

Phytochemical Characterization and Analgesic Activity of Ethanol Extract of Majapahit Stem Bark (*Crescentia cujete* L.) in Male Swiss Webster Mice

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ABSTRACT

Majapahit (*Crescentia cujete* L.) is a medicinal plant traditionally used for various therapeutic purposes and known to contain diverse secondary metabolites with potential pharmacological activities. This study aimed to characterize the phytochemical composition and evaluate the analgesic activity of a 70% ethanol extract of majapahit stem bark in male Swiss Webster mice. The stem bark was extracted by maceration using 70% ethanol. The resulting extract was characterized based on loss on drying, moisture content, total ash content, qualitative phytochemical screening, and quantitative determination of selected secondary metabolites by UV–Vis spectrophotometry. Analgesic activity was evaluated using the hot plate method at extract doses of 125, 250, and 375 mg/kg body weight, with 0.5% CMC-Na and mefenamic acid at a dose of 1.3 mg/20 g body weight used as the negative and positive controls, respectively. Loss on drying, moisture content, and total ash content of the crude plant material were 2.90%, 9.73%, and 12.51%, respectively, while the extract yield was 3.29%. Phytochemical screening detected alkaloids, flavonoids, saponins, and tannins. UV–Vis analysis yielded measured levels of 3.31 mg/kg flavonoids, 2.40 mg/kg tannins, 1.09 mg/kg alkaloids, and 0.74 mg/kg saponins. The 375 mg/kg body weight dose produced the highest analgesic response among the extract-treated groups, with 45.0% response protection and 98.0% analgesic activity. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. ANOVA indicated significant differences among treatment groups ($p < 0.05$), whereas the Tukey test showed no significant differences between the extract-treated groups and the positive control. These findings indicate that the ethanol extract of majapahit stem bark has potential analgesic activity in male Swiss Webster mice.

Keywords: *Crescentia cujete* L.; stem bark; ethanol extract; analgesic activity; hot plate

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INTRODUCTION

Pain is a common health problem that may occur as an acute or chronic condition. Chronic pain is estimated to affect approximately 20% of the global population and can impair physical function, psychological well-being, and quality of life (Cardoso & *et al.*, 2021). The International Association for the Study of Pain (IASP) defines pain as an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage. Nociceptive pain involves the processing of harmful stimuli through transduction, transmission, modulation, and perception, resulting in the subjective experience of pain (Raja *et al.*, 2020). Persistent pain can interfere with daily activities and well-being, highlighting the need for effective pain management.

Pharmacological management of pain includes opioid and non-opioid analgesics. Opioids such as morphine are highly effective for moderate to severe pain but may cause adverse effects, including tolerance, dependence, addiction, and respiratory depression (Pergolizzi *et al.*, 2020). Non-opioid analgesics, particularly nonsteroidal anti-inflammatory drugs (NSAIDs), are widely used because of their analgesic and anti-inflammatory effects. These limitations have stimulated interest in natural analgesic agents, particularly medicinal plants containing secondary metabolites with potential pharmacological activities.

One plant with potential as a natural source of bioactive compounds is majapahit, also known as berenuk (*Crescentia cujete* L.). The plant has traditionally been used for various medicinal purposes. Previous studies have reported several secondary metabolites in *C. cujete*, including flavonoids, alkaloids, saponins, tannins, steroids, and phenolic compounds. Alkaloids, flavonoids, saponins, tannins, and steroids have also been detected in ethanol extracts of *C. cujete* leaves and stem bark (Hasanah *et al.*, 2018). In addition, leaves and stem bark of *C. cujete* have demonstrated biological activities, including anti-inflammatory and antibacterial effects (Parvin *et al.*, 2015). The presence of these bioactive constituents and reported biological activities suggests that *C. cujete* may be a promising source of natural compounds with analgesic potential.

The secondary-metabolite profile of *C. cujete*, particularly its flavonoid and phenolic constituents, provides a basis for investigating its pharmacological properties. Flavonoids have been widely investigated for their biological activities and have been detected in several parts of *C. cujete*. Previous research has also demonstrated analgesic activity of *C. cujete*. Teodhora *et al.* (2020) reported that an ethanol extract of *C. cujete* fruit exhibited analgesic activity in male mice induced with 1% acetic acid using the writhing test. The study evaluated doses of 125, 250, and 500 mg/kg body weight, with the 500 mg/kg dose producing an analgesic response comparable to that of ibuprofen. However, that study used fruit and the writhing test, whereas information on the analgesic activity of *C. cujete* stem-bark ethanol extract assessed using the hot plate method remains limited. This difference in plant material and experimental model indicates a research gap regarding the analgesic potential of *C. cujete* stem bark.

