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Total flavonoid content and analgesic activity of ethyl acetate fraction of Indian jujube (*Ziziphus mauritiana* lamk) leaves

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ABSTRACT

Indian jujube leaves (*Ziziphus mauritiana* Lamk) have various potential biological activity. Previous studies have proven that ethanol extracted from Indian jujube (*Z. mauritiana* Lamk) leaves had analgesic activity. This study aimed to determine the total flavonoid content and analgesic activity of the ethyl acetate fraction of Indian jujube (*Z. mauritiana* Lamk) leaves ethanol extract. Tested fraction was prepared by maceration using ethanol as solvent followed by fractionation using ethyl acetate. Identification of active compounds was performed using color reaction, and determination of total flavonoids by colorimetric method. Ethyl acetate fraction was further tested for its analgesic activity using the writhing method. Swiss-Webster white male mice were divided into 5 treatment groups consisting of negative control contained sodium-CMC, positive control contained sodium diclofenac, while groups 3-5 were given ethyl acetate fraction of Indian jujube (*Z. mauritiana* Lamk) leaves with doses of 50, 150, and 200 mg/kg BW. All interventions were administered as a single oral dose. 0.7% (w/v) acetic acid was used as a pain inducer. Analgesic activity was measured by calculating the percentage of writhing protection as a measure of the analgesic effect produced by each intervention. The data were analyzed using one-way Anova to compare analgesic activity between treatment groups. Results showed that ethyl acetate fraction of Indian jujube (*Z. mauritiana* Lamk) leaves at doses of 50, 150, and 200 mg/kg BW gave an effect that was not significantly different from sodium diclofenac as positive control. The ethyl acetate fraction of Indian jujube (*Z. mauritiana* Lamk) leaves ethanol extract contained flavonoids, saponin, and tannins with total flavonoid content of 22.41 ± 1.16% w/w. It can be concluded that the ethyl acetate fraction of Indian jujube (*Z. mauritiana* Lamk) leaves have analgesic activity in mice.

ABSTRACT

Daun bidara (*Z. mauritiana* Lamk) mempunyai berbagai aktivitas biologi potensial. Penelitian sebelumnya membuktikan ekstrak etanol daun bidara memiliki aktivitas analgetik. Penelitian ini bertujuan untuk mengkaji aktivitas analgetik fraksi etil asetat ekstrak etanol daun bidara (*Z. mauritiana* Lamk). Fraksi uji dibuat dengan maserasi menggunakan pelarut etanol dilanjutkan dengan fraksinasi dengan etil asetat. Identifikasi kandungan kimia aktif menggunakan reaksi warna dan penetapan flavonoid total dengan metode kolorimetri. Fraksi etil asetat selanjutnya diuji aktivitas analgetiknya dengan metode geliat. Mencit jantan putih Swiss-Webster dibagi menjadi 5 kelompok perlakuan terdiri atas kontrol negatif Na-CMC, kontrol positif Na diklofenak, sedangkan kelompok 3-5 diberikan fraksi etil asetat daun bidara (*Z. mauritiana* Lamk) dengan dosis 50, 150 dan 200 mg/kgBB. Semua intervensi diberikan sebagai dosis tunggal secara oral. Asam asetat 0,7% (b/v) digunakan sebagai penginduksi nyeri. Aktivitas analgesik diukur dengan menghitung persentase proteksi geliat sebagai ukuran efek analgesic yang dihasilkan oleh setiap intervensi. Data dianalisis menggunakan Anova satu jalan untuk membandingkan aktivitas analgesik antar kelompok perlakukan. Hasil penelitian menunjukkan fraksi etil astat daun bidara (*Z. mauritiana* Lamk) dosis 50, 150 dan 200 mg/kgBB memberikan efek yang tidak berbeda signifikan dengan kontrol positif Na diklofenak. Hasil identifikasi menunjukkan fraksi etil asetat ekstrak etanol daun bidara (*Z. mauritiana* Lamk) mengandung flavonoid, saponin, dan tanin dengan kadar flavonoid total diperoleh 22,41 ± 1,16% b/b. Disimpulkan bahwa fraksi etil asetat daun bidara (*Z. mauritiana* Lamk) mempunyai daya analgetik pada mencit.

