medicine is also a challenge in the field of quality assurance. Quality assurance is essential to ensure the safety and quality of the products.

The quality of herbal medicine can be assessed by a number of methods, such as fingerprinting and marker compound analysis. Although fingerprint-based systems have gained more attention, the conventional technique of using chemical makers is commonly chosen by herbal pharmacopeia in many countries [9]. Phytochemical studies discovered that the ethanol extract of *O. aristatus* leaves contained 34 compounds, and 14 of them were successfully absorbed and retained in mice's blood plasma. Among the fourteen phytochemical constituents, tanshinone IIA, salvianolic acid B, salvigenin, sinensetin, and rosmarinic acid are the compounds that are responsible for the pharmacological activity and are suggested to be the marker compounds of *O. aristatus* [10]. Sinensetin has been used as chemical marker of *Orthosiphon aristatus* by Indonesia's Herbal Pharmacopoeia (2017) and European Medicines Agency (2010) [11], [12]. However, in 2021, the European Medicines Agency updated the *O. aristatus* marker into rosmarinic acid [13]. This change is undoubtedly a challenge in the analysis field. Therefore, it is necessary to know the level of rosmarinic acid and sinensetin in *O. aristatus* along with the correlation between the two.

The content of sinensetin and rosmarinic acid in *Orthosiphon aristatus* extract is variable and can be influenced by some factors such as the origin and extraction methods. Phytochemical study reported that the different location of *O. aristatus* effect of the level of sinensetin [14]. On the other hand, the choice of solvents is important due to the targeted compound to be extracted from the plant. The most common solvents used for *O. aristatus* extraction are methanol, ethanol, and distilled water [11], [15], [16]. Although methanol gives the high yield of rosmarinic acid and sinensetin than ethanol, but the use of methanol is frequently questioned because of its toxic to humans. Thus, the industry finds ethanol more appealing for the extraction because less toxic and shares similar chemical properties with methanol[17]. According to a literature search, no one has reported the effect of the combination of ethanol concentration and growing location on the content of rosmarinic acid and sinensetin in cat whisker extract or the relationship between the two compounds. Therefore, this study aims to determine the levels and correlation of rosmarinic acid and sinensetin in *O. aristatus* from various locations and ethanol concentrations.

## 2. MATERIALS AND METHODS

### *2.1. Materials*

#### 2.1.1 Plant materials

The *Ortoshipon aristatus* leaves were collected from 3 different locations (Sleman, Bantul, Klaten) on March 2024. The third to eights leaves from the shoots were picked by hand. The leaves are green, with a lanceolate leaf blade and a serrate margin. The venation is reticulate-pinnate, the petiole is reddish purple in colour, and the leaves range in size from 7 to 11 cm (Figure 1). The leaves were dried in an oven at 45 $^{O}$C for 24 hours. Afterward, the dried leaves were ground and sifted with a 40-mesh sieve to obtain brownish green powder of *O. aristatus.*

#### 2.1.2 Reagent

Rrosmarinic acid (Markherb, purity ≥ 95%), sinensetin (Chemfaces, purity ≥ 98%), ethanol p.a (Merck, Germany), acetonitril HPLC grade (Merck, Germany), trifluoroacetic acid HPLC grade (Sigma Aldrich), and Milli-Q water was prepared in the laboratory.

*2.2 Methods*

2.2.1. Sample extraction

The preparation of extract was modified according to previous report [14]. Approximately 500 mg of *O. aristatus* leaves powder was added with 5 mL of ethanol (p.a., 75%, 50% and 25%), then extracted using *ultrasound-assisted extraction method* for 15 minutes at room temperature and filtered through a 0.45 PVDF syringe filter. After that, each *O. aristatus* extract solution was dissolved in ethanol to obtain a concentration 10 mg/ml. The sample solution was filtered through a 0.45 PTVE syringe filter, and 10 μL was injected into HPLC.

2.2.2 Preparation of The Standard Solutions

A standard solution was prepared for rosmarinic acid and sinensetin. Each compound was made into a stock solution with concentration of 500 μg/ml in methanol. The combined working solution was obtained by mixing the stock solution of rosmarinic acid and sinensetin for 120 μL and 20 μL, respectively. The combined working solution was further diluted to five series concentration 3-60 μg/ml (rosmarinic acid) and 0.5-10 μg/ml (sinensetin).

2.2.3 HPLC Conditions

The analysis conditions for the quantification of rosmarinic acid and sinensetin compound in *O. aristatus* leave were conducted according to Ramadhani et al [18]. A Shimadzu prominence-high performance liquid chromatography system (Shimadzu Corporation, Kyoto, Japan) equipped with ultraviolet (SPD-20A/20AV) detector was used for the analysis. Separation was archived on cosmosil C18-MS-II column (4.6 i.d x 250 mm, 5.0 μm) using a 0.1% TFA in water (A) and acetonitrile (B) as a mobile phase

2.2.4 Data analysis

A nested analysis of variance (nested-ANOVA) was performed to determine the significant effect of the ethanol concentration on sinensetin and rosmarinic acid from three different locations of O. aristatus. Then, a spearman correlation analysis using Minitab 21.4.1 software was performed to determine the association between rosmarinic acid and sinensetin levels in O. aristatus at different ethanol concentrations and locations.

## 3. RESULTS AND DISCUSSION

In this study, extraction was performed using four different ethanol concentrations (p.a, 75%, 50%, and 25%) and three growing locations (Sleman, Bantul, and Klaten). Table 1 provides a detailed description of the sample's location. The results showed that different ethanol concentrations affected the present of the bioactive compound in *O. aristatus* extract. Figure 1 displays each extract's chromatogram at various concentrations and locations.

**Table 1.** A details of the *O. aristatus* locations

| Origins | Latitude, Longitude | Elevation (meter) |
|---|---|---|
| Sleman | 7°37'42.0"S and 110°25'34.8"E | 619 |
| Bantul | 7°49'29.7"S and 110°21'15.8"E | 82 |
| Klaten | 7°43'31.6"S and 110°30'12.1"E | 194 |

The fact that some peaks are absent in the beginning retention times and present in the end retention time indicates that the use of ethanol p.a tends to extract non-polar compounds rather than

polar compounds. Meanwhile, the use of binary solvents (ethanol and water) significantly extracted polar compounds, as evidenced by the appearance of many peaks at the initial retention time. This may indicate that a binary solvent is suitable for optimizing the extraction of polar compounds.

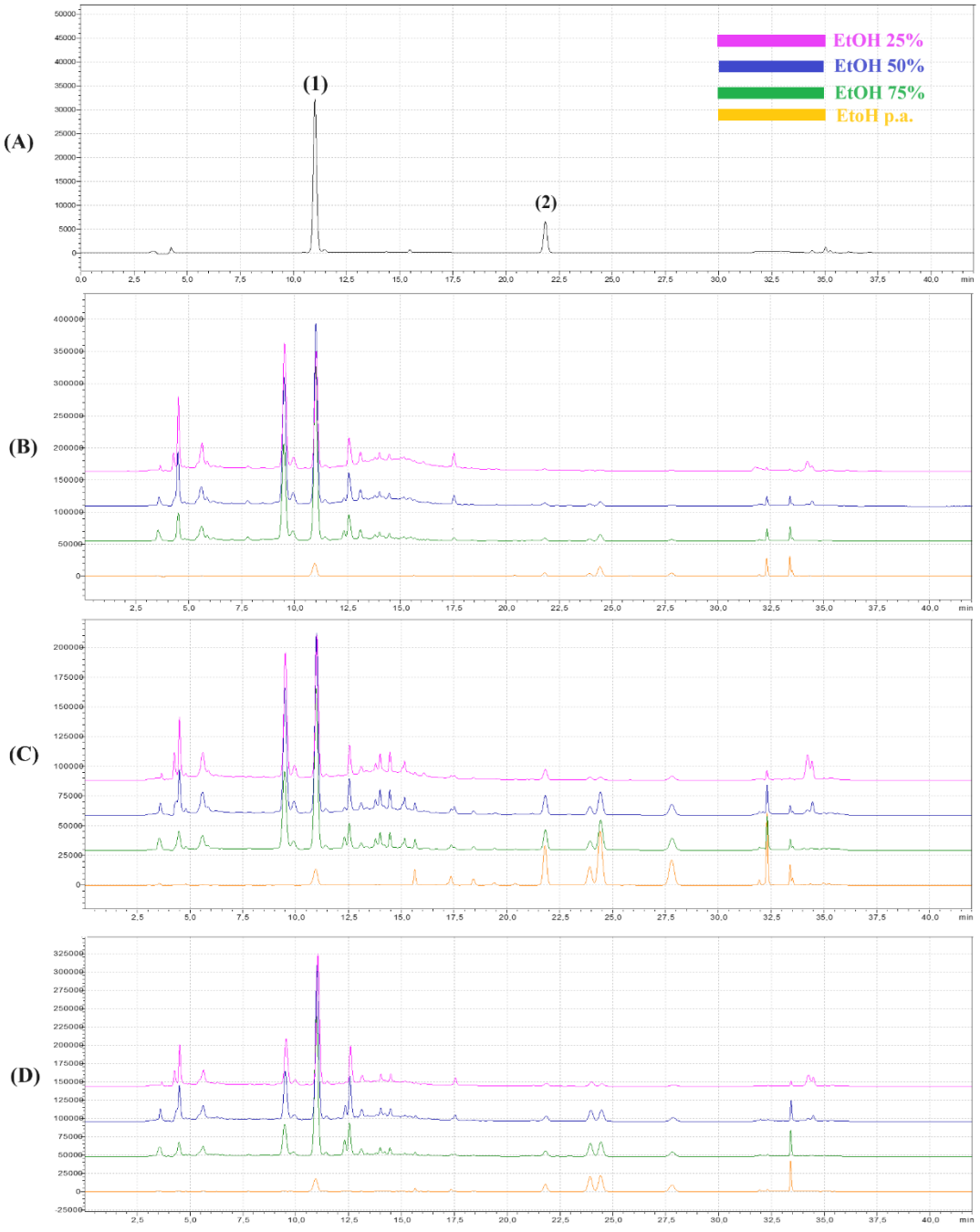


**Figure 1.** (A) Rosmarinic acid (1) and sinensetin (2) chromatogram, (B) HPLC chromatogram of *O. aristatus* extract from Sleman, (C) HPLC chromatogram of extract from Klaten, (D) HPLC chromatogram of *O. aristatus* extract from Bantul

Sinensetin is a flavone group compound with five methoxy groups bound to the benzene ring (Figure 2). The methoxy groups cause sinensetin to be relatively less polar than rosmarinic acid, which has four hydroxyl groups. The use of a C18 reversed phase column and acetonitrile as a solvent

causes less polar compounds bond to the stationary phase more strongly and move more slowly through the column, so the peak could be seen at the end of the retention time. The chromatogram showed that the peak for sinensetin was visible at a retention time of 21.827, whereas the peak for rosmarinic acid was apparent at 10.995.

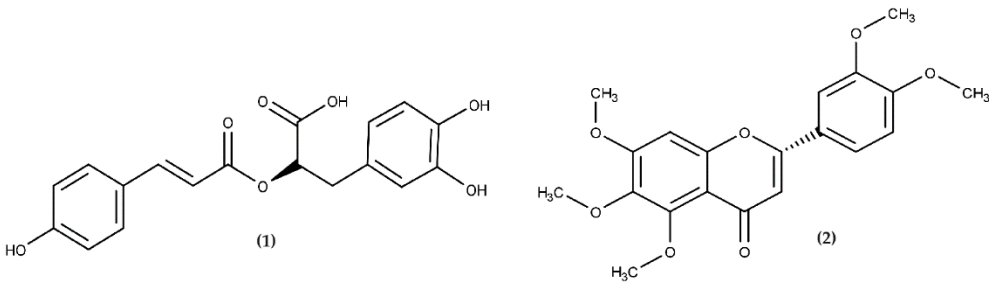


**Figure 2.** Structure of Rosmarinic Acid (A) and Sinensetin (B)

The comparation of rosmarinic acid and sinensetin content in *O. aristatus* from three different location with varying ethanol concentration are presented in Figure 3 (A) and (B). A nested ANOVA revealed that the solvent-nested growth location variable had a *p-value* less than 0.05. These results indicate that the ethanol concentration and growing location had a significant effect on the rosmarinic acid and sinensetin content.

Among all location, hydro-ethanol achieved a significantly higher rosmarinic acid content than ethanol p.a. The results of this study are similar to those of Suhaimi [19], who found that variation in the concentration of ethanol had a significant effect on the levels of rosmarinic acid in the *O. aristatus* extract. The content of rosmarinic acid increases as the ratio of water to ethanol increase. However, when the ethanol percentage was less than 70%, rosmarinic acid levels decreased. The presence of water in the extraction acts as a swelling agent, which can increase extraction efficiency by increasing the contact surface area of the plant material and solvent [20]. The high presence of water in the solvent reduces the rosmarinic acid content because rosmarinic acid is a relatively polar compound but insoluble in water [21]. The Hildebrand solubility value (δ) of 21.18 tends to make rosmarinic acid more soluble in ethanol (δ = 26.6) than in water (δ = 47.8) [22]. Taken together, in this study, we conclude that binary ethanol (50-75%) was the most efficient solvent to extract rosmarinic acid.

Among all locations, *O. aristatus* from Klaten had a higher sinensetin content compared to others. A nested-ANOVA revealed that the growing locations variable had the most significant effect on sinensetin content. Therefore, it can be concluded that the growing location influences the level of sinensetin in O*. aristatus.* These results are similar to the previous study, which showed that the level of sinensetin in *O. aristatus* varies depending on the growing location [14], [16], [23]. The amounts of sinensetin found in this study are similar to those found by Kartini et al. (2022), who analysed *O. aristatus* samples from 14 different areas in Indonesia and found levels ranging from 0.0238 to 0.1533 mg/g.

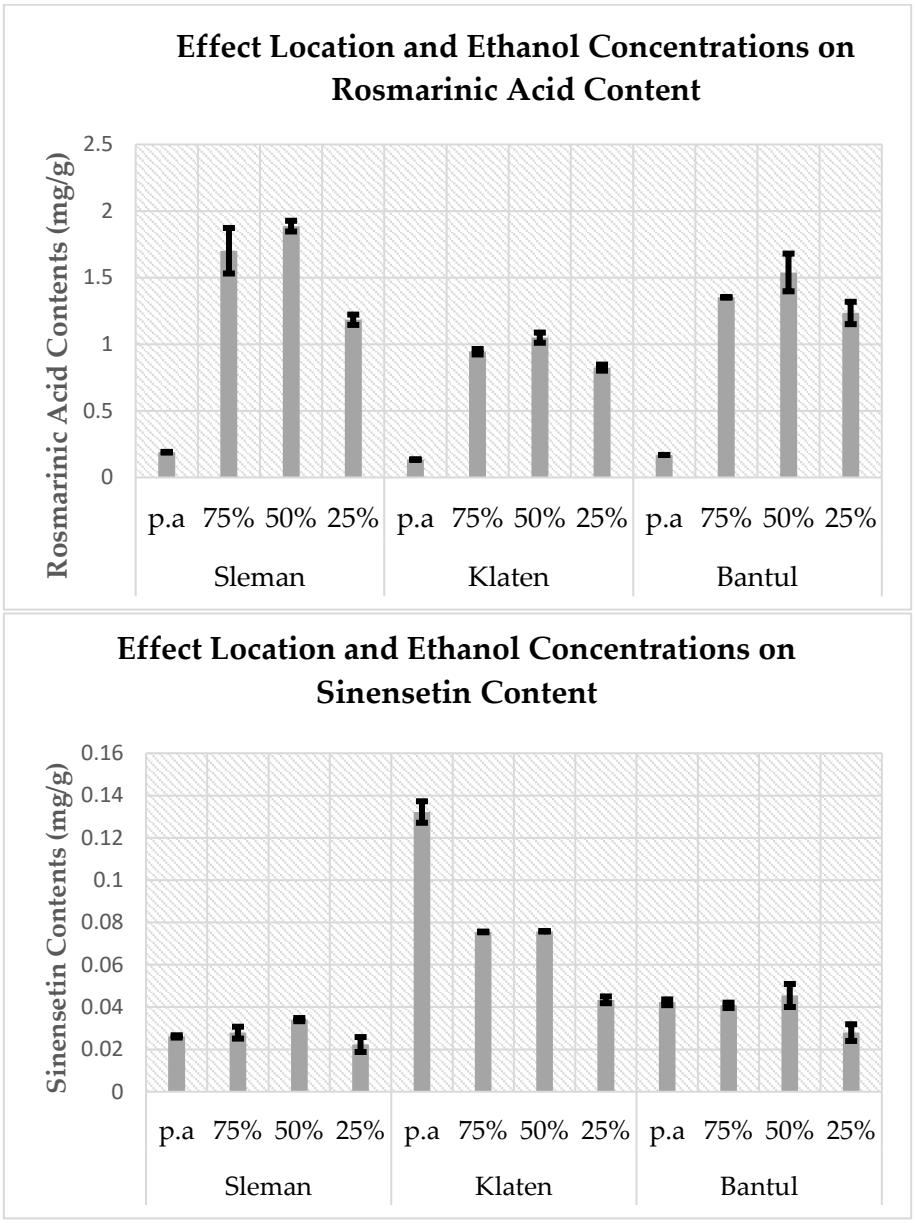


**Figure 3.** Effect of ethanol concentrations and locations on rosmarinic acid content (A) and sinensetin content (B)

This study evaluated the correlation or relationship between rosmarinic acid and sinensetin levels by combining the effects of growing location and ethanol concentration. According to the Spearman correlation test, the correlation coefficient between rosmarinic acid and sinensetin levels was -0.403 (Figure). The negative correlation value indicates that the two variables have an inverse relationship. However, the closeness of the relationship between the two compounds is weak, as evidenced by a coefficient value close to 0. The results of this analysis indicate that the sinensetin sinensetin levels had no correlation with rosmarinic acid levels in *O. aristatus* extract.

