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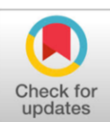
Original Study

Isolation and identification of an alkaloid compound from Bebuas leaves (Premna serratifolia) as an anti-inflammatory in white rats (Rattus norvegicus)



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Abstract: Bebuas (*Premna serratifolia*) is traditionally used by Malay people as a traditional medicine to restore health. This indicates it contains chemicals that potentially have an anti-inflammatory activity. The phytochemical screening showed that the ethanol extract contained phenolics, alkaloids, flavonoids, steroids and saponins. Meanwhile, hexane extract contained steroids only. Anti-inflammatory activity evaluation on crude extracts showed that the ethanol extract was more active than the hexane extract. Therefore, the isolation was carried out to ethanol extract with the targeted compound was an alkaloid. The extraction was conducted by using acid-base and partition techniques to give the alkaloids fraction (EaBW). EaBW was then further separated by liquid vacuum column technique using gradual polarity elution, with eluent of ethyl acetate - methanol and 5 fractions were obtained. The isolate was obtained from F.3. The isolate showed it had anti-inflammatory activity with ED_{50} = 5.45 mg/kgbw. The UV-Vis and FT-IR spectra patterns of the isolate showed similarities with ciprofloxacin as well as its physical characteristics which were hygroscopic yellowish crystal, and fluorescence property. Therefore, the isolated compound is thought to have a similar structure with ciprofloxacin.

Keyword: Bebuas; Anti-inflammatory; Alkaloid; Ciprofloxacin

INTRODUCTION

Bebuas is a shrub that belongs to the verbenaceae family [1] which is traditionally used by the Malay community as a vegetable, as well as a traditional medicine to cure various diseases such as colds, eliminating bad breath, treating intestinal worms, treat diarrhea, lung infections, rheumatism, headaches, febrifuge and can help restore the health of women after childbirth [2,3]. This indicates that bebuas contains bioactive chemical compounds.

The phytochemical screening of the ethanol extract of bebuas leaves showed that these leaves contain secondary metabolites from flavonoid, tannin, alkaloid, phenolic, and saponin groups [4]. Meanwhile, the secondary metabolite compounds that are active as anti-inflammatory are mostly derived from alkaloid, phenolic, and steroid groups [5]. This shows that bebuas leaves contain a class of compounds that are active as anti-inflammatory. The plant of the same genus, *Premna cordifolia*, has also been reported to be active as an anti-inflammatory [6]. This supports the notion that bebuas leaves also contain active compounds as anti-inflammatory. This is because chemotaxonomically, a plant that is in the same genus will contain the same chemical compounds [7]. The fact that bebuas leaves

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ethnobotanically can reduce fever and restore women's health after childbirth also reinforces the notion that bebuas leaves contain active anti-inflammatory compounds.

Inflammation is a natural response to tissue injury or infection [8]. The inflammatory process is mediated by arachidonic acid, prostaglandins, histamines, and eicosanoids. Although it is a natural process, inflammation must be addressed immediately because if it prolongs it can cause rheumatoid arthritis, atherosclerosis, fever and ischemic heart disease [9]. In Indonesia itself, the death rate due to inflammation, namely ischemic heart disease in 2017 reached 10,408 people. This makes inflammation one of the top 50 causes of death in Indonesia [10]. Therefore an effective anti-inflammatory drug is needed.

Anti-inflammatory drugs are compounds that can treat diseases caused by inflammation. Anti-inflammatory drugs are divided into steroid and non-steroidal anti-inflammatory drugs. Steroid anti-inflammatory drugs work by inhibiting the formation of arachidonic acid. Meanwhile, non-steroidal anti-inflammatory drugs work by inhibiting the formation of prostaglandins. These anti-inflammatory drugs are commercially available today. However, these anti-inflammatory drugs still have side effects in the form of stomach ulcers and muscle development disorders when consumed in a long term [11]. Therefore, the search for new anti-inflammatory drug compounds that work better is still being continued.

MATERIAL AND METHOD

Materials

The plant material used in this study was the leaves of bebuas (*Premna serratifolia*) obtained from the Tanjung Jabung Timur Regency, Jambi Province, Indonesia. The chemicals used during the research included ethanol, n-hexane, ethyl acetate, silica gel, methanol, ammonia 0.05 N, chloroform, 2N sulfuric acid, Mayer's reagent, concentrated hydrochloric acid, concentrated sulfuric acid, anhydrous acetic acid, Mg powder, 1% ferric chloride solution, 1% carrageenan, Na diclofenac, Na CMC 1%, Aquadest.

Sample Preparation

Two kilograms of bebuas fresh leaves were cleaned and washed. Furthermore, the leaves were cut into small pieces to increase their surface area, and then the leaves were dried for several days to reduce the moisture content.

Extraction of Sample

Five hundred grams of dried leaves were macerated with hexane (nonpolar) and ethanol (polar) for 3 days and carried out several times. The macerate obtained was concentrated by using a rotary evaporator to 1/10 of its original volume. The concentrated extract obtained from each solvent was combined and evaporated on a water bath to evaporate the solvent, then the dry extract was weighed.

