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Research Article

Comparison of Topical Analgesic Activity of *Mentha piperita* Extract with Ethanol Variations Using Hargreaves Test in Mice

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Pain can sometimes significantly disrupt a person's quality of life. Pharmacological pain management, often using synthetic analgesics, remains the primary option, but long-term use can lead to serious side effects and the risk of dependency. Mint leaves (Mentha piperita) are known to contain active compounds such as alkaloids and flavonoids, which have analgesic effects, making them a potential alternative to topical analgesics. This study aims to compare the topical analgesic activity of mint leaf ethanol extract with variations in ethanol solvent concentration (50%, 70%, and 96%) as an extraction solvent using the Hargreaves test method in white mice (Mus musculus). The laboratory experimental research used a posttest-only control group. Mint leaf extract was formulated in the form of a topical gel, then tested using the Hargreaves test method which measures the latency time of pain response to heat stimulation on the soles of the feet of mice. The results showed that the maximum topical analgesic activity occurred at 150 minutes for all three groups of mint leaf ethanol extract gels. The LSD test results showed that the 96% ethanol extract solvent produced significantly higher topical analgesic activity than the 70% and 50% ethanol extracts (p-value <0.05). The results of this study demonstrated that the 96% ethanol extract of peppermint leaves produced superior analgesic activity compared with the 50% and 70% ethanol extracts.

Pain is an unpleasant sensation that functions as the body's protective mechanism against stimuli that cause tissue damage. Pharmacologically, pain management is commonly achieved through the use of synthetic analgesics, such as NSAIDs (Non-Steroidal Anti-Inflammatory Drugs) and opioids. Although effective, long-term use of synthetic analgesics often leads to adverse effects, including gastrointestinal irritation, nephrotoxicity, hepatotoxicity, and the risk of dependency. This concern has encouraged the development of natural-based analgesic alternatives that are considered relatively safer, one of which is derived from medicinal plants [1]–[3]. Peppermint leaves (*Mentha piperita*) are herbal plants traditionally used to relieve pain, headaches, and digestive disorders [4]. The main components of *M. piperita* are essential oils, particularly menthol, menthone, and methyl acetate, which have been

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reported to exhibit pharmacological effects such as analgesic, anti-inflammatory, and muscle relaxant activities. Menthol exerts its analgesic effect by modulating the TRPM8 receptor, which induces a cooling sensation and reduces the transmission of pain impulses in the peripheral nervous system [5]. In addition, the local vasodilatory properties of menthol also contribute to enhancing topical analgesic effects [6].

In topical formulation development, the choice of solvent plays a crucial role in determining the extract's effectiveness. Ethanol is a polar solvent widely used in the extraction of crude drugs due to its ability to dissolve both polar and semi-polar active compounds [7]. Different ethanol concentrations can influence the composition of extracted compounds, potentially leading to variations in biological activities, including analgesic effects [8]. Therefore, it is important to compare the analgesic activity of peppermint leaf extracts obtained using various ethanol concentrations to determine the optimal concentration that produces the best pharmacological activity.

The Hargreaves test is one of the pain assessment methods using mice as experimental animals, based on thermal stimulation to evaluate analgesic responses. This method can be employed to assess the effectiveness of topical analgesic preparations, including those derived from herbal extracts [9], [10]. Therefore, the Hargreaves test method is suitable for measuring the topical analgesic activity produced by the ethanol extract of peppermint leaves.

Based on this background, the present study was conducted to compare the topical analgesic activity of peppermint leaf (*Mentha piperita*) extracts obtained using different ethanol concentrations through the Hargreaves test in mice. The results of this study are expected to provide scientific insight into the effect of solvent concentration variation on the analgesic activity of peppermint leaf extracts and to support the development of herbal medicines as safer topical analgesic alternatives.

Results and Discussion

The mint leaves used in this study underwent a determination process at the Batu Herbal Materia Medica Laboratory, and the results confirmed that the scientific name for mint leaves is Mentha piperita L. After sorting and drying, the mint leaves were blended into powder. 100 mg weight of the mint leaf powder was then macerated using three different concentrations of ethanol solvent: 50%, 70%, and 96% for 72 hours. The yields of mint leaf extract with ethanol concentrations of 50%, 70%, and 96% were 16.03%, 19.32%, and 25.57%, respectively. The calculation results of the percentage yield from maceration extraction can be seen in **Table 1**.