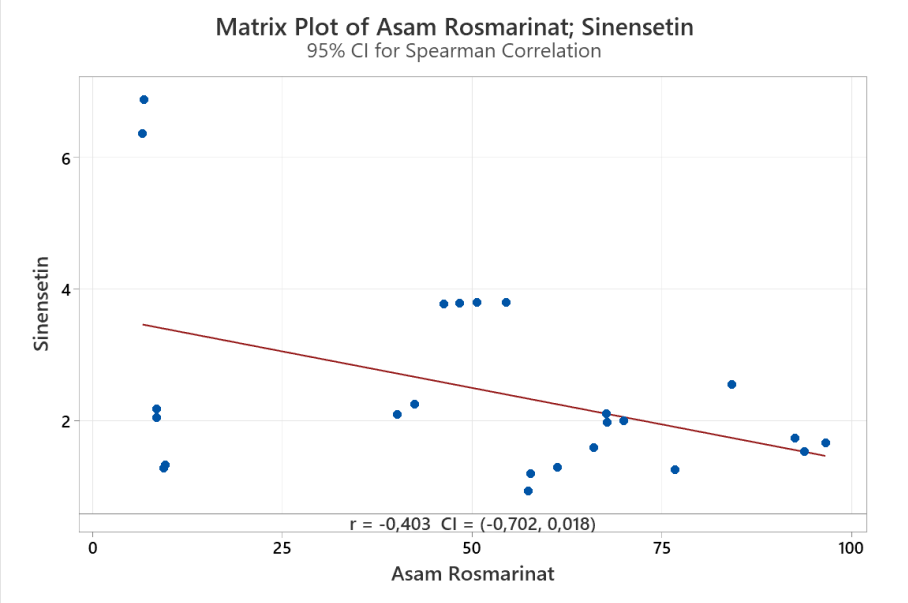


**Figure 4.** Spearman correlation test results of rosmarinic acid and sinensetin in the extract of *O. aristatus*

## 4. CONCLUSION

Variations in ethanol content and growing location significantly influenced the levels of rosmarinic acid and sinensetin in cat's whisker extract. The concentration of ethanol is the primary factor that has a significant impact on rosmarinic acid extraction. Binary ethanol (50-75%) was the most efficient solvent to extract rosmarinic acid. Meanwhile, the growing location variable had the most significant effect on sinensetin content. In addition, this study discovered that sinensetin levels had no correlation with rosmarinic acid levels (r = - 0,423). To verify current results, studies for additional places should be carried out in future research.

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**Acknowledgments:** -

**Conflicts of interest:** The authors declare no conflict of interest.

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*Original Article*

# The Effect of Temperature on Physicochemical Properties of Moringa Leaf Ethanolic Extract (*Moringa oleifera* L.) Patch and Anti-Inflamatory Test

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**Abstract:** Moringa leaf extract contains flavonoids confirmed as anti-inflammatory at 200 mg/Kg BW. For topical anti-inflammatory drug delivery system, the patch matrix was chosen. Patch is continuous for an extended treatment period compared with cream, lotion and gel. Previous research has developed a patch matrix formula using a combination of polyvinyl alcohol (PVA) and alpha-cellulose (1: 2) that produced a patch required for good patch preparation. However, the effect of temperature resistance on physicochemical properties and anti-inflammatory activity needs to be confirmed. Moringa leaves were macerated in 96% ethanol and dispersed into a patch matrix of PVA and alpha-cellulose combination. Patches were treated at various storage temperatures, namely 4°C, 25°C, and 40°C each for 8 hours for six cycles. Mice were divided into three groups, namely blank patch, brand patch product, and moringa leaf patch. Mice were given 0.05 mL of carrageenan suspension intraplanar on the sole of the left foot, and the edema volume was measured using a plethysmometer. Statistical analysis uses One-Way ANOVA and T-test. The results showed that temperature no affected to the moisture, folding endurance, and organoleptic, but effected to pH, thickness, and weight properties of patch matrix. The average edema volume of mice in the brand product group was not significantly different from the moringa leaf patch group, with a significance value of 0.066 ($p > 0.05$). The Moringa leaf ethanolic extract patch confirmed it can reduce the volume of mouse foot edema by 5 hours.

**Keywords:** moringa leaf, flavonoid total, anti-inflamatory, temperature resistance, patch

## 1. INTRODUCTION

Inflammation is a local protective response from damage to tissue caused by physical trauma, damaging chemicals, or microbiological substances. Inflammation functions to destroy, reduce, or localize both the damaging agent and the damaged tissue. Inflammation or phlogosis is a pathophysiological response of living tissues to injuries that leads to the local accumulation of plasmatic fluid and blood cells. Although it is a defence mechanism, the complex events and mediators involved the inflammatory reaction can induce, maintain or aggravate many diseases. Therefore, the use of anti-inflammatory agents is helpful in the therapeutic treatment of these pathologies. Signs of inflammation are swelling or edema, redness, heat, pain, and changes in function. Inflammation often occurs in areas of the skin. The anti-inflammatory activity of active ingredients is an activity that can inhibit or reduce the degree of edema. The mechanism of topical

anti-inflammatory agents is by inhibiting cyclooxygenase enzyme. Topical preparations will deliver the drug into the skin and enter the circulation or be absorbed into tissue to inhibit the cyclooxygenase enzyme. Based on the side effects caused by oral administration of drugs, it can increase absorption drug efficacy and avoid first-pass metabolism in the liver [1].

Medicinal plants are widely used in folk medicine of many countries to treat different inflammatory conditions and, in particular, skin inflammations. However, for many of the plants in use the real efficacy and/or the relevant active principles are unknown. Consequently, experimental studies aimed to demonstrate the pharmacological properties of these plants and to identify the relevant active principles are needed. Medicinal plants develop as anti-inflammatory alternatives besides synthetic agents. Flavonoids in medicinal plants are reported to inhibit cyclooxygenase or lipoxygenase enzyme and inhibit the accumulation of leukocytes so that they can develop as anti-inflammatory agents [2]. Moringa leaf contains flavonoids, which are reported to have anti-inflammatory properties. The Moringa plant grows well in tropical areas and is widely known as a vegetable and traditional medicine. Based on phytochemical analysis, the ethanol extract of Moringa leaf contains flavonoids and polyphenolic compounds [3]. Another research conducted by Saleem [4] on the anti-inflammatory activity test on Moringa leaf using the red blood cell membrane stabilization method showed effective anti-inflammatory activity at a concentration of 1000 ppm (1 mg/mL) by providing strong red blood cell membrane protection that induced by hypotonic solution.

Oral and topical NSAIDs demonstrated similar efficacy for treatment of both acute and chronic injuries. There were more gastrointestinal side effects in patients receiving oral NSAIDs, while local skin reactions occurred more frequently in patients treated with topical NSAIDs. Topical NSAIDs may be considered as comparable alternatives to oral NSAIDs and are associated with fewer serious GI reaction as adverse events when compared with oral NSAIDs [22]**.** The development of topical anti-inflammatory preparations has been widely. The advantages of topical anti-inflammatory preparations are quick effect, sustained drug release, use directly on the site of inflammation so that the duration of the effect can be longer, reduced frequency, and increased level of patient compliance [5]. A patch is a topical preparation containing an active substance that is placed on the skin to deliver a specific dose of medication through the skin, and this system can be discontinued if the drug is no longer desired [6, 7]. The advantage of patch compared to other topical preparations is that they are easy to use and remove, preventing water loss from the skin surface, which can increase skin permeability [8]. Research by Ermawati [9] has succeeded formulate a patch matrix of Moringa leaf ethanolic extract with a combination of polyvinyl alcohol (PVA) and alpha-cellulose polymer in a ratio of 1:2, which produces a patch preparation that meets the requirements of a good patch preparation. The patch matrix released total flavonoids with a transported weight of 119,461 μg for 5 hours. The penetration flux value is $3.75 \times 10^{-6}$ mg. $sec^{-1}$ .$cm^{-2}$, and the membrane permeability is $8.80 \times 10^{-8}$ $sec^{-1}$ .$cm^{-2}$. The patch quality needs to be tested, including releasing active substances, temperature resistance, and in vivo anti-inflammation.

PVA is often thermally processed at high temperatures to induce ester bond-mediated crosslinking between pristine hydroxyl groups from PVA and carboxyl groups from polycarboxylic acid. Alfa-cellulose contains three carboxylic acid groups, where the two terminal groups are often involved in crosslinking reactions. Meanwhile, the temperature resistance test is carried out by storing the patch preparation at cold temperature (4°C), hot temperature (40°C), and room temperature (25°C), with each storage time of 8 hours for six cycles.

To this aim to verify their topical anti-inflammatory potential of patch matrix of Moringa leaf ethanolic extract. An anti-inflammatory effect test was carried out using the plethysmometer method. The plethysmometer has a measurement principle based on Archimedes' law, which states that if an object is placed in a liquid, it will cause an upward force or pressure [10].

## 2. MATERIALS AND METHODS

Materials: Moringa leaves (Moringa oleifera Lam.) Mojogedang, Karanganyar, Central Java, Indonesia, technical 96% ethanol (repackaged by PT. Bratachem), PVA (Sigma Aldrich, Saint Louis, Missouri), Alfa Cellulose (Sigma Aldrich, Saint Louis, Missouri), 70% ethanol (repackaged by PT. Agung Jaya), penoxyethanol (repackaged by Cipta Kimia), PEG 400 (DOW, United States), propylene glycol (DOW, United States), and distilled water (repackaged by PT. Agung Jaya), brand patch product with analgesic-antipyretic. Instrument: digital scales (Sartorius, BP 110, d = 0.001 g; Gottingen, Germany), rotary evaporator (Buchi Labortechnik), hotplate (maspion), Moisture Analyzer, magnetic stirrer (IKA C-MAG HS 7, Germany), Petri dishes 5 cm diameter (normax, Portugal), pH-meter (Ohaus Starter300; Newark, New Jersey), caliper (TOKI, Japan), Spectrophotometry UV-VIS (Thermo Scientific Genesys 10S UV Vis; Waltham, MA), micropipette (DLab, United States), plastimometer.

### *2.1. Sample Preparation*

Determination of Moringa plants, including roots, stems, and leaves, was carried out at the Biology Laboratory, Faculty of Mathematics and Natural Sciences, Sebelas Maret University, Surakarta, Indonesia. Moringa leaf of 500 mg was macerated with 2.5 liters of 96% ethanol until the surface was completely submerged in a place protected from light while stirring occasionally. The maceration process lasts for three days, then the solution is filtered, and the solvent is evaporated at a temperature of 45-50 °C using a rotary evaporator, then concentrated in a water bath until the extract is thick. The specific parameters of extract include extract water content, extract yield percentage, and extract organoleptic observations [11].

### *2.2. Analysis of Flavonoid Total of Extract*

Quercetin standard of 10 mg was added with 0.3 mL of 5% sodium nitrite (NaNO2) solvent. After 5 minutes, add 0.6 mL 10% aluminum chloride (AlCl3), wait 5 minutes, and 2 mL of 1 M sodium hydroxide (NaOH). The solution was added to 10 mL of a measuring flask with distilled water. The solution was transferred into a cuvette, and the absorbance was measured using a UV-VIS spectrophotometer at a wavelength of 510 nm. The sample was weighed as much as 50 mg and put in a test tube 10 mL, and 0.3 mL of 5% sodium nitrite (NaNO2) was added. After 5 minutes, 0.6 mL of 10% aluminum chloride (AlCl3) was added and waited for 5 minutes. The solution was added with 2 mL of sodium hydroxide (NaOH) 1 M, and the absorbance was measured using a UV-VIS spectrophotometer at a wavelength of 510 nm [11].

### *2.3. Moringa Leaf Patch Formulation*

Alpha Cellulose (AC) is dissolved into 70% ethanol and hot water at a temperature of ±50 °C. PVA is dispersed in hot water at a temperature of ±50 °C. The solution is stirred until it reaches room temperature. The PVA solution was added to the AC solution and stirred until homogeneous. Phenoxyethanol and Moringa leaves ethanolic extract were added to the mixture, PEG 400 and

propylene glycol were added, and the mixture solution was stirred until homogeneous. The homogeneous mixture was poured into a petri dish and then dried using an oven at 40°C for ± 8 hours and at room temperature for 3-4 days until the dry patch is moist [12].

**Table 1**. The formula of Moringa leaf ethanolic extract patch using combination of patch polymer

| Ingredients | Weight (grams) | Function |
|---|---|---|
| Moringa extract | 0.30 | Active substance |
| Polyvinyl alcohol | 0.20 | Hydrophylic polymer |
| Alpha cellulose | 0.40 | Hydrophylic polymer |
| Polyethyelene glycol 400 | 0.60 | Plasticizer |
| Propylene glycol | 0.60 | Penetration enhancer |
| Phenoxyethanol | 0.04 | Preservative |
| Ethanol 70% solvent | 0.60 | Co-solvent |
| Aquadest | 6.50 | Solvent |

*2.4. Temperature Resistance Test*

Patches were stored at cold temperature (4 °C), hot temperature (40 °C), and room temperature (25 °C) with each storage time of 8 hours. Repetition is carried out for six cycles, with one cycle lasting 24 hours [12]. An organoleptic test includes observing the patch's shape, color, and aroma. The pH test of the patch matrix is carried out using a pH meter, where the patch is soaked in water with a ratio of 1:9, and then the pH value of the solution is measured. The patch thickness was measured using a micrometer with a Scrub Micrometer instrument with an accuracy of 0.01mm. Measurements were made on five sides, and the average value was calculated. Three patches were weighed, and then the average patch weight was calculated. The folding endurance test is a parameter for the film's flexibility and the matrix film's strength matrix. The patch is folded at one part repeatedly until the patch tears. The folding endurance value of a good patch matrix is more than 200 folding times. Moisture content analysis aims to evaluate the water absorption level from patches conditioned at 75% humidity using a moisture analyzer.

*2.5. Anti-inflamatory Test*

The ethical clearance application was conducted at Moewardi Hospital, Surakarta, Indonesia. The selected patch matrix formula was tested for anti-inflammatory activity on test animals. Nine male mice, aged 4-8 weeks and weighing 20 grams, were fasted for 18 hours but still given drinking water. Mice were divided into three treatment groups of mice each. Treatment groups include a negative control group, a positive control group, and a treatment group. The initial volume of the mice's feet was measured before treatment, using a plethysmometer, by inserting the soles of the mice's feet, which had been marked up to the ankles (up to the mark), into the plethysmometer. Anti-inflammatory effectiveness testing was carried out using the method of forming edema on the legs of test animals by induction of a 0.5% carrageenan solution. All received treatment measurements were repeated at 0, 5, 10, 15, 30, 45, 60, 90, 120, 150, 180, 210, 240, 270 and 300 minutes. The edema volume is the difference in edema volume at mice's feet after being induced with a 0.5% carrageenan solution compared with volume before being injected with the carrageenan solution [10].

*2.6. Data Analysis*

Data from the temperature resistance test results against temperature variations in patch preparations were analyzed using the SPSS program with the Shapiro-Wilk test. Continue with the One-Way ANOVA test if the data was normally distributed.

## 3. RESULTS AND DISCUSSION

The determination results with document number 071/UN27.9.6.4/Lab/2021 show that the Moringa plant used is the *Moringa Oleifera* Lam species. The percentage yield of Moringa leaf ethanolic extract was 8.74%. Previous research conducted by Ambarsari [13] obtained a yield of 9.32%. This shows that the resulting yield was close to previous research. The results of the organoleptic evaluation showed that the Moringa leaf ethanolic ethanol extract had a thick consistency with a dark green color, a bitter taste, and a distinctive smell of Moringa leaf. The organoleptic and randement test results have met the description of Moringa leaf ethanolic extract according to the Indonesian Herbal [11]. The water content of the extract affects the quality of the extract, where the water content of more than 10% makes it easy for fungus or mold to grow, so that it can reduce the biological activity of the extract during storage. The percentage of water content of Moringa leaf ethanolic extract was 0.5%. These results meet the water content requirements, namely less than 10%.

Determination of total flavonoid content using shear reagents AlCl3, NaNO2, and NaOH. The purpose of adding a shear reagent is to determine the presence of groups of additions attached to the leading group of flavonoids, which have reactions so that it can be known based on the shift in wavelength maximum [14]. The method is based on ion reactions of aluminum (Al 3+) with flavonoids in alkaline media, which will form a chelate red [15]. From the results of determining the standard calibration curve, the regression equation y = 0.0032x –0.0026 with a coefficient of determination (R2) of 0.9992 or coefficient correlation (R) of 0.9996. According to Miller and Miller (2018), these results state a linear relationship between concentration and absorbance because the correlation coefficient value is more than 0.992. The total flavonoid content in the ethanol extract of Moringa leaves was 142.1 mgQE/g extract or 14.21% w/w. There is a possibility that this level decreases during the process of patch formulation due to temperature factors. Total flavonoid levels in this study were adequate requirements in the Indonesian Herbal Pharmacopoeia due to total flavonoid content in the thick extract of Moringa leaves not less than 6.30% w/w calculated as quercetin [11].

### *3.1. Temperature Resistance Test*

The organoleptic test results of the three patches showed no changes in color, odor, or condition of the patches. This shows that variations in storage temperature do not affect the organoleptic properties of the Moringa leaf ethanolic extract patch (Figure-1)**.** The pH requirement for topical preparations, according to SNI 16-3499-1996, is 4.5-8. The pH test results show that storage temperature variations affect the patch's pH. Before the temperature variation treatment, the three patches had an average pH of 6.18; after the temperature variation treatment, the three patches had an average pH of 5.69. The paired sample t-test shows a significant difference with a significance value of 0.005 < 0.05. It can be concluded that variations in storage temperature affect the pH value of the Moringa leaf ethanolic extract patch.

A light patch matrix will be preferred because it will be comfortable [16]. The results of the statistical analysis of the paired sample t-test showed a significant difference between the weights before and after the variation of the temperature resistance test was carried out, with a significance value of 0.015 < 0.05. It can be concluded that variations in storage temperature affect the weight of Moringa leaf ethanolic extract patches.

The thickness of the patch matrix affects the release of the active substance; thick patches may take a long time to release the active substance, so the therapeutic effect is delayed. The statistical analysis results of the paired simple test show a significant difference in value significance 0.038 < 0.05. It can be concluded that variations in storage temperature affect the thickness of the Moringa leaf ethanolic extract patch.