Phytochemical Screening

Alkaloid Test. A total of 1 mL of sample was dissolved in a few drops of 2N sulfuric acid, then tested with three alkaloid reagents, namely Dragendorff's reagent, Meyer reagent and Wagner reagent. The test results are stated positive if with Dragendorff's reagent a red to orange precipitate is formed, with Meyer reagent a yellowish-white precipitate is formed and with Wagner's reagent a brown precipitate is formed [12].

Flavonoid Test. A few samples were mixed with a few drops of concentrated HCl and then Mg powder is added. A positive result is shown by the formation of foam and the change in color of the solution to orange [12].

Saponin Test. Saponins were detected by a foam test in hot water. A stable foam that can last a long time and does not disappear when 1 drop of 2N HCl is added indicates the presence of saponins [12].

Tannin Test. A number of samples were mixed with $FeCl_3$ then the mixture was homogenized. A positive reaction is shown by the formation of a greenish black color in the mixture [12].

Steroid and Triterpenoid Test. A few samples were added with anhydrous acetic acid and concentrated sulfuric acid (Liebermann-Burchard reagent). If it forms a blue or green color it indicates the presence of steroids. If a purple or orange color is formed, this indicates triterpenoids [12].

Compound Separation and Purification

Alkaloid Extraction. The concentrated ethanol extract was further separated using the acid-base method of liquid-liquid extraction. The concentrated ethanol extract was dissolved in 500 mL of water, then acidified with 2 M HCl until the pH of the solution became 3. The acidic solution was partitioned using 100 mL hexane for 5 repetitions. The partition gave 2 layers, namely the acidic water layer below and the hexane layer above. Then the two layers were separated. The acidic water layer was re-partitioned using 100 mL ethyl acetate for 5 repetitions. The partition provided 2 layers, namely the acidic water layer below and the ethyl acetate layer above. After the two layers were separated, the acidic water layer was basified by adding 2 M NH_4OH until the pH of the solution became 9. Then it was re-extracted using 100 mL of ethyl acetate for 5 repetitions. The partition provided 2 layers, namely the basic water layer below and the ethyl acetate layer above. After being separated, the ethyl acetate layer was combined and concentrated using a rotary evaporator to obtain crude alkaloid extract. The crude alkaloid extract was then weighed and further separated using VLC [13].

Thin Layer Chromatography (TLC). A 1 x 5 cm TLC plate was prepared with a lower limit of 0.5 cm and an upper limit of 0.5 cm so that the eluent distance traveled was 4 cm. Then the eluent was made by using multilevel polarity of organic solvent. The extract was spotted at the lower boundary of the plate with a capillary tube, then eluted with the mobile/eluent phase. After the developer solution moved to the upper limit, the elution process was stopped. Then the shape of the stain was noted directly under UV light of 254 and 395 nm. After the column chromatography was carried out, all fractions were subjected to a TLC test to see the stain components. The fractions having the same spots were put together and re-analyzed by TLC.

Column Chromatography. Liquid vacuum column chromatography (VLC) was performed using the stationary phase of silica gel with a ratio sample to silica gel 1:20. The extract was impregnated with silica gel, then added to the column which already contained the stationary phase. The mobile phase used was ethyl acetate - methanol with various ratios. The fraction obtained was collected in vials, the eluate was collected based on each band obtained and then evaporated. The fractions from the column chromatography were monitored with TLC. Eluates that having identical stain patterns were combined based on the R_f value on the chromatogram. The fraction which had one spot was then tested using 3 different eluents to see the stain pattern. Isolates were purified by recrystallization using n-hexane, ethyl acetate and ethanol as solvents. Furthermore, phytochemical screening, characterization, and anti-inflammatory activity evaluation were performed.

Anti-Inflammatory Activity Evaluation

Preparation of Extract Suspensions. The ethanol and hexane extracts were suspended with Na CMC in distilled water. Na CMC was sprinkled over hot water in the mortar using water as much as 20 times the weight of Na CMC and left for 15 minutes until the Na CMC developed. Then the extract was inserted gradually into the mortar while being crushed homogeneously and distilled water was added until the total volume was 10 mL [14].

Anti-Inflammatory Activity Evaluation. Anti-inflammatory activity evaluation was carried out on positive control (formulated drug of Na diclofenac), negative control (Na CMC), ethanol extract, hexane extract, VLC fraction, and the isolate.

Na diclofenac was used for the positive control as it is a medication for inflammation that has been used commercially. Before the testing, the rats were fasted for 18 hours (not eating but still given a drink). Each group consisted of 4 rats. The categories of each group, namely, C0 = negative control (-) for 1% Na CMC; C1 = control (+) for 10 mg Na diclofenac; C2 = hexane extract 250 mg/kgbw; C3 = hexane extract 500 mg/kgbw; C4 = hexane extract 1000 mg/kgbw; C5 = ethanol extract 250 mg/kgbw; C6 = ethanol extract 500 mg/kgbw; and C7 = ethanol extract 1000 mg/kgbw. Anti-inflammatory evaluation was also performed on the five fractions of VLC with code F.1; F.2; F.3; F.4; and F.5 and the test dose of each was 10 mg/kgbw. Meanwhile, the anti-inflammatory activity of the isolate was evaluated at doses of 3, 5, and 10 mg/kgbw.