Table 1. Mint Leaf Extract Yield Calculation

No.	Extraction solvent concentration	Mint leaf powder weight (g)	Thick extract weight (g)	% Yield
1	Ethanol 50%	100,12	16,05	16,03%
2	Ethanol 70%	100,06	19,34	19,32%
3	Ethanol 96%	100,08	25,58	25,57%

The yields obtained from the extraction met the requirements for an ideal yield of a thick extract, which is not less than 10% [11]. The superior extraction efficiency of 96% ethanol is attributed to its ability to solubilize compounds across a broad polarity range, including nonpolar, semipolar, and polar metabolites. In addition, its enhanced penetration into plant cell walls compared with lower ethanol concentrations promotes the liberation of

intracellular bioactive constituents, leading to the production of a more concentrated extract [12].

The extracts obtained from the three solvents were then formulated into a gel preparation for easy application to the skin of mice's paws. The gel preparation is known to have a high-water content, which increases the solubility and diffusion of the active compounds into the skin. Furthermore, the gel provides an additional cooling sensation, which harmonizes with the menthol content in the mint leaf extract, enhancing its analgesic effect [13].

Topical analgesic activity testing using the Hargreaves test method can be seen in **Table 2** below. The observational data based on the obtained latency time were then further analyzed by calculating the Maximum Possible Effect (MPE) as shown in **Table 3**.

As shown in **Table 3**, the positive control group containing diclofenac sodium exhibited the greatest overall maximum possible effect (MPE). Among the peppermint leaf extract formulations, the gel containing the 96% ethanol extract demonstrated the highest MPE. The peak MPE values for the positive control group and for the gels containing 50%, 70%, and 96% ethanol extracts of peppermint leaves were reached at 150 minutes after administration. In this study, the negative control group was treated with the gel base alone, without any peppermint leaf ethanol extract.

Table 2. Topical Analgesic Activity Test Results Using the Hargreaves Test Method

No.	Treatment Time (minutes)	Mean Latency Time (seconds)					*p-value*
		Negative Control	Positive Control	50% Ethanol Extract Gel	70% Ethanol Extract Gel	96% Ethanol Extract Gel	
1	0 minutes	1,97 ± 0,16	2,47 ± 0,14	2,41 ± 0,15	2,51 ± 0,20	2,59 ± 0,10	0,080[1]
2	30 minutes	2,41 ± 0,15	7,47 ± 0,29[*1]	3,08 ± 0,29	3,78 ± 0,27[*1]	3,81 ± 0,18[*1]	0,000[*1]
3	60 minutes	2,01 ± 0,18	7,51 ± 0,17[*1]	3,34 ± 0,18[*1]	4,08 ± 0,32[*1]	4,48 ± 0,18[*1]	0,000[*1]
4	90 minutes	2,07 ± 0,22	8,22 ± 0,33[*2]	3,96 ± 0,44[*2]	4,91 ± 0,19[*2]	5,55 ± 0,14[*2]	0,000[*2]
5	120 minutes	3,26 ± 0,35	9,38 ± 0,34[*2]	4,27 ± 0,08[*2]	6,23 ± 0,12[*2]	7,15 ± 0,16[*2]	0,000[*2]
6	150 minutes	2,34 ± 0,21	10,00 ± 0,21[*1]	5,00 ± 0,21[*1]	7,53 ± 0,26[*1]	8,53 ± 0,07[*1]	0,000[*1]

Description: - [1.]Statistical test using One-Way ANOVA followed by LSD test
- [2.]Statistical test using Kruskal Wallis followed by Mann-Whitney test
- *There is a significant difference compared to the negative control group.

Table 3. Results of Calculation of Maximum Possible Effect (MPE)

No.	**Treatment Group**	**Maximum Possible Effect (MPE) (%)**					
		0 minutes	**30 minutes**	**60 minutes**	**90 minutes**	**120 minutes**	**150 minutes**
1	Negative Control	0	3,39	0,32	0,78	9,92	2,78
2	Positive Control	0	39,90	40,23	45,88	55,17	60,11
3	50% Ethanol Extract Gel	0	5,28	7,39	12,25	14,78	20,50
4	70% Ethanol Extract Gel	0	5,63	8,19	15,13	26,23	40,20
5	96% Ethanol Extract Gel	0	1,96	7,77	17,20	31,22	47,84

The maximum %MEP occurred at the 150th minute for the three groups of mint leaf ethanol extract gel.