The hot plate method is an experimental model used to evaluate nociceptive responses to thermal stimuli. In this model, characteristic responses such as hind-paw licking or jumping are used to assess changes in sensitivity to thermal stimulation (Menéndez *et al.*, 2002). The method can therefore provide information on the analgesic effects of test substances under controlled thermal stimulation.

Accordingly, this study aimed to evaluate the analgesic activity of a 70% ethanol extract of majapahit stem bark (*Crescentia cujete* L.) in male Swiss Webster mice using the hot plate method and to determine the dose that produced the highest observed analgesic response. The findings are expected to provide scientific evidence regarding the potential of *C. cujete* stem bark as a natural source of analgesic compounds and support further investigation of its bioactive constituents.

MATERIALS AND METHODS

Materials

The materials used in this study included majapahit stem bark (*Crescentia cujete* L.), male Swiss Webster mice weighing 20–30 g, 70% ethanol, distilled water, 0.5% CMC-Na, mefenamic acid, Mayer's reagent, Dragendorff's

reagent, HCl, magnesium powder, FeCl₃, chloroform, ammonia, H₂SO₄, tannic acid standard, quinine standard, quercetin standard, saponin standard, DMSO, bromocresol green, phosphate buffer, NaNO₂, AlCl₃, NaOH, diethyl ether, NaCl, n-butanol, and silica gel. The chemical reagents were obtained from Merck, while mefenamic acid was obtained from Afi Farma.

The equipment included a blender (Miyako), No. 80 mesh sieve (Merck), maceration vessel, Pyrex glassware, rotary evaporator (Stem Heated Evaporator), furnace (Merck), desiccator (Merck), UV-Vis spectrophotometer (Merck), centrifuge (Merck), analytical balance (Kern, Germany), animal weighing scale (OEM), mortar and pestle (OneMed), syringe (OneMed), separatory funnel, and analgesic tester (Cold Hot Plate Meter).

Methods

Plant Material Collection and Identification

Majapahit stem bark (*Crescentia cujete* L.) was collected from Kalidawir District, Tulungagung Regency, East Java, Indonesia. Plant identification was conducted at the Herbal Laboratory Unit of Materia Medica Batu, Malang, East Java. Samples were collected using purposive sampling based on the following criteria: healthy and mature plants, stem bark that was intact and free from visible fungal infection, pest damage, or other physical defects, and bark collected from the main stem at a similar developmental stage.

Preparation of Plant Material

Fresh majapahit stem bark was separated from the outer bark to the cambium layer and washed under running water to remove impurities. The material was cut into smaller pieces and dried under sunlight while covered with black cloth until a constant weight was obtained, with the drying process carried out under ambient environmental conditions. The dried material was ground using a blender and passed through a No. 80 mesh sieve to obtain a fine crude drug powder.

Standardization of the Crude Drug

Standardization included determination of loss on drying, moisture content, and total ash content. Loss on drying was determined gravimetrically by drying the sample at 105 °C to constant weight. Moisture content was determined using an oven-drying method at 100 °C, with repeated weighing until a constant weight was achieved. Total ash content was determined gravimetrically by incinerating the sample in a furnace at 600 °C for 5 h, followed by cooling in a desiccator and weighing.

Preparation of the Stem-Bark Extract

A total of 300 g of powdered majapahit stem bark was extracted by maceration using 70% ethanol at a crude drug-to-solvent ratio of 1:10. The mixture was placed in a closed maceration vessel and allowed to stand for 5 days with periodic agitation. After maceration, the mixture was filtered, and the filtrate was concentrated using a rotary evaporator to obtain a concentrated extract.

Phytochemical Screening

Phytochemical screening was performed to identify alkaloids, flavonoids, saponins, and tannins. For alkaloid identification, 0.5 g of extract was treated with chloroform and ammonia, filtered, and subsequently treated with H₂SO₄. The filtrate was then reacted with Mayer's and Dragendorff's reagents. The formation of a precipitate indicated the presence of alkaloids.