At the time of testing each animal was weighed and marked on the tail. After that, the rat's left paw was subjected into a pletysmometer, then the volume was recorded as the initial volume (V_0), namely the volume of the paw before being given the test substance. Each rat's paw was injected subplantarly with 0.1 ml carrageenan 1% solution. After thirty minutes, measurements were taken by dipping the rats' paw into a plethysmometer tube and each rat was given a suspension of the test substance orally according to its group. The changes in fluid volume that occurred were recorded as the volume of the rats' paw at each observation time (V_t). Measurements were carried out every 60 minutes for 300 minutes [15]. After the measurement, the volume of rat paw edema and AUC (Area Under the Curve) was calculated from the average edema against time curve and the percent of inflammation inhibition [16].

Calculation of volume of rat paw edema, AUC from the time-average edema curve, and the percent anti-inflammatory effect [16] are as follows:

$$Vu = V_t - V_0$$

Where:

V_u : volume of rat paw edema each time t

V_t : volume of rat paw after 1% carrageenan induction at time t

V_0 : Initial volume of rat paw before induced by carrageenan 1%

$$AUC_{t_{n-1}}^{t_n} = \frac{Vu_{n-1} + Vu_n}{2}(t_n - t_{n-1})$$

Where:

V_{un-1} : volume average edema at t_{n-1}

V_{un} : volume average edema at t_n

$$\%\ the\ percentage\ of\ inflammation = \frac{AUC_k - AUC_p}{AUC_k} \times 100\%$$

Where:

AUC_k : AUC of time-to-mean edema volume curve for negative control

AUC_p : AUC of the edema volume versus time curve for the treatment group in each individual

The data were then tested with One-Way ANOVA and Post Hoc LSD test with a 95% confidence level by using SPSS 25 software [17].

ED_{50} Determination.

The percentage of inflammation inhibition of each dose variation was transformed into a probit (probability unit). Meanwhile, the dose value was converted into the logarithmic form. The probit of inhibition was then plotted against the logarithm value of the dose. The linear equation of the graph was determined using the Microsoft Excel 2010 software. From the linear equation, the ED_{50} value was calculated by entering 5 as y and x as the calculated variable. The value of x obtained was then converted into its anti-algorithmic value. The antilogarithmic value of x obtained is the ED_{50} value [18].

RESULTS AND DISCUSSION

The extractIon of bebuas leaves resulted in 5.12 grams of dry extract, with a yield of 1.024%. Dried hexane extract of bebuas leaves had a sticky and tough character like sap and had a yellowish green color. Meanwhile, the ethanol extract gave a dry extract of 30.31 grams with a yield of 6.062%. The dry ethanol extract had a resin-like sticky character and was blackish green color. The extraction results showed that the percent yield of ethanol extract was higher than that of hexane extract. This can happen because ethanol can dissolve almost all organic compounds from various groups of compounds, both polar and nonpolar. Meanwhile, hexane can only dissolve organic compounds which tend to be nonpolar [19].

Phytochemical screening was carried out on both dry extracts obtained. The results of phytochemical screening can be seen in table 1.

Table 1. Results of Phytochemical Screening of Bebuas leaf extract

	Hexane Extract	Ethanol Extract
Alkaloids		
Meyer	-	-
Dragendorff	-	+
Flavonoids	-	+
Phenolics/Tannins	-	+
Saponins	-	+
Steroids	+	+
Terpenoids	-	-

Note: - : negative, + : positive

The results of the phytochemical screening in this study gave the same results as the results obtained by Oktaviani et al. [20]. Phytochemical screening showed that the compounds extracted in ethanol solvent were more diverse than those extracted in hexane solvents, because hexane tends to only extract nonpolar compound groups such as steroids.

Plants belong to the *premna* genus have several characteristic secondary metabolites, which consist of diterpenoids, iridoid glycosides, and flavonoids as the most common secondary metabolites, followed by sesquiterpenes, lignans, phenylethanoids, megastigmanes, glyceroglycolipids, and ceramides [21]. Based on the phytochemical screening, the ethanol extract positively contained phenolic and flavonoid compounds, according to the typical compound groups contained in the genus of *premna*. Phytochemical screening also showed that the extract contained classes of compounds that have anti-inflammatory activity, including alkaloids, flavonoids, and steroids. This data provides scientific evidence and supports the fact that bebuas leaves can be used as anti-inflammatory drugs, as is often used by the Malay community to cure various diseases associated with inflammation.

Based on the results of the anti-inflammatory evaluation (Fig. 4), the ethanol extract had a lower ED_{50} value than the hexane extract. Therefore, the extract that was continued to the isolation stage was ethanol extract. Since most of the active anti-inflammatory NSAID compounds come from the alkaloid group [22], and in accordance with the results of phytochemical screening that ethanol extract contained alkaloids, so the targeted compound isolated was an alkaloid. Therefore, a specific method to isolate alkaloids was used. The concentrated ethanol extract was dissolved in distilled water and was acidified by adding 4 M HCl dropwise until the pH became 3. Before acidification, the initial pH of the extract solution was 4. This indicated that the ethanol extract of bebuas leaves was acidic, which can be caused by the presence of acidic phenolic compounds. Acidification using HCl to pH 3 aimed to convert alkaloids into alkaloid salt, so that their solubility in water increased, and their solubility in organic solvents decreased.