The number of times the patch film can be folded in the same area without tearing is the value of folding endurance [17]. The test is carried out manually by folding the patch along the same line repeatedly until it breaks or folding it up to 300 times [18]. The folding endurance of Moringa leaf ethanolic extract patches from the three patches met the requirement of the patch with a folding endurance value > 300 times during storage at various temperatures. The results obtained have good integrity when applied to the skin. Low water (moisture) content will cause the patch to become brittle quickly, but if the moisture content is high, it is susceptible to the growth of fungi and bacteria [19]. In addition, humidity can also affect the skin barrier and help drugs penetrate through the skin membrane.

Research conducted by Ermawati et al. (2022) [12] reported that formula with a combined ratio of PVA and alpha-cellulose polymers of 1:2 in Moringa oleifera leave ethanolic extract has a pH value of 6.3-7.0; thickness 0.06-0.10 mm; folding endurance more than 300 times; and moisture content of 21.00%-22.81%. A higher concentration of PVA will increase the moisture content, thickness, and pH of the patch.

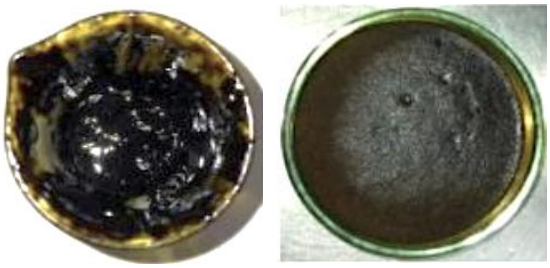

**Figure 1.** The Moringa leaf ethanolic extract and patch

**Table 2**. The results of resistance temperature test of Moringa leaf ethanolic extract patch after variaton temperature treatment in six cycles

| Treatments | Temperature resistence test in variation temperature of 4 °C, 40 °C and 24 °C for 8 hours respectively | |
|---|---|---|
| | Before treatments | After treatments |
| Weight uniformity (grams) | 1.94±0.16 | 1.68±0.17 |
| pH value | 6.18±0.15 | 5.69±0.16 |
| Folding endurance (times) | >300 | >300 |
| Moisture content (%) | 14.00±0.88 | 11.80±2.38 |
| Patch thick (mm) | 0.73±0.15 | 0.65±0.13 |

*Mean±SD, replicatin three times

The various temperature process is expected to increase the average molecular weight of the polymer, and it might raise the melting point of the crosslinked PVA polymer. However, because the hydrogels that we studied are a composite, the interaction between the two polymer such as a glass transition temperature before the first melting, the two polymers can be regarded as a miscible mixture with no indication of separation of the individual constituent.

*3.2. Anti-inflamatory Activity*

The anti-inflammatory test begins with registering an Ethical Clearance (EC) letter with document number 541/VIII/HREC/2021 issued by the Ethics Commission. It is intended for research that uses test animals. The anti-inflammatory test is the formation of artificial edema on the soles of mice's feet using a carrageenan solution of 0.5% as an edema inducer. This method was chosen because it is simple, and observational data is obtained more quickly. Percent inflammation is the amount of inflammation that occurs due to carrageenan-induced tissue on the soles of mice's feet. After the formation of edema, the soles of the mice's feet were treated by dividing them into three groups. The treatment group consisted of a positive control using a patch brand product with analgesic and antipyretic action, a negative control using an empty patch preparation, and a treatment control using a patch preparation of Moringa leaf ethanolic extract.

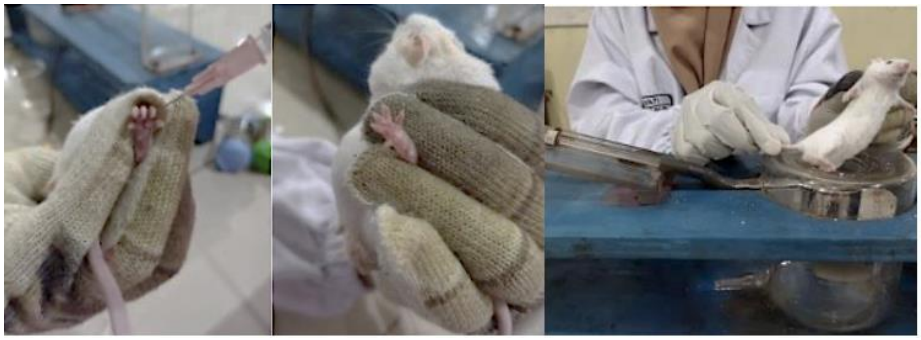

**Figure 2**. Anti-inflamatory process of Moringa leaf ethanolic extract where the edema induced, edema in mice's feet and measure the edema volume using plestimometer

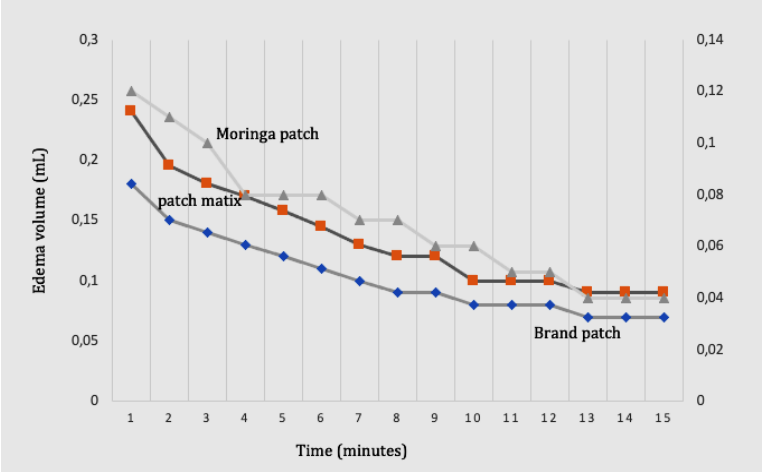


**Figure 3**. The profile of edema volume of mice's feet during treatment of patch (negative control), brand patch (positive control), and Moringa patch

The results of the one-way ANOVA statistical analysis showed a significance value of 0.000 < 0.05, meaning that treatment affected the volume of edema in the mice's feet. There is a significant difference between the negative and treatment control, with a significance value of 0.024 < 0.05. The resulting Asyimp sig value is 0.066, which is greater than 0.05. It can be concluded that the treatment control and positive control have the same average volume, and the negative control has the highest average edema volume. Moringa leaf ethanolic extract contains flavonoids, which have an anti-

inflammatory mechanism by inhibiting cyclooxygenase through the hydrogel layer on the patch. The results of this research can provide initial information that Moringa leaf ethanolic extract patches can be further developed as a delivery system with anti-inflammatory effects (figure 3) [20].

The concentrations of flavonols and flavones obtained from 100 g of dry samples were 5.53 mg luteolin, 409.06 mg quercetin, and 84.48 mg kaemferol. The results showed that the large amounts of flavonols and total flavones compounds are quercetin. Moringa oleifera leaves also contain low amounts of luteolin. Spot elution results observed using visible light, UV 254 nm, and 366 nm. Wavelength 254 nm used to see spots on silica gel, while at a wavelength of 366 nm used to see spots that can't observe at length 254 nm wave. The results show that the standard spot of quercetin and Moringa oleifera leaves ethanolic extract has Rf values of 0.91 and 0.95 respectively. Based on the requirements, the Rf value of the quercetin standard is 0.91. The Rf value of Moringa oleifera leaves ethanolic extract is close to the Rf value of quercetin standard, it may in the Moringa oleifera leaves ethanolic extract in this study contained total flavonoid counted as quercetin [12].

## 4. CONCLUSION

Temperature affected the pH, thickness, and weight properties but did not affect the moisture, folding endurance, and organoleptic properties. The average edema volume of mice in the brand product group was not significantly different from the moringa leaf patch group, with a significance value of 0.066 ($p > 0.05$). The Moringa leaf ethanolic extract patch confirmed it can reduce the volume of mouse foot edema by 5 hours.

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**Acknowledgments:** -

**Conflicts of interest:** The authors declare no conflict of interest.

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*Original Article*

# Formulation and Evaluation of Wound Healer Waterproof Gel Spray containing *Cymbopogon nardus* L. and *Musa paradisiaca* L. Leaves Wax

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**Abstract:** Red lemongrass *(Cymbopogon nardus* L.) is an Indonesian plant which has potential as a wound healer. Red lemongrass contain essential oil which has the potential effect to treat wounds, such as citral. The citral content in every 1 mL of this essential oil is 32.28%. A wound is an injury-induced disturbance of the tissues' normal anatomical relationships. Wound is basically prone to infection, and it can infect bacteria, such as *Staphylococcus aureus* bacteria. Water splashes exposed the *Staphylococcus aureus* bacteria, which then caused an infection. Water is a medium for bacterial growth. In this research, kepok banana leaves wax *(Musa paradisiaca L.)* is added as a waterproof base to prevent wounds from being splashed by water, because wounds on skin tissue are protected by a layer of wax originating from kepok banana leaves *(Musa paradisiaca L.)*. This research aimed was to make a waterproof spray from red lemongrass essential oil using wax of banana leaves as a waterproof base. The result showed waterproof gel spray preparation has yellowish white colour; a distinctive aromatic odor, pH 6.28; viscosity 135 cps, and dry time 7.5 minutes and it has waterproof properties.

**Keywords:** red lemongrass, wax banana leaves, waterproof gel spray, wound healer

## 1. INTRODUCTION

Red lemongrass *(Cymbopogon Nardus* L.) is a plant originally from Indonesia and it has antibacterial effect as a wound healer. Essential oil content of red lemongrass, such as citral has potential effect as a wound healer agent. Yield of essential oil content of red lemongrass during the distillation process from eight kilogram wet red lemongrass could produce ten milli litres essential oil. One milli litre lemon grass essential oil which is harvested in three months contained 32.23% citral [1]. Essential oils are found in red lemongrass *(Cymbopogon nardus* L.*)* plants. The average amount of essential oil found in lemongrass leaves is 0.7% (it can reach 1.2% in the dry season and about 0.5% in the wet season). The colour of red lemongrass oil is a light yellow. Aldehyde compounds (citronellol-$C_{10}H_6O$), alcoholic compounds (citronellol-$C_{10}H_{20}O$ and geraniol-$C_{10}H_{18}O$), and other compounds such geraniol, citral, nerol, metal, heptonone, and dipentene are the major active components generated [2]. From 300 grams of plant material, the root of the lemongrass plant contains about 0.52% alkaloids. The flavonoids luteolin, luteolin 7-O-glucoside (cynaroside), isoscoparin, and 2''-O-rhamnosyl isoorientin are found in the leaves and roots of lemongrass.

Quercetin, kaempferol, and apigenin are other flavonoid substances that have been identified from the aerial sections of the lemongrass plant [3]. From the results of the compound content obtained, carried out with a thin layer chromatography screening test.

Wound can be described as a disruption of the normal anatomical relationship of tissues as a result of injury. The injury may be intentional such as a surgical incision or accidental following trauma [4]. There are so many type of wound, such as incised wound, laceration, abrasion, and surgical wound. Infection of wound caused by bacterial activity including *Staphylococcus aureus.* Previous research explained that concentration of 3% essential oil obstructed *Staphylococcus aureus* activity.

The research aimed was to evaluate and formulate of waterproof gel spray containing Red Lemongrass *(Cymbopogon nardus* L.) and Kepok Banana *(Musa paradisiaca* L.) leaves wax as a waterproof base. A novel waterproof gel spray preparation was made by combining red lemongrass essential oil *(Cymbopogon nardus* L.), an antibacterial agent, combined by wax from kepok banana *leaves (Musa paradisiaca* L.). This antimicrobial helps to hasten the healing of wounds. A concentration of three grammes of wax from the extraction of kepok banana leaves *(Musa paradisiaca* L.) was shown in a research from Ramadhan et al. (2020) to have the capacity to create a waterproof layer. The wax material found in Kepok banana leaves was used to create a waterproof gel spray because it has the ability to create a waterproof coating and it shows that after spraying, the waterproof layer can stick for 30 minutes. This waterproof gel spray can protect wounds from water, and can speed up wound healing due to the presence of essential oil from red lemongrass (*Cymbopogon nardus* L.).

## 2. MATERIALS AND METHODS

### *2.1. Collection red lemongras (Cymbopogon nardus* L.)

Red lemongrass is harvested in Sorogenen, Ambarketawang, Gamping, Sleman, Indonesia. To get rid of dirt and bacteria, the stems of red lemongrass are cleaned. The red lemongrass stems are dried at 60°C in an oven until they are free of any remaining impurities *[5]*. This parameters used to detect which red lemongrass had been dried, and the weight remained consistent *[6]*.

### *2.2. Preparation of red lemongrass extract*

Extraction of red lemongrass is carried out using the steam distillation method to obtain essential oil which contains citral. It started by weighing the stems and leaves of red lemongrass which have been cut into small pieces (eight kilograms). Then put it in a distillation apparatus with the addition of distillated water as a solvent. Turn on the heater according to the temperature variable (100-105°C). The distillate is collected, then transferred into a separating funnel to separate the essential oil from the air. The essential oil is transferred into a flacon and stored in the freezer.

### *2.3. Preparation Wax Kepok Banana Leaves (Musa paradisiaca L.)*

Preparing banana leaves that have been cleansed from the midrib and allowed to air dry for a week. The leaves are then divided into little bits. 100 grams of banana leaves should be weighed before being rubbed with n-hexane solvent. The filtering and separated filtrate were left at room temperature for 2 hours after the filtering and reflux process, which took place at 60°C for 10 minutes *[7]*.

*2.4. Thin Layer Chromatography (TLC) Screening of Citral from red lemongras (Cymbopogon nardus (L.) Rendl.)*

Sample preparation started from the distillation of red lemongrass *(Cymbopogon nardus L.).* The mobile phase preparation used is n-Hexane:Ethyl acetate (8:2). Saturate the chamber using filter. Elute the sample to the limit mark. Dry the plate by airing it and then detect it with UV 254 light.

*2.5. Formulation of Wound Healer Waterproof Gel Spray*

Formulation of wound healer waterproof spray could be seen in Table 1.

**Table 1.** Formulation of Waterproof Gel Spray

| Material | Amount |
|---|---|
| PVA | 1 gram |
| Span 80 | 0.375 % |
| Tween 80 | 0.125% |
| Wax Kepok Banana Leaves | 5% |
| Essential Oil Red Lemongrass | 5% |
| Ethanol | 10% |
| Distillated water | Ad 20 mL |

*2.6. Evaluation of Wound Healer Waterproof Gel Spray*

*2.6.1. Organoleptic Test*

Organoleptic testing is carried out by sense. Organoleptic testing includes smell, color and taste which are directly tested using the senses.

*2.6.2. pH Test*

pH testing was carried out using a pH meter (Lutron pH-208), before using the pH meter, calibration was carried out using a pH 4.0 and pH 10.0 calibrator, after calibration the pH meter was dipped in the formula sample that had previously been made. The pH of a good preparation for wounds is 4.5 – 6.5 *[8].*

*2.6.3.Viscosity Test*

Viscosity testing was carried out using a Brookfield viscosimeter, when testing the viscosimeter the tool was set at a speed of 100 rpm *[9].*

*2.6.4. Dry Time Test*

Testing the preparation dry time of less than 5 minutes can minimize the growth of microorganisms and evaluation of the volume of one waterproof gel spray is carried out to estimate how many times to spray the preparation to obtain a uniform volume. Dry time testing is carried out directly on the skin by spraying the sample solution, and waiting until the sample solution dries and the drying time is recorded.

## 3. RESULTS AND DISCUSSION

Red lemongrass (*Cymbopogon nardus* L.) is a Poaceae (grasses) perennial herb [10]. In almost all continents, lemongrass has been used extensively as a traditional medicine by making "tea" or an infusion from fresh or dried leaves. It has a wide range of indications, from mild ailments like the flu, fever, cuts, coughing, and headaches to more serious illnesses like rheumatoid arthritis, bladder disorder, diabetes, and malaria [11]. It is a powerful antibacterial, antitussive, antiseptic, analgesic, and anti-inflammatory agent [12].

*3.1. Thin Layer Chromatography (TLC) Screening of Citral from red lemongrass (Cymbopogon nardus (L.) Rendl.)*

TLC was used to determine the optimal eluent for separating citral, employing Silica Gel 60 GF254 as the stationary phase and one type of eluent pair as the mobile phase, namely n-hexane: ethyl acetate (8:2) [13]. Spots formed during the elution process were identified using a 254 nm UV light. The polarity of the eluent used determines spot mobility. The optimal eluent is chosen from a mixture of eluents that can get the component up to the end line of the silica plate, resulting in a polarity difference between the solvents and a genuine spot. Figure 1 shows the spots formed on the TLC plate.

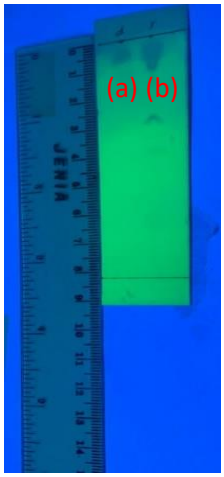


**Figure 1.** TLC Profile. (a) standard of citral (b) essential oil of Red Lemongrass

As a result of steam distillation, which yielded pure essential oil, the elution results revealed the presence of a citral compound in the red lemongrass sample. The Rf values of the sample and the reference compound were calculated; the sample's Rf value was 0.925 cm and the citral standard's Rf value was 0.925 cm.

*3.2. Formulation of Wound Healer Waterproof Gel Spray*

Topical sprays have gained popularity in clinical practice over the past few decades as a method of applying hydrogels and or cell suspensions to treat both acute and chronic wounds because of their benefits, including the ability to treat large wounds or spray over areas with unfavorable topography, the shortened application time, and the uniform distribution of the sprayed suspensions [14]. This formula use PVA as a gelling agent, Span 80 and Tween 80 as surfactant, ethanol as a solvent, wax kepok banana leaves as a waterproof base, essential oil of red lemongrass as an active substance which include citral.