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INTRODUCTION

Pain or tenderness is a symptom that indicates a problem in a certain part of the body such as inflammation, bacterial infection or muscle spasms. Pain can interfere with social function and quality of life for sufferers and is the reason patients often use analgesics.[1]and little is known about the prevalence of analgesics administration among community-dwelling older adults. The objective was to examine older adults' prescription analgesic purchases in relation to SF-36 pain in a population-based setting. Methods: One thousand four hundred twenty community-dwelling citizens aged 62–86 years self-reported SF-36 bodily pain (pain intensity and pain-related interference Pain treatment can be done both non-pharmacologically and pharmacologically.[2] Although many narcotic and non-narcotic analgesics have been established for reducing pain, a long-term use of both of them may have serious side effects.[3,4] Non-steroidal anti-inflammatory drugs (NSAIDs) has been reported to cause worsen asthma, peptic ulcers, gastric bleeding in patients taking anticoagulants, impaired renal and cardiac function such as myocardial infarction and ischemia.[5] Opioids have been reported to cause constipation, retention, and spasm as side effects, peripheral as well as central oversedation, breathing, hypotension, nausea, coughing, vasodilation and hypotension.[6]

Treatment using natural ingredients derived from plants is an interesting thing to study in overcoming pain.[7] Medicinal plants are traditionally used to prevent and treat various diseases, besides being trusted to have relatively small side effects, they are also easily obtained by people. Indian jujube (*Ziziphus mauritiana* Lamk) belongs to the Rhamnaceae family and is founded in various climatic environments.[8] The leaves are empirically used for various diseases including analgesic activity.[9] Previous studies reported that Indian jujube (*Z. mauritiana* Lamk) leaves have antioxidant activity,[10] anticancer, protective of body cells such as the kidney, liver, and brain, as well as analgesic, antipyretic and anti-inflammatory.[11] Further research has proved that flavonoids and polyphenols in ethanolic extract of Indian jujube leaves (*Z. mauritiana* Lamk) were associated with analgesic activity.[12] The ethyl acetate fraction of Indian jujube (*Z. mauritiana* Lamk) ethanol extract was determined for its flavonoid content and then tested for analgesic activity and became the focus of this study.

MATERIAL AND METHODS

Material and equipment

Materials used in this study were Indian jujube (*Z. mauritiana* Lamk) leaves, 70% ethanol (Medica), 95% ethanol (Medica), NaOH (Merck), concentrated H_2SO_4 (Mallinckrodt AR), Zinc powder (Merck), Concentrated HCl (Merck), chloroform (Merck), H_2SO_4 2N (Merck), Mayer's reagent, $FeCl_3$ (Merck), $AlCl_3$ 12% (Merck), NaOH 6% (Merck), $NaNO_2$ 8% (Merck), quercetin (Merck), aqua distillate (Brataco), acetosal (Brataco), 1% acetic acid (Brand), CMC-Na (Brataco), ethyl acetate (Merck), n-hexane (Bratachem), Mayer's reagent, Dragendroff's reagent, and Lieberman-Burchard.

Equipment used in this research were maceration vessel, 60 mesh sieve (Retsch), porcelain dish, glassware (Pyrex iwake), parchment paper, Kern analytical balance (type EW 220-3 NM), aluminum foil, rotary evaporator (Eyela Digital Water Bath SB-1000), water bath GFL (type 1042), sieve no. 45 mesh, UV-Vis Spectrophotometer (Shimadsu type W-1700), oral needle, and injection syringe.

Extraction and fractionation of Indian jujube (*Z. mauritiana* Lamk) leaves

Indian jujube leaves (*Z. mauritiana* Lamk) were collected from the Liliba area, Kupang City, East Nusa Tenggara

(NTT). The collected leaves were light green, fresh, and whole. Determination of the leaves was carried out at the Biology Department, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta (reference number BF/407/Ident/Det/XI/2018).