Table 4. Mean Difference Results of the LSD Test for % Maximum Possible Effect at the 150th Minute

Group	Negative Control	Positive Control	50% Ethanol Extract Gel	70% Ethanol Extract Gel	96% Ethanol Extract Gel
Negative Control	-	-57.32800*	-17.72000*	-37.42600*	-45.06200*
Positive Control	57.32800*	-	39.60800*	19.90200*	12.26600*
50% Ethanol Extract Gel	17.72000*	-39.60800*	-	-19.70600*	-27.34200*
70% Ethanol Extract Gel	37.42600*	-19.90200*	19.70600*	-	-7.63600*
96% Ethanol Extract Gel	45.06200*	-12.26600*	27.34200*	7.63600*	-

*Note: * = significant difference at α = 0.05*

To determine which ethanol concentration produces the most optimal % maximum possible effect, an LSD test was performed as shown in **Table 4**. Post hoc analysis using the Least Significant Difference (LSD) test was conducted at 150 minutes, corresponding to the time point at which the positive control and all extract-treated groups (50%, 70%, and 96% ethanol extracts) reached their peak MPE values. The positive control group showed a significantly greater MPE compared with all other experimental groups ($p < 0.05$). Furthermore, among the extract-treated groups, the gel formulated with the 96% ethanol extract exhibited significantly higher analgesic activity, as reflected by its MPE value, than the gels containing the 50% and 70% ethanol extracts.

Mint leaves are known to contain secondary metabolites such as alkaloids and flavonoids that act as analgesics [14], [15]. Alkaloids constitute one of the largest phytochemical groups in plants and are known to be highly effective in therapy, often used as the basis for developing potent analgesic drugs [16]. Alkaloids are known to have better solubility in 96% ethanol. Research shows that 96% ethanol is able to extract alkaloid compounds and their derivatives more effectively [17], [18]. The antinociceptive activity of alkaloids is mediated through their interaction with opioid receptors in the central nervous system, resulting in reduced pain transmission and perception [19]. Flavonoids have the ability to inhibit the action of the cyclooxygenase enzyme, which is involved in the pain mechanism, namely the release of prostaglandins [20], [21]. Previous research has shown that total flavonoid levels are higher when using 96% ethanol as the extraction solvent [17], [22]. Furthermore, saponins and tannins are believed to contribute to analgesic activity by inhibiting COX-2, thereby reducing the biosynthesis of prostaglandins, the principal mediators involved in pain and inflammatory responses [19], [23]. In addition, the analgesic effect may be partially attributed to the presence of menthol, which activates TRPM8 receptors and produces a cooling sensation that can attenuate the transmission of nociceptive signals within the peripheral nervous system [5].

The Hargreaves test is used for analgesic activity testing due to its advantages, including its ability to provide a more specific measurement of thermal pain, its quantitative nature, and its ability to objectively compare effects between doses and between treatment groups. However, this method is not without limitations, for example, results can be influenced by the physiological condition of the animal, stress levels, and technical variations during stimulation [24], [25]. A challenge in this study was the mice's constant movement, making it difficult to aim the light source directly at their paws. Therefore, before each exposure, the mice were ensured to be in a calm position first, allowing for more accurate reaction time measurements.

Conclusion

Based on the results of the research that has been carried out, it can be concluded that the concentration of 96% ethanol solvent as a solvent for mint leaf extract (Mentha piperita) produces more

optimal analgesic activity compared to 70% and 50% ethanol solvents.

Material and Methods

Research design

The laboratory experimental design used in this study was a posttest-only control group design, employing white mice (*Mus musculus*) of the Balb/C strain as the experimental subjects. The study received ethical approval from the Ethics Committee of the University of Surabaya, under approval number 611/KE/VII/2025.

Instrument

The equipment used in this study included a hot plate (Thermo Scientific®), vacuum rotary evaporator (IKA RV®), oven (Memmert®), analytical balance (Ohaus®), blender (Philips®), water bath (Memmert®), filter paper, horn spoon, parchment paper, marker, scissors, stopwatch, and various laboratory glassware.

Ingredients

The materials used in this study included peppermint leaves (*Mentha piperita*), which had been taxonomically identified at the UPT Herbal Materia Medica Laboratory in Batu; ethanol at concentrations of 50%, 70%, and 96%; Voltaren® gel; sodium carboxymethyl cellulose (CMC-Na); propylene glycol; glycerin; and distilled water. The experimental animals were 25 male white mice (*Mus musculus*), Balb/C strain, aged 30–60 days, with body weights ranging from 20–30 grams.

Preparation of Ethanol Extract of Peppermint Leaves

The peppermint leaves used in this study were required to be fresh. After selection, the leaves were dried in an oven at 50°C for three hours. The dried leaves were then ground using a blender at speed level one for two minutes and sieved with a 60-mesh filter to obtain peppermint leaf powder. A total of 100 grams of the powdered leaves were weighed and mixed with 1 liter of 50% ethanol (1:10 mL ratio). The same procedure was applied for 70% and 96% ethanol solvents. Each mixture was then macerated for 72 hours at room temperature (26–28°C).