*3.3. Physical Characterization of Waterproof Gel Spray*

Topical spray usually contains ethanol and water as solvents. Other excipients are co-solvent, which is used to dissolve the drug substance to obtain a clear solution, film forming agent, and humectants. *Cymbopogon nardus* L. in combination with *Musa paradisiaca* L. through topical spray was developed by complexing citral as an essential oil. Polyvinyl alcohol (PVA) was a film former with good adhesion as well as a stabilizing agent. The wax of banana was also an auxiliary film-forming

agent. Span 80 and Tween 80 were used to promote moisture retention on the skin. Tween 80, when applied as a surfactant, can create a stable nano emulsion [15]. Tween 80 is not only employed as a surfactant, but it can also reduce the globule size of lipophilic compounds in microemulsion and nanoemulsion systems. Essential oils prepared with tween 80 can keep active ingredients stable and effective [16].

3.3.1. Organoleptic

The human senses are used in organoleptic testing. Organoleptic testing covers smell, color, and taste, all of which are assessed directly with the nose, eyes, and mouth. The results of the tests revealed a unique fragrant odor, a yellowish-white color, and a bitter taste.

3.3.2. pH

pH testing was carried out using a pH meter (Lutron pH-208), before using the pH meter, calibration was carried out using a pH 4.0 and pH 10.0 calibrator, after calibration the pH meter was dipped in the formula sample that had previously been made. The pH of a good preparation for wounds is 4.5 – 6.5 [8]. The results of the pH test were 6.28. This result is still acceptable because it is in accordance with the pH standard for the skin.

3.3.3. Viscosity

Viscosity testing was performed using a Brookfield viscosimeter, with the toolset to a speed of 100 rpm and the spindle inserted directly into the sample solution. An excellent spray gel preparation has a viscosity of less than 150 cps [9]. The viscosity testing results were obtained 135 cps this result meets the requirements for skin spray gel formulations.

3.3.4. Dry Time

A dry time of less than 5 minutes for the preparation can minimize microbe growth, and an evaluation of the volume of one spray is performed to predict how many times to spray the preparation to get a uniform volume. Dry time testing is performed directly on the skin by spraying the sample solution and waiting until the sample solution dries before recording the drying time. The preparation test results revealed that the drying time was 7.5 minutes, which it takes longer so further testing is needed.

Citral, one of lemongrass' key components, has been proven to decrease the production of interleukin (IL)-1b, IL-6, and tumor necrosis factor (TNF)$\alpha$ [17]. Thus, the surface of banana leaves has a waxy covering that provides hydrophobic qualities that act as a water repellent. The waxy surface of the leaves, according to Jetter and Kunst (2008), serves as a protective layer against water loss, ultra-purple light, infections, and insects [18]. Wax are also valuable raw materials for industrial uses such as high-value lubricants, cosmetics and medicines, and high-powered fuels. Wax and leaf microstructure also contribute to leaf surface roughness [19]. Furthermore, lemongrass has antioxidant activity and can scavenge free radical molecules [20].

Inflammation is involved in the phases of wound healing. Inflammatory is a two-edged sword with both positive and negative repercussions, and during the inflammatory phase, leucocytes create oxygen radicals as oxygen-dependent processes. These free radicals are excreted as a weapon to destroy bacteria, but they can also be harmful to body cells. Excess free radicals and/or antioxidant deficiency can cause oxidative stress, which can lead to cellular malfunction and, in extreme cases,

cell death. In this case, antioxidants serve to protect cells from harm caused by free radicals created during inflammation [21].

## 4. CONCLUSION

The results of formulation and evaluation showed waterproof gel spray preparation has yellowish white colour; a distinctive aromatic odor, pH 6.28, viscosity of 135 cps, and dry time 7.5 minutes and it has waterproof properties.

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*Original Article*

# Effect of Corn Starch and Glycerol Additions on the Characteristics of Capsule Shells from Seaweed Carrageenan

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**Abstract:** Capsule shells are generally made by gelatinee which from animal based. However, the capsule shell has issues related to the permissibility (halalness) and safety. Carrageenan has the potential as an essential ingredient of the capsule shell to replace gelatinee. The focus of this study was to determine the optimal formulation for the preparation of capsule shells. There were 9 different formulations to test the best quality by comparing it with commercial capsule shells. The samples were manually moulded with a dipping pen for 3 s at 45℃. The concentrations of corn starch were 1%, 2%, and 3% w/v and concentration glycerol were 2%, 3%, and 4% w/v. Weight uniformity, specifications, disintegration, and FTIR were analysed to determine the best quality of the capsule shell. The results showed that formula with concentration of 95% of distilled water, 2% w/v of carrageenan, 2% w/v of corn starch and 3% w/v of glycerol fit the standard of capsule shell.

**Keywords:** Capsule shell, carrageenan, corn starch, glycerol

## 1. INTRODUCTION

Availability of medicine is very important for the society because it can help in the process of curing a disease. Various drugs have been circulating in Indonesia in multiple forms, including tablets, capsules, ointments, creams, and syrups [1,2]. Capsules are considered simpler in production compared to the other forms [3]. Capsule shells are generally made from animal-based gelatinee. However, the capsule shell has issues related to the permissibility (halalness) and safety [4]. Therefore, one alternative to overcome this issue is to utilize plants found in Indonesia as the basic material for making capsule shells.

Several research related to capsule shells made from starch has previously been conducted by Suparman et al. (2019) [5] using cacao peel pectin with carrageenan. They reported three experiments were carried out with different formulas. The purpose of formula variation is to get a suitable capsule formula composition. In addition, there is also research by Lestari et al. (2021) [6], who made a capsule shell based on potato peel starch with honey as a plasticizer. They conducted five experiments with different formulations. The best formulation of potato peel starch capsule shell with the addition of plasticizer was formula with 2% wt. starch concentration.

Several research indicates the potential of various flora, including seaweed and corn, as essential components in capsule shell development. Seaweed with the type of *Euchemma cottoni* can produce polysaccharides in the form of carrageenan with demonstrated thickening, suspending, stabilizing, and emulsifying properties [7,8]. To obtain capsule shells with the optimal quality, starch plays a key role in stabilizing carrageenan for gel formation.

In manufacturing capsule shells, the used of raw materials is shifting away from animal-derived gelatine towards plant-based polysaccharides. These versatile biopolymers offer a sustainable and ethical alternative, with properties suitable for creating effective and safe capsules. Polysaccharides can be used to make capsule shells instead of gelatinee [9]. The types of polysaccharides are carrageenan, starch, alginate, and pectin [9]. Starch is composed of two polysaccharides, amylose (15-25%) and amylopectin (75-85%), which are stored in starch granules [10,11]. Corn starch and carrageenan, promising alternatives to gelatine, were explored for their suitability in capsule shell production.

Therefore, this study focused on characterizing their properties with glycerol and determining the optimal formulation of capsule shell.

## 2. MATERIALS AND METHODS

### *2.1. Materials*

Carrageenan was obtained from Indoplant (Special Region of Yogyakarta), corn starch from Mitra Jaya Chemical (Bekasi, West Java), glycerol from CV. Indrasari (Semarang, Central Java), distilled water, HCl and vaseline. The materials used were a dipping pen, oven, magnetic stirrer, digital scale, stirrer glass, and beaker glass from Industrial Chemical Engineering Laboratory.

### *2.2. Methods*

This study begins with preparing a capsule shell solution by heating distilled water in a beaker glass. Carrageenan and corn starch were added according to the variables. The solution was stirred until homogeneous, and glycerol was added, then stirred again until the mixture was perfectly mixed.

The samples were formed with a dipping pen that had been previously smeared with Vaseline so it would be easy to retrieve the results. Next, the dipping pen is dipped into the solution for 3 s at 45℃. Then, let the dipping pen set for 10 min before it is placed in the oven to heat up. The heating process uses a temperature of 50℃ and a time of 3 h [12]. The formed capsule shell is slowly released from the pin. After that, testing was carried out to determine its quality by comparing it with capsule shells generally made of gelatine [13].

**Figure 1.** Capsule shell molding process

### *2.3. Capsule Shell Formulation*

This study is an experimental method in the laboratory with the formulation of capsule shells from carrageenan and corn starch. The concentrations of corn starch and glycerol were 1%, 2%, 3% w/v and 2%, 3%, and 4% w/v respectively. In addition, the formulation used in carrageenan, which

is 2%, refers to previous research conducted by Lestari, Indah. etc. The formula of capsule shell was following in the Table 1.

**Table 1.** Formulation of capsule shell preparation

| Formula | Distilled Water (mL) | Carrageenan (w/v) | Corn starch (w/v) | Glycerol (w/v) |
|---|---|---|---|---|
| 1. | 95 | 2% | 1% | 2% |
| 2. | 95 | 2% | 1% | 3% |
| 3. | 95 | 2% | 1% | 4% |
| 4. | 95 | 2% | 2% | 2% |
| 5. | 95 | 2% | 2% | 3% |
| 6. | 95 | 2% | 2% | 4% |
| 7. | 95 | 2% | 3% | 2% |
| 8. | 95 | 2% | 3% | 3% |
| 9. | 95 | 2% | 3% | 4% |

2.3.1. Weight Uniformity Test

This test was conducted to determine the suitability of the weight of the capsules produced based on the requirements set by Fermakope Indonesia. Capsule weight uniformity is carried out by determining the difference in the weight of the contents of each capsule whose % deviation should not exceed the predetermined one [14].

2.3.2. Specifications Test

The specification tests observed are the length, diameter, thickness, weight, and volume. The specifications of length, diameter, and thickness were measured using a caliper. For the weight of the capsule shell, it was weighed using a digital balance. Volume measurement of the capsule shell was completed by filling the capsule shell with water.

2.3.3. Disintegration Test

The disintegration time test is required for the capsule to disintegrate in the specified medium. The disintegration time of each capsule must meet the requirements of the time specification as determined by a disintegrator tester. According to the Ministry of Health (2014) [15] in Pharmacopoeia, a suitable disintegration time on distilled water is 15 to 30 min and disintegration time on acid solution is less than 5 min [16,17].

2.3.4. Fourier Transform Infrared Test

One of the analyses used to determine the structure of the compound. Types of samples that can be analysed include solutions, pastes, powders, films, and so on [18,19]. FTIR is an infrared spectrum collection technique. The energy that has been absorbed by the sample at the frequency of infrared light will be recorded and then forwarded to the interferogram [20,21].

## 3. RESULTS AND DISCUSSION

### *3.1. Effect of corn starch and glycerol on the weight uniformity test of capsule shell*

This test was conducted to determine the uniformity of the weight of the capsules produced by the requirements of Farmakope Indonesia. Capsule shells that have been made are then weighed on a digital balance sheet, and the weight of each capsule is recorded. From the results of these calculations can then be calculated % deviation. Figure 4 show the weight uniformity of capsule shell with the different concentration of corn starch and glycerol. The results showed that the capsule shells made from carrageenan and corn starch with the addition of glycerol had weights that deviated from the commercial standards set in formulas 1-4 and formula 6, which were more than 10%. This is caused by the thinness of the capsule shell formed so that it affects the capsule shell produced. While in formula 5, formula 7, formula 8, and formula 9 have complied with the set standards because the % deviation is still under the standard. The weight of the capsule shell is affected by the thickness of the capsule shell coating. It can be concluded that the thicker the capsule is, the more the weight the capsule increases.

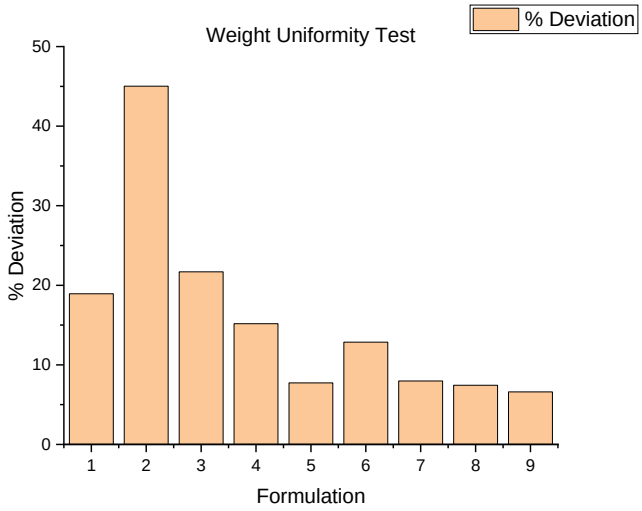


**Figure 2**. Weight Uniformity Test

### *3.2. Effect of corn starch and glycerol on the physical properties of capsule shell*

The things observed in this specific test are length, diameter, thickness, weight, and volume are sown in Table 2.

**Table 2**. Specification test of capsule shell

| Formula | Length (mm) | | Diameter (mm) | | Thickness (mm) | Weight (gr) | Volume (mL) |
|---|---|---|---|---|---|---|---|
| | Body | Cap | Body | Cap | | | |
| **1** | 16.1±0.100 | 7.21±0.006 | 7.91±0.010 | 7.65±0.006 | 0.11±0.010 | 0.045±0.005 | 0.43±0.026 |
| **2** | 16.24±0.069 | 8.5±0.010 | 7.7±0.006 | 7.74±0.006 | 0.13±0.006 | 0.02±0.002 | 0.41±0.000 |
| **3** | 16.18±0.010 | 9.82±0.015 | 7.71±0.006 | 7.62±0.015 | 0.21±0.010 | 0.04±0.002 | 0.38±0.015 |
| **4** | 16.41±0.010 | 9.67±0.058 | 7.42±0.029 | 7.7±0.006 | 0.1±0.010 | 0.056±0.004 | 0.49±0.012 |
| **5** | 18±0.006 | 10.22±0.025 | 7.52±0.029 | 7.71±0.012 | 0.11±0.010 | 0.103±0.006 | 0.58±0.104 |
| **6** | 17.6±0.090 | 10.57±0.064 | 7.5±0.000 | 7.81±0.006 | 0.21±0.010 | 0.065±0.048 | 0.53±0.021 |
| **7** | 17.54±0.036 | 10.07±0.058 | 7.53±0.026 | 7.8±0.000 | 0.31±0.015 | 0.1±0.002 | 0.53±0.036 |
| **8** | 17.67±0.113 | 10.52±0.040 | 7.61±0.017 | 7.71±0.012 | 0.33±0.006 | 0.107±0.003 | 0.28±0.072 |
| **9** | 17.67±0.029 | 10.52±0.035 | 7.7±0.006 | 7.52±0.029 | 0.31±0.010 | 0.118±0.002 | 0.19±0.040 |
| **SNI** | 18.87 | 11.23 | 7.37 | 7.7 | 0.11 | 0.099 | 0.65 |

3.2.1. Length test of capsule shell

The length of the capsule shell was recorded using a vernier caliper. The results of the length specification test can be seen in Table 2, which shows that Formula 1 has a body and cap length of 16.10 mm and 7.21 mm. Formula 2 is 16.24 mm and 8.50 mm. For formula 3 it is 16.18 mm and 9.82 mm. Formula 4 is 16.41 mm and 9.67 mm. Formula 5 is 18.00 mm and 10.22 mm. Formula 6 is 17.60 mm and 10.57 mm. Formula 7 is 17.54 mm and 10.07 mm. Formulas 8 and 9 amounted to 17.67 mm and 10.52 mm. In this research, the results of length measurements showed that formulation 5 was close to the length set by SNI.

3.2.2. Diameter test of capsule shell

Diameter size was also measured using a vernier caliper. The results of the diameter specification test can be seen in Table 2, which shows that Formula 1 has a body and cap diameter of 7.91 mm and 7.65 mm. Formula 2 is 7.70 mm and 7.74 mm. Formula 3 was 7.71 mm and 7.62 mm. Formula 4 to 7.42 mm and 7.70 mm. Formula 5 was 7.52 mm and 7.71 mm. Formula 6 was 7.50 mm and 7.81 mm. Formula 7 by 7.53 mm and 7.80 mm. Formula 8 by 7.61 mm and 7.71 mm. Formula 9 was 7.70 mm and 7.52 mm. Based on research, the results of length measurements showed that formulation 5 was close to the length set by SNI.

3.2.3. Thickness test of capsule shell

This test also used a vernier caliper to measure the capsule shell's thickness. The results of the thickness specification test can be seen in Table 2. The results showed that the formulas by SNI were formulas 1 and 5, which amounted to 0.11 mm. Formula 2 has a thickness of 0.13 mm, formula 3 of 0.21 mm, formula 4 of 0.10 mm, formula 6 of 0.21mm, formula 7 of 0.31 mm, formula 8 of 0.33 mm, and formula 9 of 0.31 mm. The thickness of the capsule shell will increase as the concentration of starch used increases. The variety of results on this thickness is because the research still uses a manual dipping system, so the results obtained are unequal. However, in contrast to the capsule shells on the market, the product is produced using a molding machine so that the thickness and weight can be uniform.

3.2.4. Weight test of capsule shell

The weight of capsule shells was measured using a digital balance and evaluated based on SNI. Table 2 show the results of this study. Formulas 1, 2, 3, 4, and 5 show values that are still below the predetermined standards. Which are 0.045 gr, 0.020 gr, 0.040 gr, and 0.056 gr. In the other formulations, the values are obtained almost close to the standard: formulas 5, 7, 8, and 9, with each weight of 0.103 gr, 0.100 gr, 0.107 gr, and 0.118 gr. It can be concluded that the more starch concentration is used, the weight produced will increase.

3.2.5. Volume test of capsule shell

The results of the volume specification test on the capsule shell can be seen in Table 2 from the different formulations; the volume results obtained were closest to the standard in Formula 5, which is 0.58 mL. Other formulations obtained values still below the predetermined standards, including formula 1 of 0.43 mL. Formula 2 amounted to 0.41 mL. Formula 3 amounted to 0.38 mL. Formula 4 amounted to 0.49 mL. Formulas 6 and 7 amounted to 0.53 mL. Formula 9 amounted to 0.19 mL. The volume produced in the study has a variety of values. This is because the concentration of starch used is different. The more starch concentration used will increase the thickness of the capsule shell, which causes less volume-produced.

*3.3. Effect of corn starch and glycerol on the disintegration time test of capsule shell*

A suitable disintegration time, according to the Indonesian Ministry of Health (1995) [22], ranges from 15-30 min in distilled water solution and 5 min in acidic solution (HCl). In this research, 9

formulations were made with different variables. The results of the measurement of the disintegration of the capsule shell are in Table 3.

