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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 3.75×10^{-6} mg. sec^{-1} .cm^{-2}, and the membrane permeability is 8.80×10^{-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.

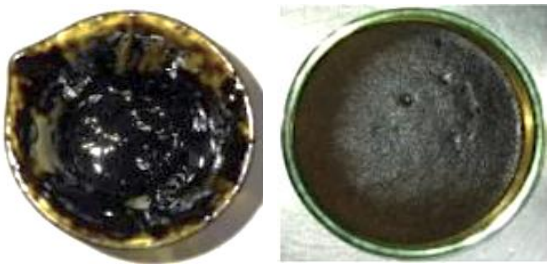

Figure 1. The Moringa leaf ethanolic extract and patch

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.

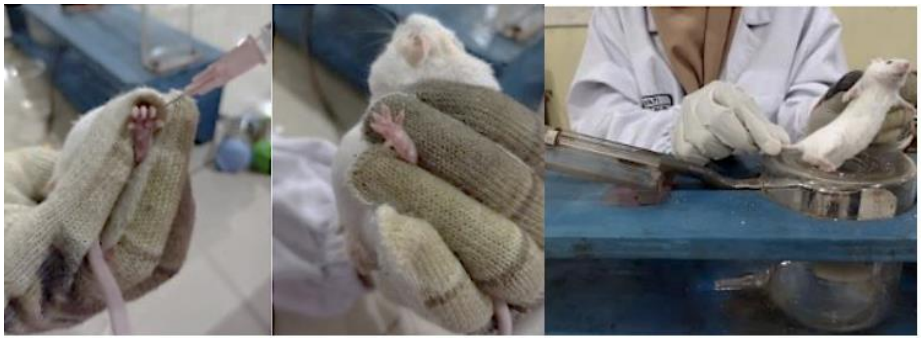

Figure 2. Anti-inflamatory process of Moringa leaf ethanolic extract where the edema induced, edema in mice's feet and measure the edema volume using plestimometer

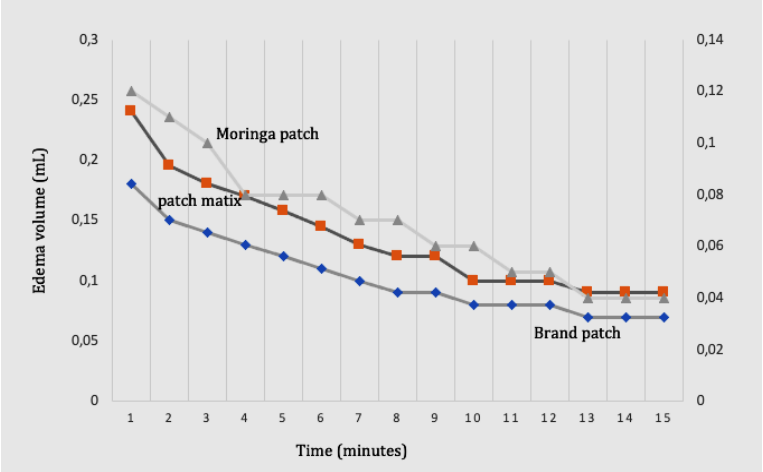

Figure 3. The profile of edema volume of mice's feet during treatment of patch (negative control), brand patch (positive control), and Moringa patch

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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