From the results of this acidification, a reddish black extract solution was obtained. Meanwhile, there was an insoluble fraction in acidic water, which was a sticky substance such as resin. The resin was then separated from the acid aqueous solution. The acid water extract was then partitioned using a hexane solvent. This partition aimed to separate polar compounds that are soluble in acidic water from non-polar compounds such as steroids and terpenoids. From the results of this partition, a green hexane layer and a reddish black acidic water extract were obtained (Fig. 1 a).

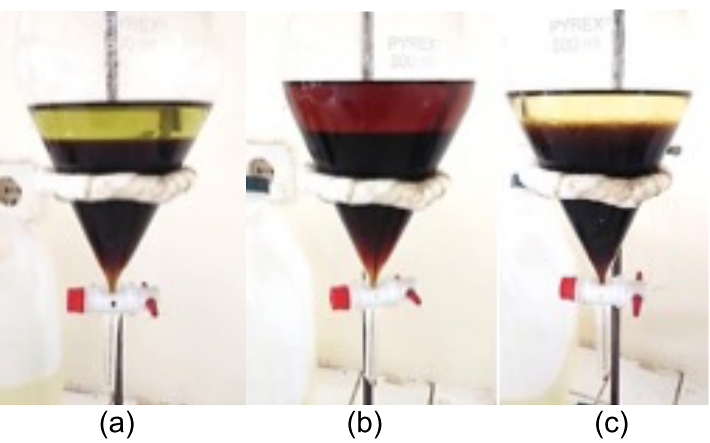


Fig. 1. Partition Results (a) acidic water extract with hexane (b) acidic water extract with ethyl acetate (c) alkaline water extract with ethyl acetate

After partitioning with hexane, then the acidic water extract was partitioned using ethyl acetate. Ethyl acetate was used to attract compounds that are semipolar and tend to be acidic, such as flavonoids, phenolics, and tannins. This can happen because in acidic water extracts these compounds tend to be in their neutral (uncharged) molecular form, so they are more soluble in semipolar organic solvents such as ethyl acetate. Meanwhile, alkaline compounds, such as alkaloids, are in the ionic form (charged) so that they are more soluble in water than organic solvents. This partition gave a layer of ethyl acetate (above) which was reddish brown in color and acidic water which was reddish black (Fig. 1 b).

After partitioning with ethyl acetate, the acidic water extract was basified with a 4 M ammonia solution until the pH was 9. The purpose of this basification was so that the alkaloids that were previously present in ionic form change back into their free, uncharged molecular form. After obtaining a pH of 9, then the basic water extract was partitioned using ethyl acetate. In a basic state, alkaloids return to their neutral molecular form so that their solubility in semipolar organic solvents such as ethyl acetate increases. From the results of this partition, a yellow ethyl acetate layer (above) and a reddish black layer of basic water were obtained (Fig. 1 c). The partition was carried out several times until the ethyl acetate appeared colorless. The ethyl acetate fraction of basic water (EaBW) is then concentrated. From the results of this concentration, 3.513 g of concentrated EaBW was obtained which was orange in color.

The hexane fraction of acidic water (HAW), ethyl acetate fraction of acidic water (EaAW), and EaBW were then screened for phytochemicals. The results of the phytochemical screening of the three fractions can be seen in Table 2.

Table 2. Results of Fractions Phytochemical Screening

	HAW	EaAW	EaBW
Alkaloids			
Dragendorff	-	-	+
Flavonoids	-	+	-
Phenolics/Tannins	-	+	+
Saponins	-	-	-
Steroids	+	-	-
Terpenoids	-	-	-

Note: HAW=hexane fraction of acidic water; EaAW=ethyl acetate fraction of acidic water; EaBW=ethyl acetate fraction of basic water

The screening results of the three fractions showed that the HAW extract was only positive for steroids. This is because steroid compounds tend to dissolve in nonpolar solvents such as hexane. Steroid compounds also tend not to form ions in either acidic or alkaline conditions. Therefore, steroids remain in a neutral molecular state which is more soluble in nonpolar organic solvents. EaAW was positive for flavonoids and phenolic. This is because in acidic conditions, the flavonoid and phenolic molecules are not charged. The aromatic –OH groups present in the flavonoid and phenolic molecules give the compound semipolar properties. Therefore, flavonoids and phenolics have large solubility in semipolar solvents such as ethyl acetate. Meanwhile, the EaBW was positive for alkaloids and phenolics as was predicted. Yet, phenolics were extracted both in EaAW and EaBW. This was possible because there were compounds that contain both phenolic and alkaloids functional groups.

Isolation was continued by using VLC to separate compounds in EaBW. Gradient elution was carried out in the VLC process using 100% ethyl acetate to 100% methanol as the eluent. The eluent was used in accordance with the results of the TLC test, which gave the result that the compounds in EaBW could only be eluted using the two eluents. The elution process was monitored using a 395 nm UV lamp (Fig. 2). This was performed so that each compound can be separated according to the band/fluorescence of the compound observed.