The collected leaves were then washed under running water, dried in an oven, and ground into powder. A total of 500 g of mashed Indian jujube (*Z. mauritiana* Lamk) leaves were put into a maceration vessel and added 70% ethanol until completely submerged. The vessel was then closed and allowed to stand for 5 days while stirring frequently. After 5 days of maceration, the homogenized mixture was filtered and the filtrate was collected. The residue was remacerated again with new 70% ethanol two times. The collected filtrates were evaporated using a rotary evaporator at a temperature of 60 °C[13], and obtained a thick extract with a yield percentage was 26.65%.

Ethyl acetate fraction was prepared by dissolving 30 g of extract in 100 mL of ethanol-water mixture. The solution was then partitioned by adding 100 mL of ethyl acetate in a separating funnel, shaken to form 2 layers after being allowed to stand for 30-60 min. Ethanol-water layer, which was the bottom layer, was added with ethyl acetate and repeated 3 times. The ethyl acetate layer that was obtained was collected and evaporated in a rotary evaporator at a temperature of 50°C until a thick extract was obtained and further identification of the chemical compound was carried out.[14]

Identification of chemical compound of ethyl acetate fraction

Identification of active compounds includes the identification of flavonoids, alkaloids, tannins, and saponins through color reactions. Identification of flavonoids was carried out by reacting 10 g of sample with 0.1 mL concentrated HCl, if it formed a brownish red layer indicating that it contained flavonoids positively. Identification of alkaloids was carried out by reacting sample with the Meyer's reagent, obtained white precipitate and used Dragendorf's reagent obtained red orange precipitate indicating that the sample contained alkaloids positively. The tannin test was carried out by reacting the sample in $FeCl_3$, if blue to black color was formed, indicating that the sample contained tannin.[15] Meanwhile, saponins were identified through foam test by observing the formation of foam due to shaking with distilled water and it disappeared with the addition of HCL.[16]

Total flavonoid content determination

The sample solution was made with a concentration of 4000 ppm then pipetted 0.5 mL and added with 0.1 mL aluminum (III) chloride 10%, 0.1 mL sodium acetate 1 M, and 2.8 mL distilled water. The solution was incubated for 30 minutes and the absorbance was measured using a UV-Vis spectrophotometer at a quercetin maximum wavelength of 522 nm. Total flavonoids from ethyl acetate fraction of Indian jujube (*Z. mauritiana* Lamk) leaves ethanol extract were calculated using linear regression equation from the previously measured quercetin calibration curve and expressed as gram of quercetin equivalent per gram of extract.[17]

Analgesic activity test

This experiment used 15 healthy Swiss-Webster white male mice (2-3 months, 20-25 g). Animals were kept under controlled conditions at a temperature of 22±25℃, a humidity of 55±10%, and a dark and light cycle for 12 h, given standard feed and water ad libitum. Experimental animals were fasted for 8 h before use and given ad libitum water. All experimental animal procedures were approved by the Research Ethics Committee at the Faculty of Medicine, Nusa Cendana University, Kupang, Indonesia (reference number 04a/UN15.16/KEPK/2018).

Animals were divided into 5

groups, each group consisting of 3 experimental animals. Treatment group (P) consisted of 1) P1 was given 1% Na-CMC suspension with the volume of administration according to body weight as the negative control, 2) P2 was given sodium diclofenac with a volume of 0.26 mL/20 kgBW as the positive control, 3) P3 was given fraction dose ethyl acetate of Indian jujube (*Z. mauritiana* Lamk) leaves ethanol extract of 50 mg/kgBW, 4) P4 was given a dose of ethyl acetate fraction of Indian jujube (*Z. mauritiana* Lamk) leaves ethanol extract of 150 mg/kgBW, 5) P5 was given a dose of ethyl acetate fraction of Indian jujube (*Z. mauritiana* Lamk) leaves ethanol extract of 200 mg/kgBW was administered orally. After 30 min, all mice in each treatment group were injected intraperitoneally with 0.7% acetic acid solution. Mice were placed in observation cages and then observed the number of writhing every 5 min for 60 min. Data on the number of writhes of each mouse were tabulated and averaged and then compared between the test group and control group.[18]