**Table 3**. Disintegration Test

| Formulation | Distilled Water (min) | HCl (min) |
|---|---|---|
| **1** | 20.04±0.075 | 4.00±0.035 |
| **2** | 23.06±0.167 | 4.16±0.060 |
| **3** | 23.27±0.261 | 4.07±0.064 |
| **4** | 24.18±0.156 | 4.53±0.289 |
| **5** | 21.34±0.053 | 4.73±0.058 |
| **6** | 25.18±0.137 | 5.69±0.081 |
| **7** | 35.89±0.248 | 5.78±0.127 |
| **8** | 36.48±0.072 | 5.12±0.240 |
| **9** | 37.26±0.047 | 5.92±0.067 |
| **SNI** | 30.00 | 5.00 |

3.3.1. Disintegration test in distilled water

Based on the results of this study, the disintegration time required in Formula 1 was 20.04 min, Formula 2 was 23.06 min, Formula 3 was 23.27 min, Formula 4 was 24.18 min, Formula 5 was 21.34 min, Formula 6 was 25.18 min, Formula 7 was 35.89 min, Formula 8 was 36.48 min, and Formula 9 was 37.26 min. The disintegration of a capsule shell is influenced by the thickness of the capsule shell itself. The thicker the capsule shell, the longer the disintegration time. The value of disintegration time in samples 1 to 6 is still in accordance with the standards set by Farmakope, which is 15-30 min.

3.3.2. Disintegration test in acid solution

The disintegration time in HCl can be seen in Figure 5. Formula 1 was 4.00 min, Formula 2 was 4.16 min, Formula 3 was 4.07 min, Formula 4 was 4.53 min, Formula 5 was 5.22 min, Formula 6 was 5.69 min, Formula 7 was 5.78 min, Formula 8 was 5.12 min, and Formula 9 was 5.92 min. The value of the destruction time in samples 1 to 4 is still by the standards set by Farmakope, which is around 5 min. The research results show that the capsule shells tested for disintegration in the acid solution were able to release the dye powder into the environment in less than 5 min [23]. This is also influenced by the thickness of a sample, where the thicker the capsule shell, the longer the destruction time required. In addition, what affects the length of the destruction time is carrageenan. Carrageenan material will affect the destruction time of a capsule to be longer because the chemical structure of carrageenan has hydrophobic properties [24].

*3.4. Fourier Transform Infrared Test of Capsule Shell*

To identify the molecular functional groups contained in a sample, a tool such as Fourier Transform Infrared Spectroscopy (FTIR) can be used. From the results of the FTIR specta in this study (Figure 3), it can be seen that there is an O-H hydroxyl absorption at 3292.97 cm-1. There is also a C-H functional group at 2940.65 cm-1 absorption. The C≡C functional group is shown in the 2110.94 absorption spectrum. The absorption peak of 1640.25 cm-1 shows the presence of C=C functional groups. Then, there is also an O-H functional group at 1420.68 cm-1. Sulfate ester bond S=O indicates all types of carrageenan content in the sample, namely at 1367 cm-1 and 1147.75 cm-1. The analysis done on samples made from carrageenan and corn starch shows that the carrageenan obtained is kappa carrageenan. The model has already fit the standard so that it is suitable for use as a raw material for capsule shells.

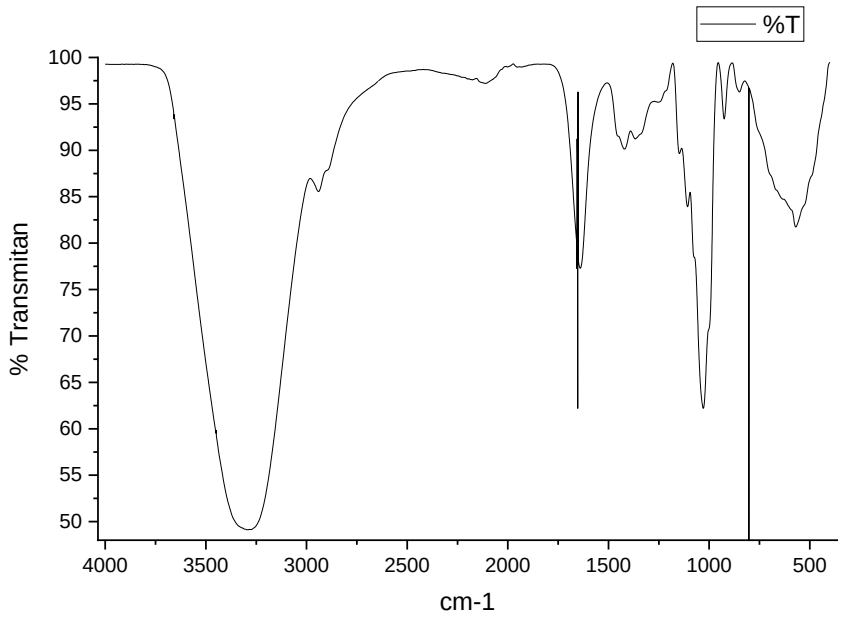


**Figure 3**. Fourier Transform Infrared Test

## 4. CONCLUSION

This study successfully fabricated capsule shells utilizing carrageenan and corn starch as primary components. Characterization and optimization of formulations containing carrageenan, corn starch, and glycerol resulted in formula 5 as the most suitable composition for capsule shell preparation, with 2% w/v of carrageenan, 2% w/v of corn starch, 3% w/v of glycerol, 95% wt. of distilled water.

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*Research Article*

# Preparation and Characterization of Red Dragon Fruit *(Hylocereus polyrhizus)* Starch as an Excipient in Solid Dosage Form

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**Abstract:** Starch is often used as a filler, crusher, and binder in solid preparations. One source of starch can be found in red dragon fruit stems. When making starch, browning often occurs which causes the flour to become brownish, which can reduce public acceptance. Efforts are made to prevent browning in the starch making process by using sodium bisulfite solution. This study aims to determine the effect of different sodium bisulfite soaking times on the characteristics of red dragon fruit stem starch *(Hylocereus polyrhizus)* to be used as a solid preparation additive and to determine the length of sodium bisulfite soaking time that can produce red dragon fruit stem starch *(Hylocereus polyrhizus)* characteristics that meet the standards of solid preparation additives. The results of the analysis showed significant differences in yield, moisture content, flow velocity, angle of repose, pH, solubility, expandability, compressibility index, bulk density, tap density, true density, and Hausner index. FTIR analysis showed that red dragon fruit stem starch contains starch functional groups. Based on the results of the red dragon fruit stem characterization test, the best treatment was obtained, namely 1 hour soaking, producing starch with physical characteristics suitable for solid preparation additives.

**Keywords:** red dragon fruit stem; starch; sodium bisulfite; characterization

## 1. INTRODUCTION

Red dragon fruit plants after harvest will be pruned to quickly stimulate the growth of new flowers. The remaining red dragon fruit stem will only be discarded because it is considered as waste and its utilization is still very minimal, so it is very necessary to handle it so that it does not become a problem if it is not handled properly, but the high water content of the red dragon fruit stem makes its shelf life very short, by making dragon fruit powder is expected to extend its shelf life. Powder from red dragon fruit stems that have been peeled off the skin contains starch, making it suitable for use as an additive to solid preparations [1].

Starch has many benefits and has long been used as a food ingredient or additive or excipient in solid preparations. The use of starch in the pharmaceutical field, especially in tablet preparation formulas, is used as a filler, crusher, or as a binder [2].

Processing of red dragon fruit stems into starch flour is done through several processes such as washing, removal of unwanted parts, size reduction, drying, crushing and sieving. In the flouring

process, browning often occurs which causes the flour to become brownish so that it can reduce public acceptability [3]. Many efforts are made to prevent browning in these foodstuffs by using sodium bisulfite and bleaching solutions.

A study needs to be conducted to determine the effect of sodium bisulfite soaking time on the characterization of starch from red dragon fruit stems *(Hylocereus polyrhizus)* as an additive to solid preparations.

## 2. MATERIALS AND METHODS

### *2.1. Materials*

The materials used in making starch and characterization testing of red dragon fruit stem starch are red dragon fruit stem *(Hylocereus polyrhizus)* from Gembong-Pati, distilled water, iodine pro analisis (Merch), $Na_2S_2O_3$ Pro Analisis (Smartlab), potato starch, cassava starch, corn starch, kaffein (Sigma Aldrich) , diclofenac sodium, ascorbic acid (Merch), paraffin liquid (ROFA).

### *2.2. Preparation of Red Dragon Fruit Stem Starch*

The red dragon fruit stems obtained were then washed and peeled off the outer skin, then sliced thinly with a thickness of about ± 0.2 mm. The cleaned red dragon fruit stems were then isolated by soaking in sodium bisulfite ($NaHSO_3$) solution with a temperature of 40°C only at the beginning of soaking (temperature is not maintained) with a concentration of 500 ppm, the weight ratio of red dragon fruit stems: solution is 1:2 with variations in soaking time of 1 hour, 2 hours, and 3 hours. Red dragon fruit stems are blended until they become a slurry, then stirred and kneaded with the aim of accelerating the release of starch from the protein or gum that covers it, then filtered with a filter cloth gradually. Settled for 24 hours until the starch separated from the soaking water. The precipitated starch was then washed with water 2-3 times until white (brownish white) starch was produced. The red dragon fruit stem starch sediment obtained was then dried at 60° C until a certain moisture content (⩽ 15%). The dried red dragon fruit stem starch was pulverized with a grinder, and sieved with a 100 mesh sieve

### *2.3. Physical Characteristics Testing*

#### 2.3.1. Organoleptical

The plants used in this study have been confirmed by plant determination conducted at the Pharmaceutical Biology Laboratory of the College of Pharmacy, Yayasan Pharmasi Semarang. The results of plant determination showed that the plant was a red dragon fruit plant (*Hylocereus polyrhizus*). Then the red dragon fruit stem starch was tested to observe the shape, odor, color and taste [4].

#### 2.3.2. Yield

Red dragon fruit stem starch with sodium bisulfite soaking at soaking times of 1 hour, 2 hours, and 3 hours was weighed and the yield was calculated.

#### 2.3.3. Moisture Content

Red dragon fruit stem starch was weighed as much as 1 gram and put into the moisture analyzers (Ohaus), the moisture analyzers was measured at 110° C.

#### 2.3.4. Identification

Red dragon fruit stem starch solution as much as 5 mL is put into a test tube and dripped with iodine solution as much as 5 drops, observe the color changes that occur [5][6].

2.3.5. Microscopy

Red dragon fruit stem starch is placed on a glass object, then covered with a cover glass, and observed the shape of the hilum and lamella of dragon fruit stem starch under a microscope at 1000x and 400x magnification.

2.3.6. pH Examination

Red dragon fruit stem starch was weighed as much as 1 gram and suspended with distilled water as much as 10 ml, pH was measured using a pH meter (WalkLAB) [7].

2.3.7. Flow Speed and Angle of Repose

Starch powder was weighed as much as 25 grams, put into a funnel whose bottom was closed, then the bottom of the funnel was opened so that the granules could flow and then the time was recorded and the height and radius were measured [8][9].

2.3.8. Water Content

The crucible was heated in an oven at 105°C for 30 minutes, and then tared to constant weight. Red dragon fruit stem starch was weighed as much as 1 gram, put into the krus, dried in an oven at 105°C for 30 minutes with the lid open, then put in a desiccator for 15 minutes. Dried until the weight of the crucible was constant [10].

2.3.9. Ash Content

The red dragon fruit stem starch was weighed as much as 1 gram, put into a crucible, then incinerated with a muffle furnace at 600°C for 3 hours. Then the crucible was cooled in a desiccator for 10 minutes and weighed [11].

2.3.10. Starch Content Analysis

Red dragon fruit stem starch was weighed as much as 50 grams and dissolved in distilled water as much as 50 ml of distilled water. The sample was pipetted 6 ml, then put in a 10 ml volumetric flask and added 1% iodine as much as 0.5 ml and then ad 10 ml. The absorbance was measured with a UV-Vis spectrophotometer (Shimadzu 1240) double beam at a maximal wavelength of 400-800 nm is (461.20 nm) obtained from the standard amylum maydis, and measured the standard series with concentrations of 300, 400, 500, 600, 700 and 800 ppm, then obtained a linear equation that will be used to determine the starch content of red dragon fruit stems [12].

2.3.11. Swelling Power and Solubility

Red dragon fruit stem starch was weighed as much as 2.5 grams, made a suspension of 50 ml of distilled water (2.5 g/50 ml), taken 10 ml and put into a test tube, heated in a waterbath at 60°C for 30 minutes. After the waterbath, it was centrifuged at 3000 rpm for 15 minutes. The precipitate was separated and weighed, then dried in an oven at 130°C for 2 hours, the dried precipitate was weighed and the swelling power and solubility were calculated.

Swelling power and solubility are calculated based on equations 1 & 2

$$\% S = \frac{A}{W} \times 100\% \quad \text{..... (1)}$$

$$\% SP = \frac{D}{W\,(1-S)} \times 100\% \quad \text{..... (2)}$$

Keterangan :

%S = Solubility

%SP = Swelling power

A = Weight of substance after oven (substance after oven)

W = Weight of dry matter

D = Sediment weight (starch wet) [13].

2.3.12. Compressibility Index, Tap Density, Bulk Density, and Hausner Ratio

Red dragon fruit stem starch was placed in a 100 ml measuring cup and the initial volume (Vo) was recorded and tested for impermeability. Determination of 10, 500, 1250 times was carried out and the compressible volume was obtained [14].

2.3.13. Analysis of Fourier Transform Infrared (FTIR) Spectrum

Red dragon fruit stem starch was weighed as much as 2 mg using an analytical balance, measured the absorption with an FTIR spectrophotometer (Agilent Technologies Cary 630 FT-IR) at a wavelength of 4000 - 370 $cm^{-1}$

2.3.14. Particle Size Distribution

Particle size testing using particle size analyzer (PSA) Laser Scattering Particle Size Analyzer LA-960.

2.3.15. Microbial Contamination Test

The media used for Total Plate Count (ALT) testing is Plate Count Agar (PCA) while the Yeast Mold Number (AKK) is Potato Dextrose Agar (PDA) [14].

## 3. RESULTS AND DISCUSSION

The characterization test of red dragon fruit stem starch *(Hylocereus polyrhizus)* which includes organoleptic test, yield test, moisture content test, amylum qualitative test, flow rate, angle of repose, pH test, water content, ash content, solubility, swelling power, compressibility index, Hausner index, tap density, bulk density, true density, microscopic test, starch content analysis, microbial contamination test, FTIR, SEM test, and particle size analyzer (PSA) test. The results of the evaluation of red dragon fruit stem starch characteristics can be seen in Table 1.

**Table 1**. Characterization Test Results of Red Dragon Fruit Stem Starch

| No | Evaluation | | Results | | | literature data *Corn Strach* |
|---|---|---|---|---|---|---|
| | | | **1 h** | **2 h** | **3 h** | [14][15] |
| 1 | Organoleptics | Shape | Powder | Powder | Powder | Powder |
| | | Color | Broken- White | Broken- White | Broken- White | White |
| | | Odor | Odorless | Odorless | Odorless | Odorless |
| | | Flavor | Flavorless | Flavorless | Flavorless | Flavorless |
| 2 | Yield (%) | | 1.43 ± 0.03 | 1.39 ± 0.02 | 1.34 ± 0.02 | - |

continued table 1…

| | | | | | |
|---|---|---|---|---|---|
| 3 | Moisture content (%) | 2.40 ± 0.23 | 3.00 ± 0.25 | 3.48 ± 0.25 | 10 - 15 |
| 4 | Identification | Dark blue | Dark blue | Dark blue | Dark blue |
| 5 | Flow rate (g/second) | 1.05 ± 0.11 | 0.80 ± 0.05 | 0.59 ± 0.03 | 7.99 |
| 6 | Angle of repose (°) | 25.90 ± 1.11 | 29.89 ± 1.69 | 32.67 ± 1.25 | 25 - 30 |
| 7 | pH | 6.13 ± 0.10 | 5.67 ± 0.20 | 5.40 ± 0.05 | 4.0 – 7.0 |
| 8 | Water content (%) | 8.44 ± 0.96 | 10.94 ± 0.88 | 12.82 ± 0.73 | - |
| 9 | Ash content (%) | 0.40 ± 0.04 | 0.53 ± 0.08 | 0.52 ± 0.06 | 0.20 – 0.38 |
| 10 | Swelling power (%) | 30.72±10.90 | 58.22 ± 15.04 | 84.01 ± 8.75 | - |
| 11 | Solubility (%) | 5.37 ± 2.66 | 12.44 ± 4.93 | 18.48 ± 8.75 | 6 - 8 |
| 12 | Compressibility index (%) | 27.5 ± 5.75 | 30.5 ± 4.51 | 36.25 ± 3.30 | 24 - 30 |
| 13 | Hausner ratio | 1.39 ± 0.12 | 1.43 ± 0.06 | 1.58 ± 0.08 | - |
| 14 | Tap density (g/mL) | 0.50 ± 0.03 | 0.53 ± 0.03 | 0.61 ± 0.03 | 0.64 – 0.83 |
| 15 | Bluk denity (g/mL) | 0.36 ± 0.01 | 0.37 ± 0.01 | 0.39 ± 0.01 | 0.47 – 0.59 |
| 16 | True density (g/mL) | 2.38 ± 0.22 | 2.83 ± 0.16 | 3.05 ± 0.25 | 1.478 |
| 17 | Particle size (μm) | 216 | 235 | 229 | 2 - 32 |
| 18 | Starch content (%) | 58.66 ± 3.6 | 58.89 ± 4.97 | 56.32 ± 5.51 | - |

Description: average result of testing 4 replicates along with ±SD

Organoleptical tests of red dragon fruit stem starch with soaking times of 1 h, 2 h, and 3 h showed almost the same results is powder, broken-white, tasteless and odorless, but the 3 h soaking showed slightly whiter starch results. The results of the yield test obtained by red dragon fruit stem starch the longer the soaking time the resulting yield will be more. The difference in the yield of red dragon fruit stem starch is due to the material that is too long soaked, the water content in the red dragon fruit stem and other components contained in the red dragon fruit stem will dissolve in the soaking water. The yield test results can be seen in Table 1.