Fig. 2. Vacuum liquid chromatography process

Gradient elution was used to increase the resolution of complex mixtures especially if the sample has a wide polarity range. This can be seen when VLC was carried out using ethyl acetate eluent, the second band cannot be eluted and was retained on silica. After the eluent polarity was increased by the addition of methanol, the bands can be eluted. From the VLC results, eleven vials were obtained. The solvent from each vial was evaporated and a TLC test was carried out with 100% ethyl acetate as the eluent. The vials that had the same stain pattern were combined. From the TLC test, it was obtained five fractions namely F.1; F.2;

F.3; F.4; and F.5 which were coded according to the VLC elution time sequence. The fraction mass obtained for F.1; F.2; F.3; F.4; and F.5, respectively were 35.35 mg; 164.85 mg; 18.3 mg; 859.95 mg; and 137.48 mg.

TLC test using ethyl acetate eluent showed that F.3 gave a single stain with R_f 0.3 (Fig. 3 a). The TLC test on F.3 was then followed by eluent ethyl acetate: methanol 1: 1 and ethyl acetate: methanol 1: 2, which gave a single stain with R_f values of 0.61 and 0.81, respectively. This indicated that F.3 was a single compound, or at least there was one dominant compound in it. F.3 was then characterized using FT-IR and UV-Vis to determine the functional groups and basic skeleton of this compound. Furthermore, F.3 and other fractions previously obtained were subjected to a phytochemical screening.

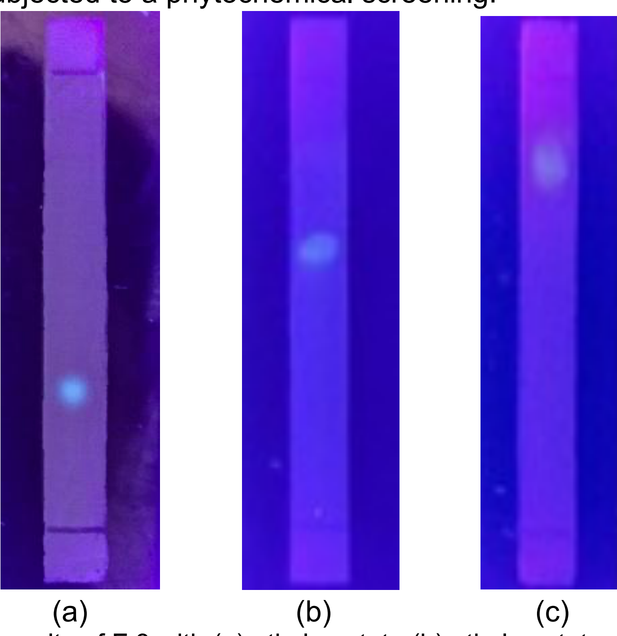


Fig. 3. TLC test results of F.3 with (a) ethyl acetate (b) ethyl acetate: methanol 1: 1 (c) ethyl acetate: methanol 1: 2 deluent

The results of the phytochemical screening of the five fractions are presented in Table 3. Phytochemical screening showed that all fractions were positively contained alkaloids. Meanwhile, in addition to alkaloids, F.1 was also positive to steroids and F.2 and F.3 were positive to phenolics. This might happen because there was a possibility that F.1; F.2; and F.4 was a mixture of compounds from different groups or the compounds in them had more than one functional group, thus giving positive results to more than two functional groups.

Table 3. Phytochemical Fraction Screening

	F.1	F.2	F.3	F.4	F.5
Alkaloids					
Dragendorff	+	+	+	+	+
Flavonoids	-	-	-	-	-
Phenolics/Tannins	-	+	-	+	-
Saponins	-	-	-	-	-
Steroids	+	-	-	-	-
Terpenoids	-	-	-	-	-

Note: F.1 to F.5 are the chromatography column factions of EaBW. The numbers represent the order in which the fractions are eluted from the chromatography column.

The five fractions were tested for their anti-inflammatory activity to determine which fraction had the best anti-inflammatory activity. The dose used to evaluate anti-inflammatory activity was 10 mg/kgbw, as the positive control dose used. The test results of the five fractions can be seen in Fig. 6.

From the test results, it can be seen that F.5 had the highest activity as an anti-inflammatory. However, based on the TLC test of F.5 using 1:1 methanol: water eluent, it consisted of several spots. This indicated that F.5 was still a mixture of compounds. Meanwhile, the TLC test on F.3 with various solvent ratios showed that F.3 consistently gave one spot, indicating that F.3 was a single compound. Therefore, characterization was carried out to F.3.

The anti-inflammatory activity evaluation was carried out on crude extract of hexane and ethanol from the leaves of bebuas. Tests were carried out on male albino rats (*Rattus norvegicus*). These rats were used as test animals because their genome is similar to the human genome (90%) and many human condition symptoms can be replicated on them [23]. Before being tested, the rats were not fed for 18 hours in order to avoid the influence of other substances on the test. The results of the anti-inflammatory evaluation are presented on the graph in Fig. 4.