Statistical analysis

This research produced qualitative and quantitative data. Phytochemical identification by the color reaction was qualitative data. Quantitative data was total flavonoid content and percentage of writhing protection presented using mean ± SD. Data were then analyzed using one-way Anova to determine whether there was or no analgesic effect based on the difference in the percentage of analgesic ability between control and treatment, while the difference in the percentage of analgesic ability between doses was tested by LSD (Least Significant Differences).

RESULTS

Characteristics of ethyl acetate fraction of ethanol extract Indian jujube (*Z. mauritiana* Lamk) leaves

In this study, the weight of ethyl acetate fraction of Indian jujube (*Z. mauritiana* Lamk) leaves ethanol extract was obtained at 40.57 g with a yield percentage of 15.23% with a thick texture, blackish green color, and characteristic odor of the leaves. Results of phytochemical identification through color reaction showed that the ethyl acetate fraction of Indian jujube (*Z. mauritiana* Lamk) leaves positively contained flavonoids, saponins, and tannins as shown in TABLE 1.

TABLE 1. Results of qualitative identification of ethyl acetate fraction Indian jujube (*Z. mauritiana* Lamk) leaves ethanol extract

Compound	Reagent	Result	Description*
Flavonoid	Extract + NaOH	There was a color change from light green to reddish green	+
	Extract + H_2SO_4	There was a color change from light green to blackish brown	+
Saponin	Extract + 10 mL aqua distillate, shaken vigorously	Foam formed	+
Alkaloid	Extract 0.5 g + 5 mL HCl. Filtrate + Mayer Reagent	There was color a change from orange to cloudy yellow	-
Tannin	Extract + $FeCl_3$	There was color a change from light green to dark green	+

* + = active compound positive indentified; - = active compound not identified

Total flavonoid levels determination

After identifying the chemical compounds, the total flavonoid content of the ethyl acetate fraction of Indian jujube (*Z. mauritiana* Lamk) leaves was determined using quercetin as standard, the results were as in TABLE 2.

TABLE 2. Total flavonoid content of ethyl acetate fraction of Indian jujube (*Z. mauritiana* Lamk) leaves

Sample concentration (ppm)	Absorbance	Total flavonoid content (QE)*		
		mg/g extract	g/g extract	% b/b
4000	0.349	216	0.216	21.60
	0.392	237.5	0.2375	23.75
	0.355	219	0.219	21.90
Mean		224.16	0.2241	22.41 ± 1.16

*QE = quercetin equivalent

On TABLE 2, it could be seen that the greater the absorbance value of the sample, the greater the total flavonoid content in the sample. The total flavonoid content of the ethyl acetate fraction of Indian jujube (*Z. mauritiana* Lamk) leaves was expressed as quercetin equivalent (QE), which was the equivalent number of g of quercetin in 100 g of extract (QE). Total flavonoids in the form of flavones and flavonols were calculated in percent w/w and the total flavonoid content of ethyl acetate fraction of Indian jujube (*Z. mauritiana* Lamk) leaves was 22.41% w/w ± 1.16 calculated against quercetin.[17]

Analgesic activity

Testing analgesic activity of ethyl acetate fraction of Indian jujube (*Z. mauritiana* Lamk) leaves using Witkin method. The principle of this method was a decrease in the number of writhing due to the administration of test substance in mice induced by chemical compounds, in this case 0.7% acetic acid was used as a single oral dose.[18] The average number of writhing in each treatment group was calculated and then the percentage of writhes protection in experimental animals was determined[19] as shown in FIGURE 1 and 2.