The results of the moisture content obtained from red dragon fruit stem starch with a soaking time of 3 h are greater than the red dragon fruit stem starch with a soaking time of 1 h and 2 h, this is because the longer the soaking time, the higher the absorbed water content will be. In the results of testing the flow rate that can be seen in Table 1 shows that the longer the immersion of the flow rate produced will be smaller, while in testing the angle of respose, the longer the immersion time will be the greater the angle of repose produced. Stationary angle testing is related to water content, the lower the water content in the sample, the less water content in the sample so that the flow properties are faster, where the faster flow rate indicates that the starch flows freely so that a small stationary angle is formed.

Red dragon fruit stem starch with a soaking time of 3 h is lower and tends to be more acidic than the pH of starch with soaking times of 1 and 2 h. The longer the soaking time and the higher the concentration of sodium bisulfite used causes the pH of the starch produced to be more acidic, because in water sodium bisulfite will break down into sulfuric acid ($H_2SO_3$) which can reduce pH.

In testing the water content, it was concluded that the longer the soaking time, the higher the water content. The difference in the amount of water content of red dragon fruit stem starch is due to the length of soaking so that the absorption of water by the tissue increases. The results of the ash

content test can be seen in Table 1, indicating that the length of soaking time of red dragon fruit stem starch has no effect on the ash content value.

From the test results of solubility and expandability, it was found that red dragon fruit stem starch with a soaking time of 3 h had the highest solubility and expandability values compared to red dragon fruit stem starch with a soaking time of 1 h and 2 h. Solubility is related to expandability, if the higher the expandability of a starch, the solubility of the starch will increase. The higher the expandability value, the more water is absorbed. The results of the expandability and solubility tests can be seen in Table 1.

Microscopic examination using a binocular microscope connected to an optilab application with a microscope magnification of 400x and 1000x showed that dragon fruit stem starch was round and flat, hilus and lamella were not clearly visible. It can be seen that red dragon fruit stem starch with a soaking time of 1 h is not much different from red dragon fruit stem starch with a soaking time of 2 h and 3 h. Microscopic test of red dragon fruit stem starch can be seen in Figure 1. Microscopic test results of red dragon fruit stem starch samples have round and flat particle shapes, hilus and lamella are not clearly visible. Scanning Electrone Microscopy (SEM) test results of red dragon fruit stem starch can be seen in Figure 2.

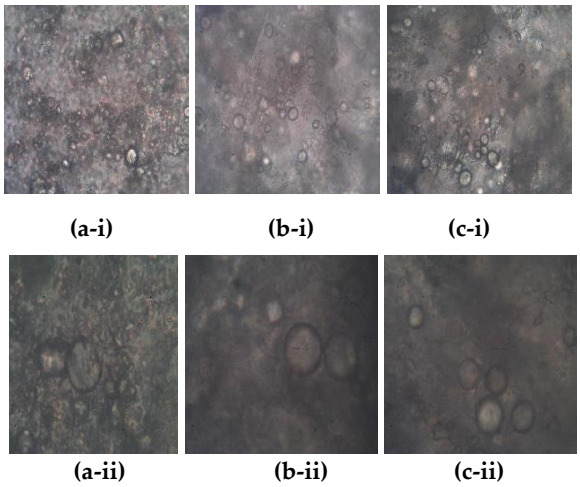



**Figure 1.** Microscopic of Red Dragon Fruit Stem Starch (a) Soaking Time 1 h, (b) Soaking Time 2 h, (c) Soaking Time 3 h with (i) 400x and (ii) 1000x Magnification

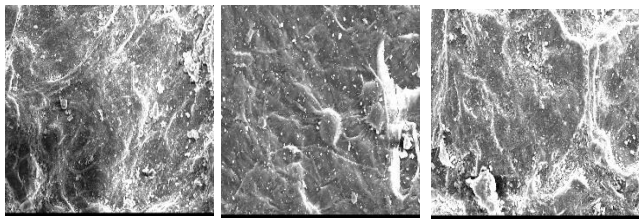



**Figure 2.** Scanning Electrone Microscopy (SEM) of Red Dragon Fruit Stem Starch (a) Soaking Time 1 h, (b) Soaking Time 2 h, (c) Soaking Time 3 h with 1000x Magnification

SEM test was conducted to determine the size of the red dragon fruit stem starch that has been made. Scanning Electrone Microscopy (SEM) test results can be seen that red dragon fruit stem starch with 1000x magnification shows the surface of the sample is not smooth and uneven and has an average particle size of 10 μm. Red dragon fruit stem starch still meets the general microgranule size requirements of 1-1000 μm [16].

The results obtained from the analysis of starch content can be seen in Table 1, the highest starch content is in red dragon fruit stem starch with a soaking time of 2 h. The differences found in the three samples can be influenced by the level of purity during the process, because the more mixtures

such as fiber, sand or impurities that participate in starch, it can affect the resulting starch content. The results of FTIR absorption of red dragon fruit stem starch can be seen in Figure 3.

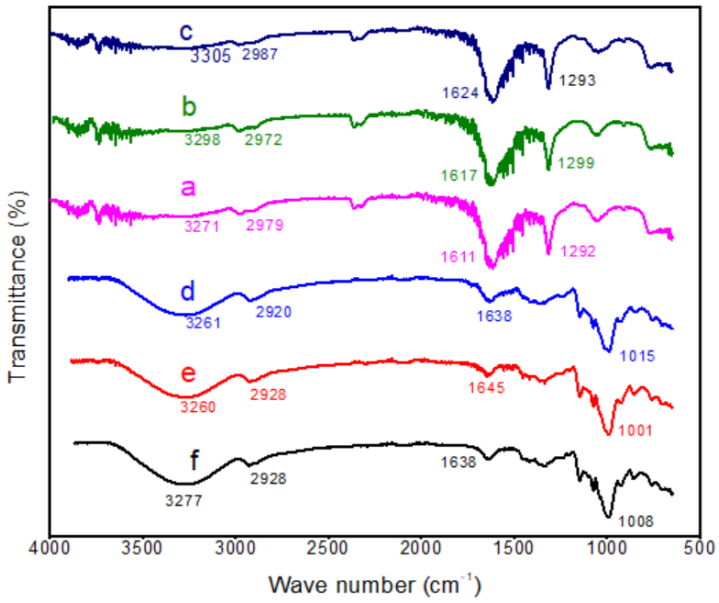
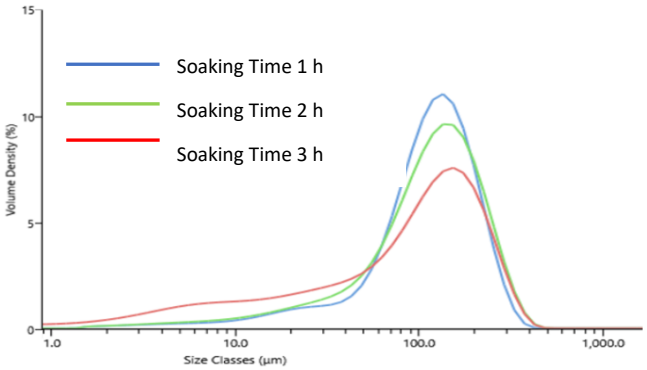



**Figure 3.** FTIR images of Red Dragon Fruit Stem Starch (a) Soaking Time 1 h, (b) Soaking Time 2 h, (c) Soaking Time 3 h, (d) Potato Strach, (e) Corn Starch and (f) Cassava Starch

Red dragon fruit stem starch with soaking time of 1 h 3271 $cm^{-1}$, 2 h 3298 $cm^{-1}$, and 3 h 3305 $cm^{-1}$ showed the peak intensity of hydroxyl group (-OH) strain. The absorption bands 2979 $cm^{-1}$, 2972 $cm^{-1}$, and 2987 $cm^{-1}$ show the absorption of (-CH3) aliphatic strain. The 1611 $cm^{-1}$, 1617 $cm^{-1}$ and 1624 $cm^{-1}$ absorption bands showed the presence of (-C=O). Carbonyl groups (C=O) are formed due to the presence of alcohol groups in starch that undergo oxidation. The 1292 $cm^{-1}$, 1299 $cm^{-1}$ and 1293 $cm^{-1}$ absorption bands show (-C-O) absorption, so it can be concluded that red dragon fruit stem starch contains starch functional groups.

The average results of compressibility index and Hausner ratio on red dragon fruit stem starch with 1 h soaking time are lower, when compared to 2 h and 3 h soaking time. A lower compressibility index or lower hausner ratio indicates better flow properties than higher ones. The percent compressibility result is influenced by particle size and its distribution [17].




| Soaking Time (h) | Dx (10) (µm) | Dx (50) (µm) | Dx (90) (µm) |
|---|---|---|---|
| 1 | 38.5 | 118 | 216 |
| 2 | 31.7 | 120 | 235 |
| 3 | 7.04 | 97.6 | 229 |
| Mean | 25.7 | 112 | 226 |
| SD | 16.5 | 12.2 | 9.90 |
| RSD (%) | 64.3 | 11.0 | 4.37 |

**Figure 4.** Particle Size Distribution Test Results

Particle size distribution testing using PSA (Particle Size Analyzer) Malven® Mastersize 3000 (Malvern Instruments, UK) the average results of particle size distribution testing can be seen in Figure 4. The higher the number of Dx used, the greater the distribution of samples in the test. Based on the results obtained, it is concluded that the particle size distribution of red dragon fruit stems

does not meet the requirements, because it does not enter the range of 2 - 32 μm [15] and the length of dragon fruit soaking time has no effect on particle size distribution.

ALT and AKK microbial contamination testing can be seen in Table 2. it can be seen that the colony results obtained are still within the range of predetermined limits, so that red dragon fruit stem starch is still safe to use as an additive to solid pharmaceutical preparation.

**Table 2**. Total ALT and AKK Contamination of Red Dragon Fruit Stem Starch

| Red Dragon Fruit Stem Starch Samples with Variation of Soaking Time Number (CFU/mL) | Total (CFU/mL) | | |
|---|---|---|---|
| | ALT | AKK Day-5 | AKK Day-7 |
| 1 h | $6.1 \times 10^2$ | $1.7 \times 10^2$ | $2.3 \times 10^2$ |
| 2 h | $5.2 \times 10^2$ | $5 \times 10^1$ | $4.8 \times 10^2$ |
| 3 h | $6.7 \times 10^2$ | $2.3 \times 10^2$ | $5.2 \times 10^2$ |

## 4. CONCLUSION

Based on the characteristic tests carried out, it can be concluded that differences in soaking time can affect the characterization of each sample of red dragon fruit stem starch, There are significant differences (p-value<0,05) in the test results of yield, moisture content, flow rate, angle of repose, pH, solubility, swelling power, compressibility index, density and hausner index.

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*Original Article*

# The Antibacterial Activity of Bajakah Tampala Extracts (*Spatholobus littoralis Hassk.*) Mouthwash Formulation Inhibited Dental Plaque against *Streptococcus mutans*

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**Abstract:** Dental plaque is the main cause of dental caries caused by *Streptococcus mutans*, with a high prevalence in Indonesia. Currently, the mouthwash market contains high levels of alcohol, which can cause long-term side effects. Tampala bajakah root (*Spatholobus littoralis*) is used in traditional medicine for the Dayak community in Central Kalimantan. Bajakah Tampala root has antibacterial activity produced by flavonoids and phenolic compounds. The development of herbal cosmetics can be achieved by Bajakah Tampala mouthwash formulations to prevent dental plaque caused by *Streptococcus mutans* infection. In this study, ultrasound-assisted extraction (UBT) and infusion (IBT) derived the active compounds of Bajakah Tampala root extract. The various concentrations of UBT (20–80%) and IBT (10%) were evaluated for antibacterial activity using the disk diffusion method. The results showed that positive control and 80% UBT have antibacterial activity higher than other extracts, with an inhibition zone of 14,01±2,70 mm. Based on these results, an effective mouthwash dosage formulation can be developed at 80% UBT. The formulation evaluation of mouthwash assessed viscosity, homogeneity, pH, and organoleptic test. The UBT mouthwash product has qualified formulation evaluation parameters. This research contributed to the innovation of herbal cosmetics by developing the potential of Indonesian medicinal plants.

Keywords: Bajakah Tampala, Antibacterial activity, Mouthwash, Herbal cosmetic, *Streptococcus mutans*.

## 1. INTRODUCTION

Dental plaque is the main cause of dental caries caused by *Streptococcus mutant* bacteria. The average prevalence of dental caries in Indonesian people is 88.8% of the total population [1].The high prevalence rate is due to a lack of knowledge about dental and oral hygiene. Dental caries can be inhibited and treated with mouthwash formulations that are used for an antiseptic effect, control plaque, and have temporary antibacterial activity [2]. Mouthwash formula is a cosmetic product that performs effectively at cleansing areas of the mouth and teeth that are challenging to reach. Mouthwash formulations widely sold on the market contain high levels of alcohol, so they can cause uncomfortable sensations and have long-term effects such as cancer of the pharynx, mouth, and throat [3].

Natural ingredients are explored for Indonesia's biodiversity. One of the natural Indonesian products is the Bajakah Tampala root (*Spatholobus littoralis*). The root of Bajakah Tampala is a typical plant of Central Kalimantan that is commonly used by the Dayak community as traditional medicine. Bajakah root, or *Spatholobus littoralis* Hassk, is a typical herbal plant that grows in Kalimantan [4]. Bajakah root extract contains flavonoid, phenolic, and saponin components that have antioxidant, antiviral, antibacterial, anti-inflammatory, and anti-allergic activities [5]. Previous research reported that Bajakah Tampala extract has antibacterial activity against *S. aureus* and *E. coli* [6], [7]. Hamzah et al. (2024) reported also that Bajakah Tampala extract has antifungal activity against *Candida albicans*. The antibacterial activity of Bajakah Tampala root identified against *Streptococcus mutants* was rarely carried out, so this study is interested in conducting research using these bacteria.

This research could contribute to the development of pharmaceutical products through various extraction methods. The ultrasound assisted extraction method (UAE) is one of the modern extraction methods for extracting active compounds using high-intensity sound waves [9]. The UAE method offers to enhance the mass transport of bioactive compounds, reduce times, and have a low temperature [10]. Bajakah tampala root extract was proven to have antibacterial activity using infusion and maceration method [11], [12], but no studies have been explained on the UAE method. The goals of research to develop a natural mouthwash formula from the Bajakah Tampala root extract. In addition, the potential of the mouthwash formulation of Bajakah Tampala root extract was accepted more easily by the community.

## 2. MATERIALS AND METHODS

### *2.1. Place and time of research*

This research was conducted at the Biology Pharmacy Laboratory of Universitas Jenderal Ahmad Yani Yogyakarta. The research was conducted from May to July 2024.

### *2.2. Tools and materials*

The materials used include the Bajakah Tampala roots, water for injection (WFI), glacial acetic acid p.a, formic acid p.a, *Streptococcus mutant*, ethanol p.a., ethyl acetate p.a, glycerin, chloroform p.a, quercetin p.a, methanol p.a, Nutrient Agar (NA) media, n-butanol p.a, n-hexane p.a, sodium benzoate p.a, paper disc blank, amoxicillin paper disc, chloramphenicol paper disc, sorbitol, peppermint oil, and Total Care® Mouthwash. The tools used are Class II BSC, autoclaves, petri dishes, CAMAG® chambers, UV detectors, micropipette (Eppendorf), infuse pan, ultrasonic bath (Cole-Parmer), analytical balance (Ohaus), rotary evaporator, Brookfield viscometer, and other glasses.

### *2.3. Sampling and Plant Determination*

The Bajakah tampala roots (*Spatholobus littoralis*) were obtained from Central Kalimantan, Indonesia. The determination of samples was carried out at the Biology Learning Laboratory, Ahmad Dahlan University, with identification number 230/Lab.Bio/B/V/2024.

### *2.4. Preparation and sample extraction of Bajakah Tampala roots*

The Bajakah tampala roots were washed and dried for two days in sunlight, then reduced in particle size using a grinder and 40-mesh sieves.

2.3.1. Infusion Method

10 g of root powder were added to 100 ml of aquadest in an Erlenmeyer. Samples were heated at 90 °C for 15 minutes. The infusion of Bajakah tampala (IBT) extract was filtered with filter paper [12].

2.3.2. Ultrasound-Assisted Extraction (UAE)

100 g of root powder were extracted into 1000 mL of ethanol p.a., then sonicated with an extraction time of 60 minutes at the optimal temperature of 45 °C. The extract was evaporated with a rotary evaporator at a temperature of 40 °C until UAE of Bajakah tampala (UBT) extract was obtained [13].

*2.5. Qualitative Analysis of Flavonoid Content*

The extracts were qualitatively tested using $AlCl_3$ and $FeCl_3$ reagents to determine the flavonoid and phenolic content. A few drops of 5% FeCl3 were added, and then the colour changed to brown precipitation, as well as $AlCl_3$. The saponin test was carried out with an extract dissolved in 10 mL of hot water and shaken for 30 seconds [14].

Thin-layer chromatography (TLC) involved a stationary phase on a 60 F254 silica gel plate and two different mobile phases with n-hexane: ethyl acetate (3:7) [7] and n-butanol: ethyl acetate: water (7:4:2), modified by [15]. 50 mg of root extract and 5 mg of standard quercetin were added to 5 mL of methanol p.a. The F254 silica gel plate was activated in an oven at a temperature of 100 °C for 30 minutes and was saturated with the mobile phase. The spots were observed under visible light, UV 254 nm, and UV 366 nm. A yellow spot indicated the presence of flavonoids in the samples under UV 366 nm light [16].

*2.6. Sterilization*

The tools were sterilized in an oven at a temperature of 171 °C for 1 hour. The materials used were sterilized by autoclave at a temperature of 121 °C for 15 minutes. Nutrient agar (NA) media was dissolved in an aqueous solution and homogenized with a stirrer at a temperature of 100 °C until boiling. The NA was sterilized by an autoclave at 121 °C for 15 minutes. Continuously, the NA was cultured with *Streptococcus mutants*, modified by[17].