Flavonoids were identified by adding HCl and magnesium powder to 0.5 g of extract. The development of a reddish orange to reddish-purple color indicated a positive reaction. For saponin identification, 10 mL of hot water was added to 0.5 g of extract, followed by vigorous shaking for approximately 10 s. The formation of foam that persisted for approximately 10 min indicated the presence of saponins.

Preparation of Test Solutions and Treatment

Mefenamic acid was used as the positive control at a dose converted from a human dose of 500 mg to a mouse dose of 1.3 mg/20 g body weight, equivalent to 65 mg/kg BW. The ethanol extract

of majapahit stem bark was administered at doses of 125, 250, and 375 mg/kg body weight. The required amount of extract was weighed, suspended in 0.5% CMC-Na, and homogenized.

Experimental Animals

Twenty-five male Swiss Webster mice weighing 20–30 g were divided into five groups, with five animals per group:

1. Negative control: 0.5% CMC-Na.
2. Positive control: mefenamic acid at 65 mg/kg body weight
3. Extract group: 125 mg/kg body weight.
4. Extract group: 250 mg/kg body weight.
5. Extract group: 375 mg/kg body weight.

Before testing, the mice were fasted according to the experimental protocol while maintaining access to water. The animal study received ethical clearance from Universitas Airlangga, Surabaya.

Analgesic Activity Test

Analgesic activity was evaluated using the hot plate method. The hot plate was maintained at 55 °C. Twenty-five male Swiss Webster mice, with five mice in each group, were administered the respective treatments described above. Each mouse was placed individually on the apparatus, and the response to thermal stimulation, particularly hind-paw licking or jumping, was observed. Responses were recorded at 0, 30, 60, 90, and 120 min after treatment. A cut-off time of [15/20] s was applied to prevent tissue injury, and each mouse was immediately removed from the hot plate when the cut-off time was reached. Analgesic activity was evaluated by comparing responses among the treatment, negative-control, and positive-control groups.

Data Analysis

Data were analyzed using SPSS. Normality was assessed using the Shapiro–Wilk test because the sample size was less than 50, while homogeneity of variance was assessed to determine whether the variances among groups were comparable. When the data met the assumptions of normality and homogeneity, one-way analysis of variance

(ANOVA) was performed to evaluate differences among treatment groups. Statistical significance was set at $p < 0.05$.

The percentage of response protection was calculated using:

$$\% \text{ Protection} = 100 - [(p/k) \times 100\%]$$

where p is the cumulative response of the treatment group and k is the mean cumulative response of the negative-control group.

The percentage of analgesic activity was calculated as:

$$\% \text{ Analgesic Activity} = (p/k_p) \times 100\%$$

where p is the percentage response protection of the treatment group and k_p is the percentage response protection of the positive-control group.

RESULTS

Plant Identification and Crude-Drug Characteristics

Plant identification conducted at the Herbal Laboratory Unit of Materia Medica Batu confirmed that the plant material used in this study was majapahit (*Crescentia cujete* L.), belonging to the family Bignoniaceae. The identification was documented in Determination Certificate No. 000.9.3/654/102.20/2024.

Processing of 3 kg of fresh stem bark yielded 1.76 kg of dried crude drug, which was subsequently ground and sieved to obtain 300 g of powder. The results of crude-drug characterization are presented in Table 1.

All measured parameters met the criteria used in this study. Loss on drying and moisture content were 2.90% and 9.73%, respectively, indicating relatively low residual moisture. The total ash content was 12.51%, which remained below the specified limit. Adequate moisture control is important because excessive moisture can promote microbial growth and accelerate deterioration of plant material.

Table 1. Characteristics of the *Crescentia cujete* L. crude drug

Parameter	Result	Requirement	Interpretation
Loss on drying	2.90%	≤ 10%	Meets requirement
Moisture content	9.73%	≤ 10%	Meets requirement
Total ash content	12.51%	≤ 16.6%	Meets requirement

Extraction Yield

Maceration of 300 g of *C. cujete* stem-bark powder with 70% ethanol for 5 days yielded 9.87 g of concentrated extract, corresponding to an extraction yield of 3.29%. The extraction characteristics are presented in Table 2.