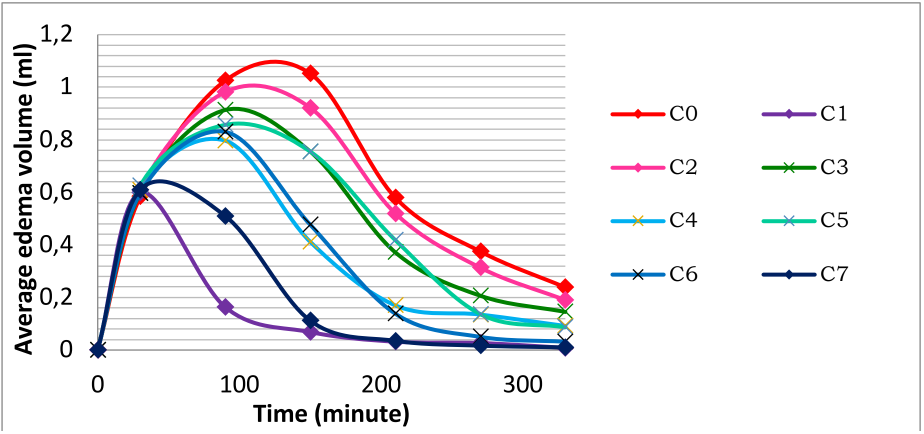


Fig. 4. Graph of edema volume against time

Note:

C0 = negative control (1% Na CMC); C1 = positive control (10 mg Na diclofenac); C2 = hexane extract 250 mg/kgbw; C3 = hexane extract 500 mg/kgbw; C4 = hexane extract 1000 mg/kgbw; C5 = ethanol extract 250 mg/kgbw; C6 = ethanol extract 500 mg/kgbw; and C7 = ethanol extract 1000 mg/kgbw.

The data in Fig. 4 shows that ethanol extract at 250, 500, and 1000 mg/kgbw doses could reduce swelling of the rats' paws at 60 minutes after induction. Hexane extract had a significant effect on reducing swelling at 60 minutes only at a dose of 1000 mg/kgbw. Meanwhile, hexane extracts at doses of 250 and 500 mg/kgbw were only able to significantly reduce swelling in 120 minutes after induction.

The average of percentage of inhibition of each dose was calculated based on the data in Fig. 4. The mean percentage of inhibition obtained for C1 was 79.30%. The average percentage of inhibition of hexane extracts, for C2, C3, and C4 were 8.37%, 22.90%, and 43.83%, respectively. Meanwhile, the average percent inhibition of ethanol extracts, for C5, C6, and C7 respectively were 25.35%, 45.42% and 68.35%. This showed that both hexane extract and ethanol extract have strong anti-inflammatory activity (percent inhibition is above 40%). However, the anti-inflammatory activity of ethanol extract was stronger than that of hexane extract. This can be seen from the percent inhibition of ethanol extract which was above 40% at a dose of 500 mg/kgbw (C6). Meanwhile, the percentage of inhibition above 40% was only achieved by hexane extract at a dose of 1000 mg/kgbw (C4).

The one-way ANOVA statistical test showed that there were significant differences between the groups of rats tested. Post Hoc LSD test shows that all doses statistically had significant differences in inhibition compared to the negative control. Meanwhile, it was also seen that there was no significant difference between the percent of inhibition of C4 and C6, which means that the two doses

had almost the same percentage of inflammation inhibition. However, for the percent inhibition value, the ethanol extract requires a lower dose. This showed that the ethanol extract was more active than the hexane extract. Compared to the positive control, all the doses given had a significant difference in the percentage inhibition value. This showed that the dose given had not been able to match the percent inhibition of C1. All doses also statistically had a significant difference with the C0 which meant that all extract showed anti-inflammatory activity.

Table 4. LSD Post Hoc Test Results on Bebuas Leaf Extracts

Sample	% Inhibition
C1	79.30 a
C7	68.35 b
C6	45.42 c
C4	43.83 c
C5	25.35 d
C3	22.90 e
C2	8.37 f
C0	0.00 g

Note:

The values followed by different letter notations indicate that there is a statistically significant difference.

The values followed by the same letter notation indicate that there is no statistically significant difference.

The percentage of inhibition of each dose was transformed into a probit (probability unit) form and plotted into a graph against the logarithmic value of the dose (Fig. *5*). From the graph, a linear equation of the probit of inhibition against the logarithm of the dose was obtained to calculate the ED_{50}. The ED_{50} for hexane and ethanol extract were 1,162.30 mg/kgbw and 571.56 mg/kgbw, respectively. This showed that the ethanol extract had a higher anti-inflammatory activity than the hexane extract because it had a lower ED_{50} value.