*2.7. Inoculation of Streptococcus mutant bacteria*

In the Bio Safety Cabinet (BSC), *Streptococcus mutants* were inoculated by the zig-zag method on NA media and then incubated for 48 hours at 37 °C. *Streptococcus mutants* were suspended in a tube containing 5 mL of a 0.9% NaCl solution. Then McFarland standard 0.5 measured turbidity with a turbidimeter, modified by[17].

*2.8. Antibacterial Activity Test by Disc Diffusion Method*

The serial concentrations of 10% of IBT and 20%, 40%, 60%, and 80% of UBT were dissolved in WFI. The blank paper disc was soaked in the UBT and IBT samples for 5 minutes. 100 µL of bacterial suspension was inserted into a petri dish containing NA media. The petri dish media was divided into four parts, such as control and samples. As a positive control, paper discs containing amoxicillin and chloramphenicol. Blank paper discs contained WFI as negative controls. After that, the petri dish was incubated at 37 °C for 24 hours, and the clear zone formed was measured by their zone inhibition diameter. This test was repeated three times modified by [17].

*2.9. Mouthwash Formulation of Bajakah Tampala Root Extract*

The formulation of mouthwash was selected based on the optimal concentration of extracts in Table 1. Ingredients of glycerol, sorbitol, and sodium benzoate were added to the homogeneous. Peppermint oil and extract samples were mixed and milled back until homogeneous. This mixture was then supplemented with water until it reached a total volume of 100 mL, modified by [17].

**Table 1.** Formula Mouthwash

| Ingredients | Units | Quantity |
|---|---|---|
| 80% UBT Extract | mL | 10 |
| Peppermint oil | mL | 0.1 |
| Sorbitol | mL | 0.25 |
| Sodium Benzoate | g | 0.1 |
| Glycerol | mL | 1.0 |
| Water | mL | ad 100 |

*2.10. Physical Evaluation of Bajakah Tampala Root Extract Mouthwash Formulation*

2.10.1. Organoleptic Test

Observations were made with the five observations directly, including aroma, colour, and taste [17].

2.10.2. pH Test

pH paper was dipped in mouthwash for a few minutes and then matched with the colour of the indicator. Measurements are taken at room temperature. The pH of mouthwash is good at pH 5-7 [17]

2.10.3. Homogeneity Test

It is done by observing the formulation that has been put into a clear bottle and given a white background. Good mouthwash formulations are homogeneous, not cloudy, and free from contamination and microbial growth [17].

2.10.4. Viscosity Test

The viscosity of this formulation was measured using a Brookfield viscometer with the spindle rotation speed set at 100 rpm and using spindle no. 2. A total of 100 mL of samples were put into beaker glass (Sulistiyono et al., 2022). The standard viscosity of mouthwash on the market is ± 7.25 cP [18].

*2.11. Data Analysis Methods*

The inhibition zone data at each concentration of extract samples can be analysed using the normality test, homogeneity test, and One-Way ANOVA method using SPSS software v.25 with a significant level of 95%. The normality test is carried out by the Shapiro-Wilk test if the number of samples is less than 50. The homogeneity test was used to determine whether the data obtained using Levene's test. The data is distributed normally and homogeneously using One-Way ANOVA with a significant (p) > 0.05 [13].

## 3. RESULTS AND DISCUSSION

### *3.1. Extraction of Bajakah Tampala roots*

Bajakah tampala root powder is extracted using two extraction methods, namely UAE (UBT) and infusion (IBT). UBT extract evaluated % yield value was 15%, but IBT didn't give % yield value. A good yield value is not less than 7.2%. It can be concluded that the research yield is eligible [13].

### *3.2. Qualitative analysis*

#### 3.2.1. Phytochemical screening

The results of phytochemical screening showed that flavonoids and phenolics were present in UBT and IBT extracts. UBT and IBT were given $AlCl_3$ to identify the content of flavonoids with their colour such as dark yellow. If contains saponins, foam 1–10 cm high is formed for no less than 10 minutes, and at the addition of 1 drop of HCl 2 N, the foam does not disappear. Meanwhile, saponins were not detected in the UBT and IBT extract [14].

**Table 2.** Phytochemical Screening Results

| No. | Active Compounds | Screening Result |
|---|---|---|
| 1 | Flavonoid | + |
| 2 | Phenolic | + |
| 3 | Saponin | - |

Notes: (+) Positive; (-) Negative

#### 3.2.2. Thin-Layer Chromatography (TLC)

The results of the TLC test were used using a mobile phase of n-hexane: ethyl acetate with a ratio of 3:7 v/v and the stationary phase of the $F_{254}$ silica plate with quercetin standard and UBT extract. The Rf (retention factor) value was calculated to be used as evidence in the identification of compounds, namely flavonoids. If the Rf value is the same, the compound has similar characteristics to the comparator (quercetin). The standard Rf result was 0.6, and the sample Rf was 0.7.

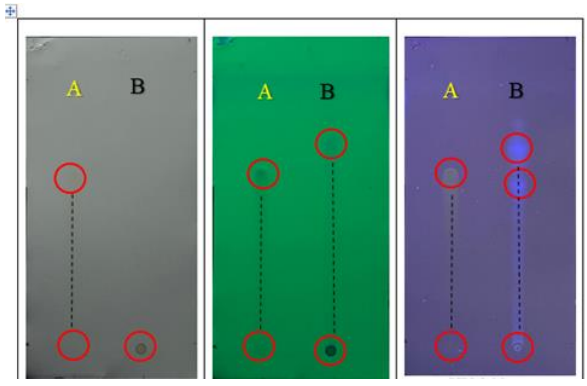
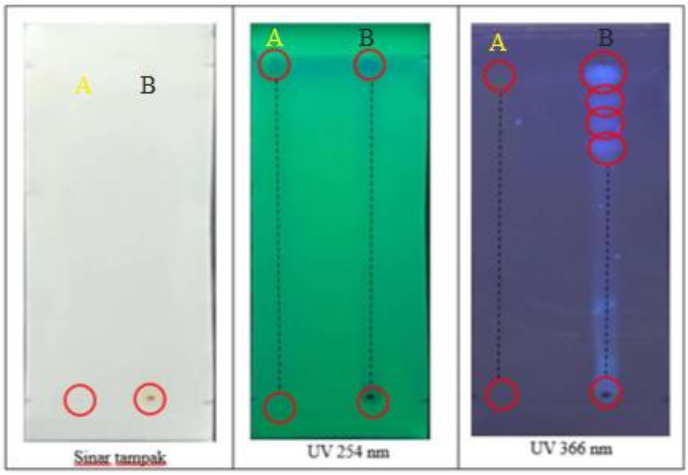



**Figure 1.** TLC results of UBT extract with n-hexane: ethyl acetate (3:7 v/v); A: Quercetin standard; B: UBT extract.

The results of the TLC test used a mobile phase of n-butanol: ethyl acetate: water with a ratio of 7:4:2 v/v/v and a stationary phase of silica plate F254 with quercetin standards and UBT extract.

The results of the UBT extract showed several fluoresced points that were visualized in UV 366 nm. The standard Rf result was 1, and the sample Rf was 0,7-1.




. **Figure 2.** TLC results of UBT extract with n-butanol:ethyl acetate:water (7:4:2 v/v/v); A: Quercetin standard; B: UBT extract

*3.3. Antibacterial activity using disk diffusion method*

Antibacterial activity was evaluated the inhibition zone in various concentration of Bajakah Tampala with UAE (UBT) and infusion (IBT). According to the analysis of the inhibition zone, the concentration of 20% UBT and IBT extract could inhibit the growth of *Streptococcus mutans* bacteria in Figures 3 and 4. The inhibition zone wasn't formed optimally clear with an average diameter of 9.84±1.17 mm and 7.51±0.13 mm by 20% UBT and IBT extracts. The 40%, 60%, and 80% UBT-formed inhibition zones formed optimally clear with an average diameter of 10.78±1.17; 13.56±1.54; and 14.01±2.70 mm.

**Table 3.** Inhibition Zone Diameter (mm) Evaluated Antibacterial Activity

| Treatments | Concentration | Replication | | | Mean ±SD | Interpretation |
|---|---|---|---|---|---|---|
| | | I | II | III | | |
| UBT | 20% | 10.33 | 10.70 | 8.50 | 9.84 ±1.17 | Medium |
| UBT | 40% | 11.53 | 11.40 | 9.43 | 10.78±1.17 | Strong |
| UBT | 60% | 14.63 | 14.27 | 11.80 | 13.56 ±1.54 | Strong |
| UBT | 80% | 15.30 | 15.83 | 10.90 | 14.01±2.70 | Strong |
| IBT | 10% | 7.66 | 7.43 | 7.43 | 7.51 ±0.13 | Medium |
| Chloramphenicol | (+) | 30.88 | 30.65 | 0 | 20.51±17.76 | Powerful |
| Amoxicillin | (+) | 30.40 | 26.53 | 24.00 | 26.97 ±3.22 | Powerful |
| Negative Control | (-) | 0 | 0 | 0 | 0.00 ±0.00 | None |

Based on Table 3, the greater the concentration of the UBT root extract, the larger the clear zone formed in the petri dish. The selection of UBT concentrations was based on the strength of the diameter of the inhibition power at concentrations of 20% and 40%, which were included in the moderate category (5–10 mm), while concentrations of 60% and 80% were included in the strong

category [19]. The higher the concentration of the Bajakah tampala root extract, the stronger the inhibitory power against *Streptococcus mutans*. Bajakah tampala extract has flavonoids that play an important role in inhibitory reactions to bacterial growth, especially *Streptococcus mutans*. Previous research conducted by [20] reported that papaya leaf and green tea infusion extracts have antibacterial activity against *Streptococcus mutans* bacteria due to their flavonoid content.

**Table 4.** One-Way ANOVA Test Results

| No | Category | P (0,05) |
|---|---|---|
| 1 | Shapiro-Wilk | 0.061 (>0.05) |
| 2 | Levene test | 0.015 (<0.05) |
| 3 | One-Way ANOVA | 0.000 (<0.05) |

The inhibition zone bacterial of Bajakah tampala root extract was evaluated normality test using the Shapiro-Wilk method that the value of significance of 0.061, shown in Table 4. The value obtained is greater than p > 0.05. These indicates that the data on the diameter of the inhibition zone of Bajakah tampala root extract against *Streptococcus mutans* was normally distributed. Furthermore, the results of the homogeneity test determined using Levene statistical test**.** The homogeneity test showed P<0.05, which means that data was not homogeneous. A One-Way ANOVA result could be performed that the data was normally distributed. The One-way ANOVA test obtained a result of 0.000, so the P value obtained is smaller than 0.05. The inhibition zone of Bajakah tampala root extract against *Streptococcus mutans* has a significant difference based on One-way ANOVA test. There is a difference in the diameter of the inhibition zone at each concentration of UBT, IBT, negative control and positive control (chloramphenicol and amoxicillin).

**Figure 3.** Inhibition zones formed on various concentrations of IBT, positive control and negative control against *S. mutans*

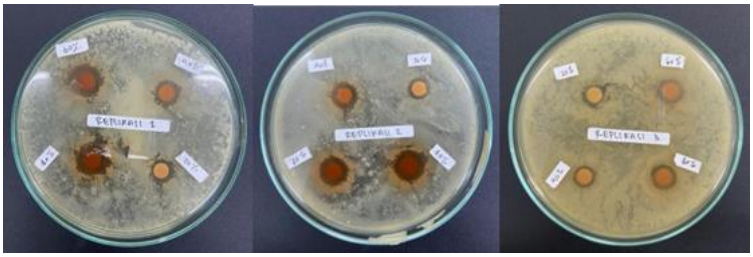


**Figure 4.** Inhibition zones formed on UBT, positive control and negative control against *S. mutans*

*3.4. Mouthwash Formulations*

In this study, one formula was made with the strongest concentration of extract for bacterial inhibition, which is at a concentration of 80%. The mouthwash made is composed of 80% UBT

extract (as an active substance), glycerol (as a humectant), sorbitol (a sweetener), sodium benzoate (as a preservative), peppermint oil (as a flavor enhancer or freshner), and water (as a solvent). The mouthwash that has been made must have the effectiveness of the active ingredient content, comfort when using, and viscosity of the solution. In addition, mouthwash must have a fresh taste when used for gargling.

*3.5. Mouthwash Evaluation*

3.5.1. Organoleptic and Homogeneity test

The organoleptic extract formulation has the appearance of a clear chocolate and peppermint fragrance with a mint taste. The homogeneity test on the mouthwash formula showed that there were no particles, it was not cloudy, and it was clear. The homogeneity test of the mouthwash formula obtained was acceptable.

**Table 5.** Organoleptic Results of Mouthwash

| | **80% UBT** | **Total Care®** |
|---|---|---|
| **Fragrance** | Peppermint fragrance | Mint |
| **Taste** | Mint | Mint |
| **Color** | Clear chocolate | Clear yellow |
| **Texture** | Liquid | Liquid |
| **Appearance** |  |  |

3.5.2. pH Test

The pH value of mouthwash needs to be known so that it doesn't irritate the mouth or taste sour when used for gargling. The pH of mouthwash is neutral at pH 5-7. A mouthwash whose pH is acidic will cause irritation in the mouth, while a pH that is alkaline will trigger the growth of fungus that causes canker sores or aphthous stomatitis. From Table 6, the pH results of the mouthwash formula were similar to those of conventional mouthwash (Total Care).

**Table 6.** Mouthwash Evaluation

| **Formula** | **pH** | **Homogeneity** | **Viscosity** |
|---|---|---|---|
| Mouthwash (80% UBT) | 5 | Clear | 7.20 cP |
| Total Care ® | 5 | Clear | 7.25 cP |

3.5.3. Viscosity test

Viscosity describes the amount of resistance that a liquid has to flow. The greater the viscosity value, the more difficult it is for a liquid to flow, and vice versa. Viscosity measurement is important to do because it can affect how easily the formulation flows out of the container so that it is easy to apply. The viscosity of mouthwash formulations is most important because it can affect

the comfort of use. A good viscosity of Bajakah tampala mouthwash evaluated not too thick. The closer the viscosity of the mouthwash to the viscosity of the water, the better and more comfortable it is when used. The standard viscosity of mouthwash on the market is ± 7.25 cP (Rowe et al. 2009). The result of the viscosity observation in Table 6 showed that Bajakah Tampala mouthwash was similar value with conventional mouthwash (Total Care®) [17].

*3.6. Research limitations*

The limitation of this study was that the raw material of Bajakah Tampala roots (*Spatholobus littoralis*) might not be easily available in several areas. Additionally, there were no saponins in the Bajakah Tampala extract in the results of the phytochemical screening test, which may limit the applicability of the research findings. The potential future of herbal mouthwash formulations is to optimize formulation, combination of other herbal medicines, stability of the product, and preclinic trials compared to other market mouthwashes.

## 4. CONCLUSION

Studies revealed that 80% UBT had antibacterial activity greater than other extracts with an inhibition zone of 14,01±2,70 mm. These findings allowed for the development of an efficient mouthwash dose formulation at 80% UBT. The organoleptic, pH, homogeneity, and viscosity test were evaluated in the mouthwash formulation assessment. UBT mouthwash product qualified evaluation parameters. In conclusion, this study enhanced the potential of Indonesian medicinal herbs, which led to innovation in herbal mouthwash formulation.

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**Conflicts of interest:** The authors declare no conflict of interest.

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Journal of
Food and Pharmaceutical Sciences

*Original Article*

# Aerobe Fermentation Enhanced Antioxidant Activity Index of *Citrus limon* Leaves

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**Abstract:** The chemical composition and antioxidant activity of a natural product are directly correlated with the preparation process which can alter certain enzymatic pathways, leading to the alteration of the production of secondary metabolites affecting its biological activity. This study aims to observe the effects of the pre-extraction process of *Citrus limon* on its TFC, TPC, and antioxidant activity. Samples were prepared by two different pre-extraction techniques (solar drying and aerobe fermentation) and extracted using ultrasound-assisted extraction with ethanol 96%. The TFC and TPC were measured by a colorimetric method using a spectrophotometer UV-Vis. The antioxidant activity was tested on DPPH and calculated as AAI with ascorbic acid as a standard. The result showed that solar dried sample exhibited higher TFC and TPC than the fermented sample with TFC and TPC values of 32.09 ± 0.45 mg QE/g and 335.80 ± 0.80 mg GAE/g extract. In contrast, the antioxidant activity assay revealed that fermented samples provided higher antioxidant activity than solar-dried samples and even better than ascorbic acid with an $IC_{50}$ value of 2.23 ± 0.19 µg/mL. The pre-extraction process significantly influences the TFC, TPC, and antioxidant activity of *Citrus limon*.

**Keywords:** Pre-extraction process; *Citrus limon*; Flavonoid; Phenolic; Antioxidant activity index

## 1. INTRODUCTION

Free radicals are one of the main causes of various degenerative diseases, such as cancer [1], diabetes mellitus [2], and hypertension [3]. The emergence of various degenerative diseases is directly related to oxidative stress due to the presence of the reactive oxygen species (ROS) that oxidizes cells and tissues in the body [4]. The negative impact of ROS can be tackled by donating the electron to the empty orbital of the radicals, resulting in reducing the reactivity of ROS, leading to cell and tissue oxidation can be avoided [5].

Antioxidants are those molecules that can transfer their electron to neutralize the ROS in which various chemical compounds have been synthetically developed [6]. However, the use of synthetic antioxidants faces several adverse events emerging new health problems for the community. Natural products have been known as a source of numerous phytochemicals that are biologically and pharmacologically active, one of which is as antioxidants [7]. *Citrus limon* is one of the terrestrial plants that is known and scientifically proven to possess excellent antioxidant activity [8]. This antioxidant activity was reported in view of the fact that the presence of flavonoids and

phenolic compounds found in several parts of *Citrus limon* provide plentiful hydroxyl groups acting as the source of electrons to be donated to the free radicals [9].

Total flavonoid content (TFC) and total phenolic content (TPC) in a natural product are directly linked to their antioxidant capacity in which the higher the TFC and TPC, the greater the antioxidant activity [10]. However, the TFC and TPC of plants vary depending on numerous factors that can influence their antioxidant capacity. The pre-extraction process is pointed out to be one of the factors affecting the TFC and TPC of plants that has been widely reported in various scientific articles. Dried-powdered plants are reported to have higher TFC and TPC than the fresh samples [11]. In contrast, the fermented samples exhibited better antioxidant activity than dry samples [12]. This justified that the diversity of pre-extraction treatment of plants might give different results. However, until the present time, there have been no reports regarding the best pre-extraction process for Citrus limon to obtain high TFC, TPC, and antioxidant activity. Therefore, this work was undertaken to evaluate the influence of the pre-extraction process of *Citrus limon* leaves on its TFC, TPC, and antioxidant activity.