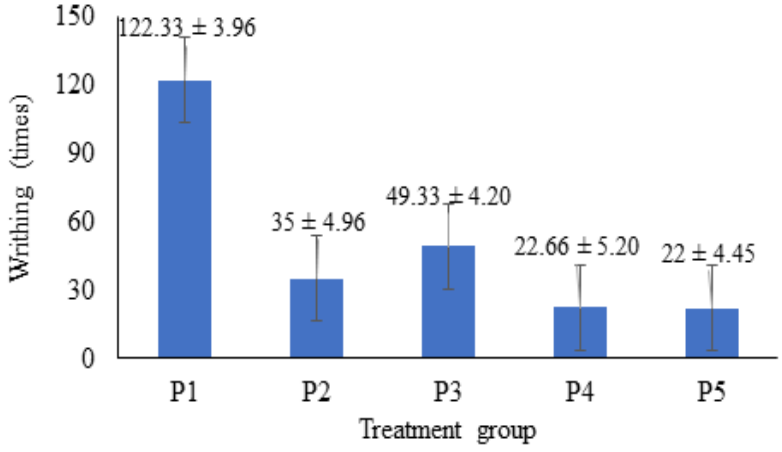

FIGURE 1. Amount writhing in experimental animals

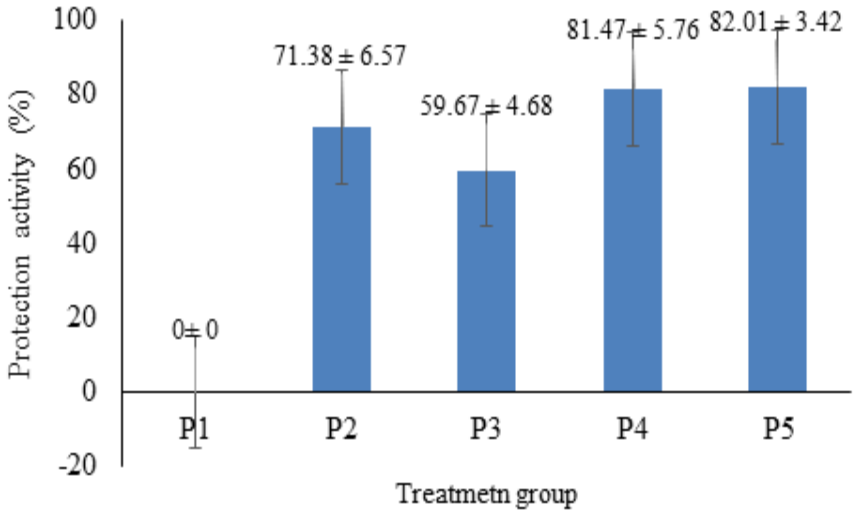

FIGURE 2. Percentage protection of ethyl acetate fraction of Indian jujube (*Z. mauritiana* Lamk) leaves. P1: negative control of sodium CMC; P2: positive control of sodium diclofenac; P3: ethyl acetate fraction of Indian jujube (*Z. mauritiana* Lamk) leaves 50 mg/kg BW; P4: ethyl acetate fraction of Indian jujube (*Z. mauritiana* Lamk) leaves 150 mg/kg BW; P5: ethyl acetate fraction of Indian jujube (*Z. mauritiana* Lamk) leaves 200 mg/kg BW.

DISCUSSION

This study identified the presence of chemical compounds as shown in TABLE 1. Indian jujube (*Z. mauritiana* Lamk) leaves were positive for flavonoids, this was also reported for the presence of flavonoids in the ethyl acetate fraction of Indian jujube (*Z. mauritiana* Lamk) leaves.[20] Indian jujube (*Z. mauritiana* Lamk) leaves contained rutin, quercetin, catechins, naringenin (2, 3-dihydro-5, 7-dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one which were classified as flavonoid compounds that act as analgesics.[21,22] Other studies had shown that flavonoids were able to inhibit the action of cyclooxygenase enzyme, thereby reducing the production of prostaglandins by arachidonic acid which would reduce the pain, besides that flavonoids also inhibited neutrophil degranulation so that it would inhibit the release of cytokines, free radicals, and enzymes that played a role in inflammation.[23] Results identification also showed that the leaves contained tannins. In other studies, In-vivo studies had shown that tannins had a mechanism of inhibiting cyclooxygenase enzyme so that it could reduce the amount of writhing in mice.[24]