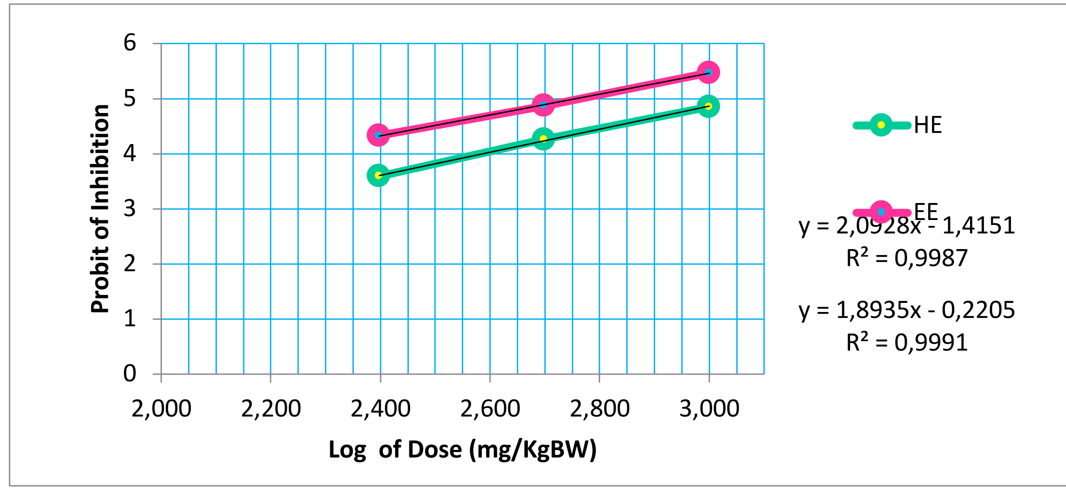


Fig. 5. Graph of Probit inhibition vs log of extract dose

Where: HE = Hexane Extract; EE = Ethanol Extract

The active anti-inflammatory compounds found in hexane extract could be steroid compounds which was detected in previous phytochemical screening. Meanwhile, the anti-inflammatory active compounds in ethanol extract could come from the phenolic or alkaloid groups [5].

Based on the anti-inflammatory evaluation, ethanol extract had a higher percent of inhibition than hexane extract for the same dose. Therefore, the extract that was continued to the isolation stage was the ethanol extract. Since most of the active NSAID compounds come from the alkaloid group [22], and in accordance with

the results of phytochemical screening that ethanol extract contained alkaloids, the targeted compound to be isolated was an alkaloid. Therefore, special extraction and isolation methods for alkaloids were used. From the method, the EaBW was obtained and it was positive for alkaloids.

Each fraction resulting from VLC of EaBW was evaluated for anti-inflammatory to determine which fraction has the best activity as anti-inflammatory. The dosage used for all fractions was 10 mg/kgbw, as the dose used for positive control. The results of the anti-inflammatory evaluation for each fraction can be seen in Fig. 6.

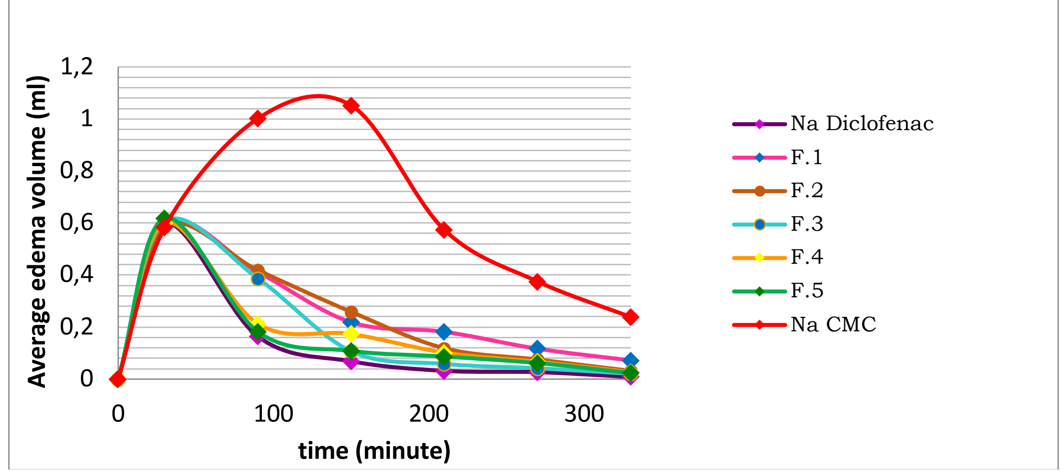


Fig. 6. Fraction's anti-inflammatory evaluation curve

From the results of the anti-inflammatory evaluation, the percent of inhibition for F.1; F.2; F.3; F.4; and F.5, respectively were 60.26%, 62.78%, 70.29%, 71.43% and 74.32%. It showed that all fractions had strong anti-inflammatory activity (% inhibition > 40%).

The one-way ANOVA statistical test showed that there were significant differences between the groups of rats tested with the five fractions. This can be seen from the calculated F value which was greater than the $F_{0.05\ (6.35)}$ table (889.408 > 2,372) and the p value was smaller than the α value (0,000 < 0.05).

Table 5. Post Hoc LSD test results for each EaBW fraction from VLC

Sample	% Inhibition
C1	79.30 a
F.5	74.54 b
F.4	71.67 c
F.3	70.53 c
F.2	63.09 d
F.1	60.59 d
C1	0.00 e

Note:

The values followed by different letter notations indicate that there is a statistically significant difference.

The values followed by the same letter notation indicate that there is no statistically significant difference.

The post hoc LSD test data in Table 5 shows that all fractions statistically had a significant difference in percentage of inhibition compared with negative controls. This shows that all fractions were active as anti-inflammatory agents. There was also no significant difference between the percentage of inhibition of F.1 and F.2, as well as the percentage inhibition of F.3 and F.4. This indicates that F.1 and F.2 as well as F.3 and F.4 have the same inflammation inhibition strength. This similarity of inhibitory strength occurred because there was the possibility that

the same chemical compound spread in F.1 and F.2, as well as in F.3 and F.4, which is possible because the fractions were in close order according to their separation in VLC.

Based on the TLC test, F.3 gave one single stain. This showed that F.3 was a pure compound (isolate). Therefore, the anti-inflammatory activity evaluation with various doses was carried out on F.3, hereinafter referred to as isolates. The results of the isolate anti-inflammatory evaluation is depicted in Fig. 7.