## 2. MATERIALS AND METHODS

### *2.1. Chemicals and reagents*

All chemicals and reagents used in this work were analytical grade: Ethanol 96% (Bratachem, Indonesia), Ethanol absolute (Merck, Germany), Acetic acid (Merck, Germany), Sodium carbonate (Merck, Germany), $FeCl_3$ (Merck, Germany), $AlCl_3$ (Merck, Germany), Magnesium powder (Bratachem, Indonesia), DPPH (Sigma-Aldrich, Singapore), Quercetin (Sigma-Aldrich, Singapore), Gallic acid (Sigma-Aldrich, Singapore), Ascorbic acid (Merck, Germany).

### *2.2 Sample collection and identification*

Fresh leaves of Citrus limon were collected in Karang Endah, Muara Enim, South Sumatera-Indonesia in April 2023. The samples were then identified by the staff of Dr. Angga Dwihartama, School of Life Sciences and Technology, Bandung Institute of Technology.

### *2.3 Sample preparation and extraction*

Fresh leaves of *Citrus limon* were prepared using two different techniques including solar drying and aerobe fermentation. The leaves were solar-dried using a direct solar drying method for three days. On the other side, some leaves were aerobe fermented in a dark and isolated chamber until wilted. The samples were then grounded, and 100 g of each sample were ultrasound-assisted extracted using 1 L of ethanol 96% for 30 mins at room temperature. The filtration using a Wathman filter paper was applied to separate the liquid extract from the residues. The residues were then re-extracted as much as two times using the same method as mentioned. The liquid extracts were collected and evaporated using a vacuum rotary evaporator (Buchi, Germany) at 70 rpm and 45°C. The extract yield was calculated using the formula described by Setiawansyah et al. (2018) [13]:

$$Yield\ (\%) = \frac{Weigh\ of\ crude\ extract\ (g)}{Weigh\ of\ dry\ powder\ sample\ (g)} x100$$

*2.4 Total falvonoid content analysis*

TFC was measured using a colorimetric method in a spectrophotometer UV-Vis (Shimadzu, Japan) as described by Nurlinda et al. (2021) [14] with minor modification. Approximately 15 mg of *Citrus limon* leaf extracts were dissolved in 15 mL ethanol and transferred 1 mL of extract solution to the test tube, mixing with 1 mL of $AlCl_3$ 5% and 1 mL of 120 mM acetic acid. The mixture was then measured at 370.5 nm after 30 mins incubation. The experiment was carried out in triplicate and TFC was calculated as quercetin equivalent using the following formula:

$$TFC = \frac{c\ x\ V\ x\ f}{m}$$

Where

TFC : Total flavonoid content (mg QE/g)
c : Quercetin equivalence (μg/mL)
V : Total volume of extract (mL)
f : Dilution factor
m : Extract mass (g)

*2.5 Total phenolic content analysis*

TPC determination was carried out using a method described by Sumaiyah et al. (2018) [15] with slight change by dissolving 10 mg of *Citrus limon* leaf extracts in 10 mL of ethanol. A 0.1 mL extract solution was then taken and mixed with 7.9 mL of distilled water and 0.5 mL of $FeCl_3$ 5%, then vortex for 1 min. The mixture was measured in a spectrophotometer UV-Vis (Shimadzu, Japan) at wavelength 745 nm after 30 mins incubation. The experiment was done in triplicate and TPC was calculated as gallic acid equivalent using the equation below:

$$TPC = \frac{c\ x\ V\ x\ f}{m}$$

Where

TPC : Total phenolic content (mg GAE/g)
c : Gallic acid equivalence (μg/mL)
V : Total volume of extract (mL)
f : Dilution factor
m : Extract mass (g)

*2.6 Antioxidant activity assay*

Antioxidant activity of *Citrus limon* leaf extract was determined using DPPH as explained by Setiawansyah et al. (2023) [16] with modifications. A stock solution of 50 μg/mL of DPPH was reacted with six different concentrations of *Citrus limon* leaves extract solution (1:1). The mixture was incubated in a dark room at 27°C for 30 mins and then measured in a spectrophotometer UV-Vis (Shimadzu, Japan) at the wavelength 517 nm. The experiment was run in three independent replications using ascorbic acid as standard. The antioxidant activity was calculated using the following formula:

$$Inhibition\ (\%) = \frac{Abs\ control - Abs\ sample}{Abs\ control} x\ 100$$

The $IC_{50}$ calculation used the equation of linear regression obtained from the inhibition versus concentration and expressed as antioxidant activity index (AAI) using the following equation:

$$AAI = \frac{Final\ concentration\ of\ DPPH}{IC50}$$

*2.7 Data analysis*

The effect of the pre-extraction process of *Citrus limon* leaves extract on its TFC, TPC, and antioxidant activity index was analyzed statistically using One Way ANOVA followed by a Tukey's test in a GraphPad Prism 10 version.

## 3. RESULTS AND DISCUSSION

*3.1. Effect of pre-extraction process on extract yield*

The samples were ultrasound-assisted extracted and provided a significantly different yield of extract. Figure 1 depicts the effect of the pre-extraction process on the extract yield of *Citrus limon* leaf extract. It shows that different sample preparations influenced the extract yield in which the solar-dried *Citrus limon* gives the higher yield (17.3 gram) compared to the aerobe-fermented samples (14.3 gram).

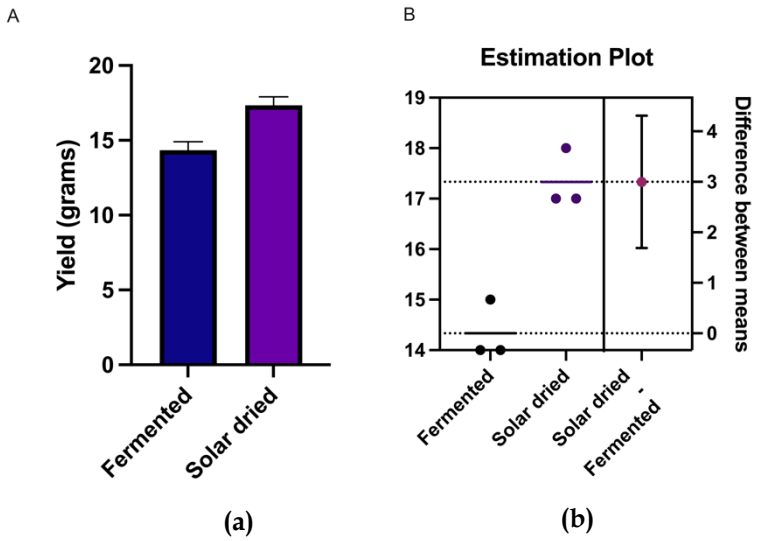


**Figure 1.** Effects of pre-extraction process on extract yield of *Citrus limon*. (**a**) Extract yield and (**b**) Unpaired independent t-Test result

The effect of the pre-extraction treatment was observed on the extract yield showing a significant difference between the fermented and solar-dried samples of *Citrus limon* leaves in which fermentation decreases the extract yield of *Citrus limon* leaves. This is not in line with previous work that reported natural fermentation increases the nutritional content of plant products, leading to an elevation of its extract yield [17]. However, this result is higher than other fermented plants including *A. artilis* (1.62-7.75%) [18]. The yield of the extract is directly correlated with the chemical constituent being extracted. It seems that fermentation alters the production of certain secondary metabolites production of *Citrus limon* leaves by enzymatic reaction being elevated which leads to the decomposition of the products. In contrast, solar drying decreases the water content of *Citrus limon*

leaves which causes degradation of enzymes responsible either for oxidizing or hydrolysis of constituents of *Citrus limon* leaves.

*3.2 Effect of pre-extraction process on TFC and TPC*

The flavonoids and phenolic content of *Citrus limon* were qualitatively analyzed prior to the quantitative determination. Table 1 describes the presence of flavonoids and phenols in *Citrus limon* leaves extract determined by colorimetric reaction using $Mg^{2+}$ for flavonoids and $FeCl_3$ for phenols.

**Table 1.** Qualitative screening of flavonoids and phenols of *Citrus limon*

| Secondary Metabolites | Samples | |
|---|---|---|
| | **Fermented** | **Solar dried** |
| Flavonoids | + | + |
| Phenols | + | + |

The concept of flavonoid identification using magnesium ($Mg^{2+}$) is by harnessing the chemical properties of $Mg^{2+}$ that can change the electron resonance of the gamma-benzopyrone within the C ring of the flavonoid. Changing the electron resonance causes the change of its structure to become a cyanidin structure that provides yellow, orange or reddish color [19] as illustrated in Figure 2b. Not all flavonoids can be identified by this reagent, but only applies to certain flavonoids that have gamma-benzopyrone structure including flavones, flavonols, and isoflavones.

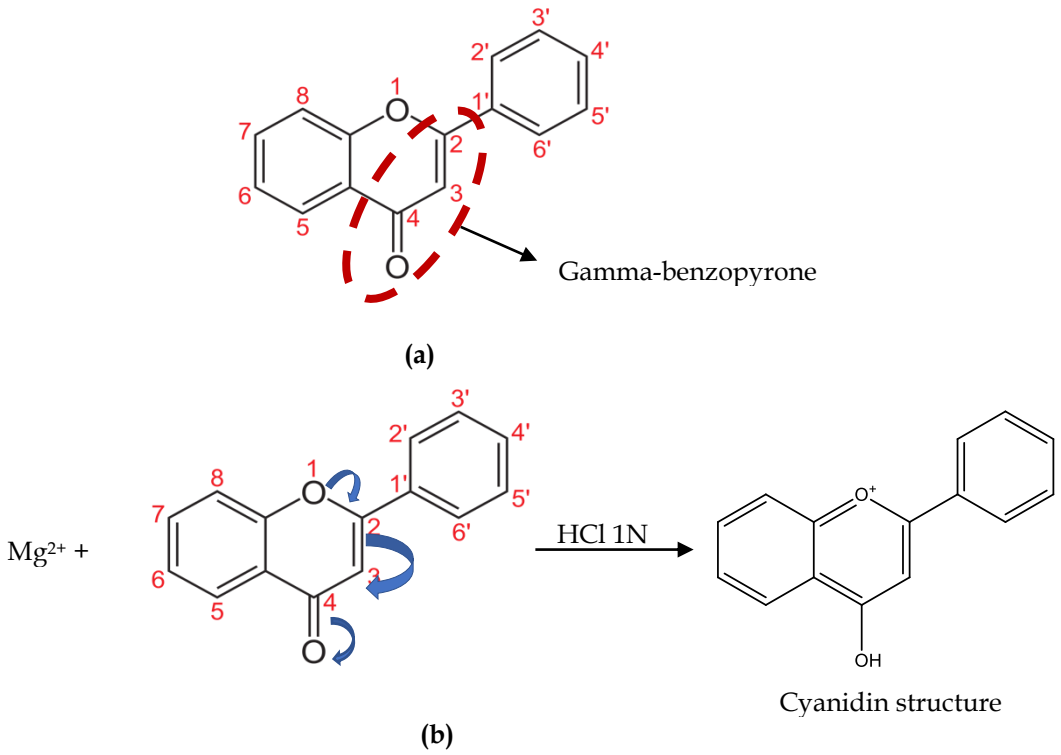


**Figure 2.** Schematic concept of flavonoid identification using $Mg^{2+}$. (A) a gamma-benzopyrone responsible for the reaction, and (B) the mechanism of reaction of a gamma-benzopyrone flavonoid with $Mg^{2+}$

While the concept of phenol identification using $FeCl_3$ is by harnessing the ability of the phenol compounds to form a phenol-iron complex with $Fe^{3+}$ by direct reaction with the hydroxy (OH)

groups within the phenol structure (Figure 3) [19], [20]. This reagent is very non-specific because it can react with all compounds that have OH groups within their structure without any compromises.

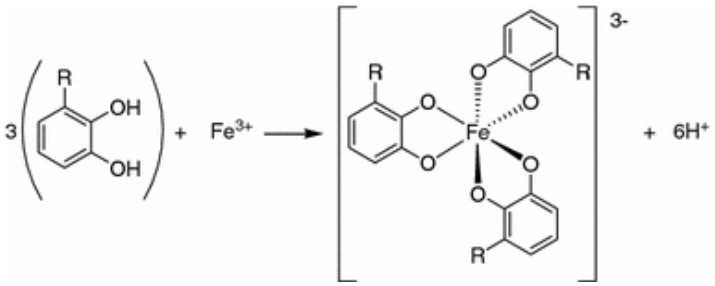


**Figure 3.** Polyphenol-iron complex resulted from reaction of $FeCl_3$ with the OH groups in phenol identification [20].

The existence of flavonoids and phenols in all samples was considered as a basic reference to be quantified in further experiments. The quantitative analysis of TFC and TPC of fermented and solar-dried *Citrus limon* leaves extract showed a diverse result in which solar dried sample provided a higher TPC (335.80 ± 0.80 mg GAE/g extract) (significantly different at $p<0.05$) than that of fermented sample (248.79 ± 0.25 mg GAE/g extract) as illustrated in Figure 4. However, the pre-extraction process did not significantly ($p>0.05$) affect the TFC of *Citrus limon* leaf extract from both aerobe-fermented and solar-dried samples with TFC values of 31.91 ± 0.19 mg QE/g extract and 32.09 ± 0.45 mg QE/g extract, respectively. *Citrus limon* provides higher phenolic compounds than flavonoids as reported by Makni et al. (2018) [21] which stated that *Citrus limon* contains phenolics and flavonoids content ranging from 105 – 204 mg GAE/g and 27 – 56 mg QE/g extract, respectively. However, our work is not in line in which the TFC and TPC were lower than other reports influenced by differences in the ecological environment affecting the production of secondary metabolites. The chemical composition of plants highly correlates with the ecological zone where the plants grow caused by whether the biotic or abiotic-related factors of the environment alters their production [22], [23].

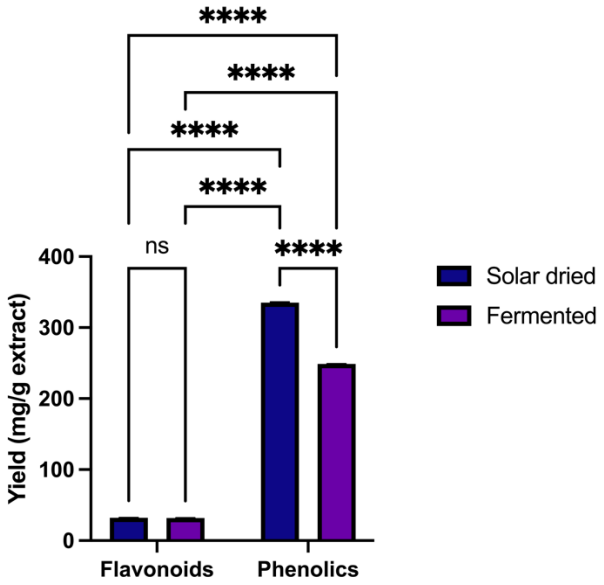


**Figure 4.** TFC and TPC of solar dried and fermented *Citrus limon* leaves extract

As depicted in Figure 4, it seems that fermentation diminishes the production of phenolic compounds in *Citrus limon* leaves. In this work, aerobe fermentation was used which is known to

retain the water content in the samples which causes the enzyme responsible for catalytic phenols to remain active. Additionally, high water content is the major contributor to bacterial growth which alters the production of phenol components. Certain bacterium encoded several genes coding for enzymes like phenyl phosphate synthase and phenyl phospate carboxylase involved in phenol degradation through phosphorylation and carboxylation [24]. The solar drying reduces the water content in the samples inhibit the bacterial growth and inactivates the enzymes, leading to the degradation of phenolic compound being avoided.

*3.3 Effect of pre-extraction process on antioxidant activity index*

The antioxidant activity of *Citrus limon* leaf extract was tested on DPPH using an ascorbic acid as a standard. All samples both fermented and solar-dried showed a significant free radical scavenging activity by reducing the absorbance of the test solution. $IC_{50}$ calculation revealed that fermented *Citrus limon* leaf extract provides stronger antioxidant activity than solar-dried samples, even better than ascorbic acid as summarized in Table 2. In this work, we also utilized the antioxidant activity index (AAI) to observe the capacity of *Citrus limon* to scavenge the radical from DPPH. The AAI was used to avoid different results when tested on diverse DPPH concentrations. Statistical analysis indicated that the pre-extraction treatment significantly influences the antioxidant activity of *Citrus limon* leaf extract.

**Table 2.** Antioxidant activity of *Citrus limon* leaves extracts

| **Samples** | **$IC_{50}$ (µg/mL) ± SD** | **AAI** |
|---|---|---|
| Solar dried | 35.11 ± 1.23 | 1.42 ± 0.05 |
| Fermentation | 2.23 ± 0.19 | 21.91 ± 1.90 |
| Ascorbic acid | 4.13 ± 0.25 | 12.10 ± 0.72 |

Apart from being a quite plentiful source of phenolic compounds, *Citrus limon* leaves are reported to contain ascorbic acid ranging from 317 – 580 mg per 100 grams leaves [25]. Fermented *Citrus limon* leaves provide high antioxidant activity due to the presence of high content of ascorbic acid. Anyiam et al. (2023) [26] reported that fermentation elevates the production of ascorbic acid in plants by approximately 125%. Additionally, the fermentation process results in the production of new bioactive compounds that might increase the antioxidant activity [27].

## 4. CONCLUSION

The pre-extraction process highly influenced the TPC and antioxidant activity of *Citrus limon* leaves but did not affect the TFC. Fermentation significantly decreases the TPC, while increasing the antioxidant activity. This indicates that fermentation can be applied to enhance the biological activity related to the antioxidants of *Citrus limon* leaves. However, further research is needed to prove this hypothesis.

**Conflicts of interest:** The authors declare no conflict of interest.

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