All mixtures were filtered using a Buchner funnel with Whatman No. 4 filter paper. The ethanol filtrate was evaporated at 50°C under vacuum using a rotary evaporator to remove the ethanol solvent. The extract was then concentrated by evaporation in a water bath at 80°C for 180 minutes until a thick extract was obtained, resulting in the 50%, 70%, and 96% ethanol extracts of peppermint leaves [26], [27].

Preparation of Peppermint Leaf Ethanol Extract Gel

The standard formulation used a CMC-Na gel base. The formulation of the analgesic gel containing peppermint leaf ethanol extract is presented in **Table 5** below [28] :

Table 5. Formula of Peppermint Leaf Ethanol Extract Gel

No	Ingredient	Negative Control	Extract Concentration in Formula		
			F1 (10%)	F2 (10%)	F3 (10%)
1	50% Ethanol Extract	-	5 gram	-	-
2	70% Ethanol Extract	-	-	5 gram	-
3	96% Ethanol Extract	-	-	-	5 gram
4	CMC Na	2,5 gram	2,5 gram	2,5 gram	2,5 gram
5	Glycerin	5,0 mL	5,0 mL	5,0 mL	5,0 mL
6	Propylenglycol	2,5 mL	2,5 mL	2,5 mL	2,5 mL
7	Distilled Water ad	50 mL	50 mL	50 mL	50 mL

The gel preparation began with the formation of the gel base. CMC-Na was dissolved in a portion of water heated on a hot plate to 50°C. Next, the 50% ethanol extract of peppermint leaves at a 10%

concentration was added and stirred using a magnetic stirrer until a homogeneous mixture was obtained. Subsequently, glycerin, propylene glycol, and the remaining water were gradually added while continuously stirring until the gel was formed. The same procedure was applied for the preparation of gels containing the 70% and 96% ethanol extracts [28].

Preparation of Test Animals and Analgesic Activity Evaluation

A total of 25 male *Mus musculus* mice of the Balb/C strain, aged between 30 and 60 days, weighing 20–30 grams, healthy, and exhibiting normal activity (not under stress), were prepared and divided into five groups. Each mouse received only one treatment. The number of mice used in this study was determined using the Federer formula as follows :[29]

(n-1) (t-1)	**≥ 15**	…… Equation 1
(n-1) (5-1)	≥ 15	
(n-1) (4)	≥ 15	
4n-4	≥ 15	
4n	≥ 19	
n	≥ 4,75 (rounded up to 5 mice per group)	

Description:
n = sample size
t = number of groups

Prior to treatment, the test animals were trained to adapt to their cages for seven days. Afterward, their body weight was measured, their urine output was measured, and their activity was observed. On the day of testing, each animal was given the appropriate treatment for its group. The treatment groups for the topical analgesic testing were as follows:

1. Group I: Mice's feet were rubbed with base gel (Negative control)
2. Group II: Mice's feet were rubbed with Voltaren gel (Positive control)
3. Group III: Mice's feet were rubbed with gel containing 50% ethanol extract of peppermint leaves
4. Group IV: Mice's feet were rubbed with gel containing 70% ethanol extract of peppermint leaves
5. Group V: rubbed with gel containing 96% ethanol extract of peppermint leaves

Each mouse was administered 0.1 mL of the gel corresponding to its treatment group, which was uniformly applied to the plantar surface of the hind paw [30]. After 30 minutes of administration of the test material, each mouse in groups I, II, III, IV, and V had its feet irradiated using light with an intensity of 40% from a Hargreaves apparatus. The time required for the mouse to withdraw its foot or jump from the heat from the light that was fired was recorded as the reaction time [31]. The reaction time was recorded before administration (0 minutes), 30 minutes, 60 minutes, 90 minutes, 120 minutes and 150 minutes after gel administration. During each observation, a cut-off time of 15 seconds was applied to prevent tissue damage [32]. Next, the analgesic activity is calculated using the following formula:

$$\textbf{\% Analgesic Activity} = \frac{T-K}{C-K}\ x\ 100\% \quad \text{……Equation 2}$$

Description:
T = Response time after gel administration
K = Response time of the negative control group
C = Cut-off time (15 seconds)

Data Analysis

Observation data are presented in tabular form and analyzed statistically using the Shapiro-Wilk test to determine normality, followed by the Levene test to assess homogeneity. If the data meets the assumptions of normality and homogeneity, an analysis is performed to determine significant differences using One Way ANOVA at a 95% confidence level, followed by the LSD (Least Significant Difference) test. If the distribution is not normally distributed and not homogeneous, the non-parametric Kruskal-Wallis test is used, followed by the Mann-Whitney test. All data analysis is performed using SPSS software.

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