The extraction yield was relatively low compared with the >10% criterion used in this study. Extraction yield can be influenced by solvent type and polarity, particle size, extraction time, solvent-to-material ratio, and concentration conditions. Therefore, a low extraction yield does not necessarily indicate low biological activity. Ethanol can extract secondary metabolites with a broad range of polarities

Table 2. Extraction characteristics of *Crescentia cujete* L. stem bark

Parameter	Result
Mass of crude drug powder	300 g
Solvent	70% ethanol
Extraction method	Maceration
Extraction time	5 days
Mass of concentrated extract	9.87 g
Extraction yield	3.29%

Phytochemical Screening

Qualitative phytochemical screening detected alkaloids, flavonoids, saponins, and tannins in the ethanol extract of *C. cujete* stem bark. Positive reactions were indicated by characteristic precipitate formation or color changes. The results of the phytochemical screening are presented in Table 3.

Table 3. Phytochemical screening of *Crescentia cujete* L. stem-bark ethanol extract.

Metabolite group	Observation	Result
Alkaloids—Mayer	White precipitate	+
Alkaloids—Dragendorff	Orange precipitate	+
Flavonoids	Red color development	+
Saponins	Stable foam	+
Tannins	Bluish-black color	+

Note: (+) = detected.

The positive reactions indicate that the extract contains all four secondary-metabolite groups examined. This finding is consistent with previous reports describing the occurrence of flavonoids, alkaloids, saponins, and tannins in *C. cujete* and other plant preparations.

Quantitative Determination of Secondary Metabolites

Quantitative determination was performed using UV–Vis spectrophotometry. Flavonoids showed the highest measured level, followed by tannins, alkaloids, and saponins. The measured levels of the secondary-metabolite groups are presented in Table 4.

Flavonoids showed the highest measured level at 3.31 mg/kg, whereas saponins showed the lowest at 0.74 mg/kg. The predominance of flavonoids is relevant because this group has been widely investigated for anti-inflammatory and analgesic activities through several molecular pathways. However, the higher measured flavonoid level does not establish flavonoids as the sole constituents responsible for the observed analgesic activity because the extract contains multiple metabolite groups.

Table 4. Measured levels of secondary-metabolite groups in *Crescentia cujete* L. stem-bark ethanol extract.

Metabolite group	Measured level (mg/kg)
Flavonoids	3.31
Tannins	2.40
Alkaloids	1.09
Saponins	0.74

Analgesic Activity

Analgesic activity was evaluated using the hot plate method at 55 °C in 25 male Swiss Webster mice divided into five experimental groups. The treatment groups consisted of a negative control (0.5% CMC-Na), a positive control (mefenamic acid), and three extract doses of 125, 250, and 375 mg/kg body weight. The mean response counts recorded at each observation interval are presented in Table 5.

The positive-control and all extract-treated groups showed lower mean response counts than the negative-control group. Among the extract-treated groups, the 375 mg/kg BW group had the lowest overall mean response count (14.00 ± 6.37), followed by the 250 mg/kg BW group (14.08 ± 7.32) and the 125 mg/kg BW group (14.48 ± 7.66). The positive control showed a mean response count of 13.76 ± 7.91 . The calculated response protection and analgesic activity are presented in Table 6.

The 375 mg/kg BW group showed the highest response protection (45.0%) and analgesic activity (98.0%) among the extract-treated

groups. These values were close to those of the positive control, which showed 45.9% response protection and 100% analgesic activity.

The reduction in responses observed in the extract-treated groups suggests potential antinociceptive activity. The highest dose produced the strongest response descriptively, although the differences among the three extract doses were relatively small. Therefore, the results suggest an increasing trend in observed analgesic activity with increasing dose, but they do not by themselves establish a statistically significant dose–response relationship.

The hot plate test evaluates nociceptive responses to thermal stimulation. Responses such as hind-paw licking or jumping can be used to assess changes in sensitivity to thermal stimuli (Menéndez *et al.*, 2002). The positive control, mefenamic acid, produced the highest analgesic activity. As an NSAID, mefenamic acid exerts analgesic effects partly through cyclooxygenase inhibition, thereby reducing prostaglandin formation involved in nociceptor sensitization (Bannon, 2021).