The presence of flavonoids in the ethyl acetate fraction of Indian jujube (*Z. mauritiana* Lamk) leaves was determined using the $AlCl_3$ colorimetric method with UV-Vis spectrophotometry as shown in TABLE 2. Quercetin was used as a comparison so that the total flavonoid content of Indian jujube (*Z. mauritiana* Lamk) leaves ethanol extract was expressed as QE, which was the equivalent number of g of quercetin in 100 g extract with a value of 22.41 ± 1.16 % w/w, while total flavonoid in ethanol extract was 1.5312% w/w.[17] This showed that flavonoid dissolved in ethyl acetate fraction was higher and further affects the analgesic activity.[25]

Analgesic activity test was carried out using the writhing test method or what was known as the chemical stimulation

method. Acetic acid was chosen in this method as a pain inducer originating from local acute inflammatory pain. This response resulted from the release of arachidonic acid from tissue phospholipids via the cyclooxygenase pathway and produced prostaglandins in peritoneal fluid. Experimental animals responded by showing writhing, which was observed for abdominal retraction and bending of the head and legs back.[26]

It could be seen that each mice in negative control group, positive control group, dose of 50, 150, and 200 mg/kg BW had different amounts of writhing (TABLE 1). The less amount of writhing indicated the more effective analgesic ability of test material. Meanwhile, in negative control group, sodium CMC suspension did not showed any change in the amount of writhing before and after test material administration. This indicated that sodium CMC did not had ability to treat pain.[27]

Amount of writhing data was then used to calculated writhing protection ability or also called as percentage of analgesic power. Percentage of analgesic power was obtained by comparing average number of writhing of test material group with negative control group. TABLE 2 showed the greater dose of ethyl acetate fraction of Indian jujube (*Z. mauritiana* Lamk) leaves ethanol extract, the greater percentage of analgesic power. Test compound was said to had an analgesic effect if it was able to reduced 50% of total writhing of negative control group.[27] The highest percentage of analgesic power was in ethyl acetate fraction of Indian jujube (*Z. mauritiana* Lamk) leaves at dose of 200 mg/kgBW with value of 82.01 ± 3.42% while the lowest value was in 200 mg/kgBW dose group with value of 59.67 ± 4.68%.

In Shapiro-Wilk test, data for each treatment group was normally distributed with p value > 0.05. Then the homogeneity test showed that results of analgesic ability data were inhomogeneous with a significance value of 0.141 p>0.05. The results of the one-way Anova test with p = 0.000 showed that there was effect of ethyl acetate fraction of Indian jujube (*Z. mauritiana* Lamk) leaves as analgesic on male white mice.[28] The next test was assessed by LSD analysis and showed that negative control group had significant difference with positive control group, at dose of 50, 150, and 200 mg/kgBW. Other evidence also showed that positive control did not show significant difference in all groups of doses of test material.

From the results of the study, it was shown that all treatment groups with a dose of ethyl acetate fraction of Indian jujube (*Z. mauritiana* Lamk) leave ethanol extract had analgesic activity. It was suspected that the presence of flavonoids and tannins could inhibit prostaglandins and inhibit the activity of the cyclooxygenase enzyme which was an initial pathway for the formation of prostaglandins as pain mediators.[28,29] The high number of flavonoids in the ethyl acetate fraction supports the ability of Indian jujube (*Z. mauritiana* Lamk) leaves to provide protection against the writhing of experimental animals.

CONCLUSION

In conclusion, the ethyl acetate fraction of Indian jujube (*Z. mauritiana* Lamk) leaves has analgesic activity in male white mice. The fraction contains flavonoids, saponin and tannins. Further study will be conducted to isolate analgesic active compounds from the ethyl acetate fraction.

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