The analgesic response observed with the extract may be associated with its secondary-metabolite composition. Flavonoids were the most abundant measured metabolite group and have been reported to possess anti-inflammatory properties and to modulate pathways associated with pain and inflammation (Ferraz *et al.*, 2020). Alkaloids, tannins, and saponins may also contribute through different biological mechanisms. Thus, the observed activity may reflect the combined effects of multiple secondary metabolites rather than the action of a single metabolite group (Balogun & Ashafa, 2021).

Table 5. Mean response counts of mice during the hot plate test

Group	0 min	30 min	60 min	90 min	120 min	Mean $\pm$ SD
Negative control	27.8	26.2	23.2	23.8	26.4	25.48 ± 1.92
Positive control	25.0	17.4	13.2	8.6	4.6	13.76 ± 7.91
Extract, 125 mg/kg BW	24.6	19.6	13.2	9.4	5.6	14.48 ± 7.66
Extract, 250 mg/kg BW	23.2	18.4	14.4	8.8	5.4	14.08 ± 7.32
Extract, 375 mg/kg BW	22.4	17.2	14.6	9.8	6.0	14.00 ± 6.37

Table 6. Response protection and analgesic activity

Group	Response protection (%)	Analgesic activity (%)
Negative control	0.0	0.0
Positive control	45.9	100.0
Extract, 125 mg/kg BW	43.1	93.8
Extract, 250 mg/kg BW	44.7	97.3
Extract, 375 mg/kg BW	45.0	98.0

Statistical Analysis

The data met the assumptions of normality based on the Shapiro–Wilk test and homogeneity of variance. Therefore, one-way ANOVA was performed. The ANOVA indicated a significant difference among the treatment groups ($p < 0.05$).

The Tukey post hoc analysis showed that the 375 mg/kg BW extract group, 250 mg/kg BW extract group, positive control, and 125 mg/kg BW extract group were included in the same homogeneous subset. The reported significance value for this subset was $p = 0.969$. The negative control was separated from the extract 375 mg/kg BW group. These results indicate that the responses of the extract treated groups were statistically comparable with that of the positive control, while the negative control differed from the highest-dose extract group.

Although the 375 mg/kg BW group showed the highest response protection and analgesic activity descriptively, the differences among the three extract doses were relatively small and were not statistically significant. Therefore, the 375 mg/kg BW dose should be described as producing the highest observed response, rather than as a statistically confirmed optimal pharmacological dose. The Tukey post hoc test results are presented in Table 7.

Table 7. The Result of Post Hoc Tukey

Group	Subset 1	Subset 2
Negative control	11.840	-
Positive control	13.720	13.720
Extract, 125 mg/kg BW	13.760	13.760
Extract, 250 mg/kg BW	14.480	14.480
Extract, 375 mg/kg BW	-	25.480
Significance	0.969	0.076

Phytochemical Analgesic Relationship and Study Limitations

The combined findings indicate an association between the phytochemical profile of the ethanol extract and its observed analgesic activity. The extract contained flavonoids, tannins, alkaloids, and saponins and produced measurable analgesic responses in male Swiss Webster mice. Previous research has likewise reported biological activities of *C. kujete* stem bark, particularly anti-inflammatory activity.

Nevertheless, the phytochemical characterization performed in this study does not identify individual compounds or establish which constituents are directly responsible for the analgesic response. Qualitative phytochemical screening and UV–Vis spectrophotometry provide information at the metabolite-group level rather than definitive structural identification. Further studies using LC–MS/MS, GC–MS, or NMR would be useful for identifying and characterizing individual compounds. Bioactivity-guided fractionation and testing of isolated compounds or active fractions would also be necessary to establish relationships between specific constituents and analgesic activity.

CONCLUSIONS

The 70% ethanol extract of majapahit stem bark (*Crescentia kujete* L.) contained alkaloids, flavonoids, saponins, and tannins and exhibited analgesic activity in male Swiss Webster mice evaluated using the hot plate method. The measured levels of flavonoids, tannins, alkaloids, and saponins were 3.31, 2.40, 1.09, and 0.74 mg/kg, respectively. Among the extract-treated groups, the 375 mg/kg BW dose produced the highest observed response, with a mean response count of 14.00 ± 6.37 , 45.0% response protection, and 98.0% analgesic activity. One-way ANOVA indicated significant

differences among treatment groups, whereas the Tukey test showed that the extract-treated groups and positive control were statistically comparable. Thus, the ethanol extract of *C. cujete* stem bark shows potential analgesic activity under the experimental conditions used in this study.

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