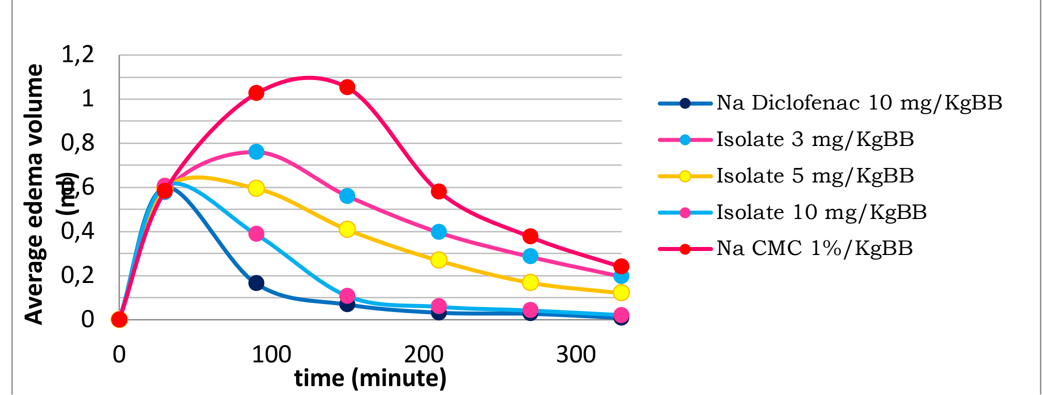


Fig. 7. The isolate anti-inflammatory evaluation curve

Based on the anti-inflammatory evaluation, the percentage of inhibition of isolates for doses of 3, 5, and 10 mg/kgbw, respectively were 29.48%, 45.88% and 70.53%. The graph of the probit of inhibition against the logarithm of dose can be seen in Fig. 8. Based on the calculation of the linear equation in Fig. 8, the ED_{50} value of the isolate was 5.45 mg/kgbw.

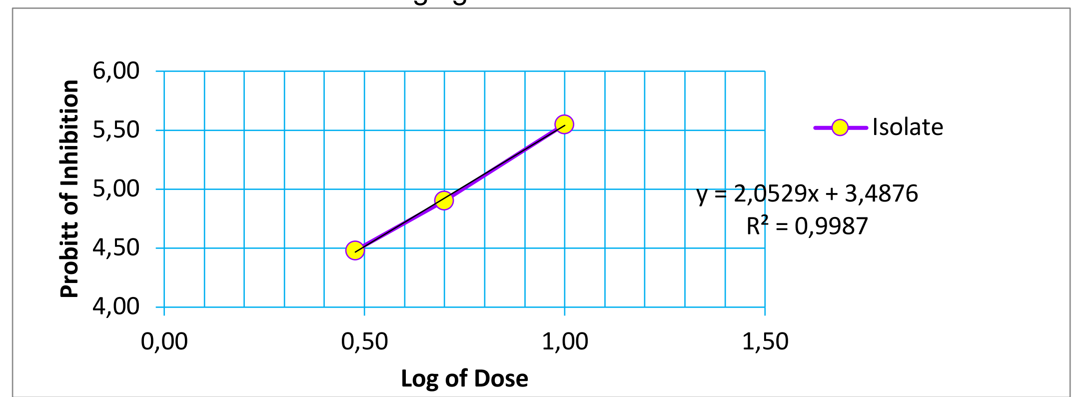


Fig. 8. Graph of probit vs log dose of the isolate

One-way ANOVA statistical test showed that there was a significant difference between the groups of rats tested and the isolates. This can be seen from the calculated F value which was greater than the $F_{0.05(4,21)}$ table (1847.997 > 2.840) and the p value was smaller than the α value (0.000 < 0.05). Based on the LSD post hoc test data presented in Table 6, it can be seen that all isolate doses were statistically significantly different in inhibition compared to the negative control.

Table 6. LSD Post Hoc Test Results on the Isolate

Sample	% Inhibition
C1	79.30 a
Isolate 10 mg/kgbw	70.53 b
Isolate 5 mg/kgbw	45.88 c
Isolate 3 mg/kgbw	2948 d
C0	0.00 e

Note:

The values followed by different letter notations indicate that there is a statistically significant difference.

The values followed by the same letter notation indicate that there is no statistically significant difference

The isolate was characterized using FT-IR and UV-Vis instruments. UV-Vis characterization was carried out to determine the basic skeleton of the compounds from these isolates. The UV-Vis spectrum (Fig. 9) showed that the isolate gave two absorption peaks at λ = 281 nm and λ = 307 nm. The absorption at 281 nm indicates the presence of a conjugated diene/double system in the structure. Meanwhile, the absorption at 307 indicates the presence of an aromatic system with certain substituents [24].

The UV-Vis spectrum of the isolates has a similar pattern to the spectra of compounds containing the quinolone skeleton. The two UV absorption bands of the isolate approach the typical quinolone absorption area, specifically the presence of two peaks at λ around 269 nm and 314 nm [25]. Based on the literature, the UV spectrum of the isolate has a similar pattern to the UV spectrum of ciprofloxacin, and both absorption bands are close to each other. This shows that isolate and ciprofloxacin have the same basic skeleton. However, the absorption peak of the isolates did not appear to be too intense. This could be due to the relatively low concentration of the isolate when characterized by using UV-Vis.

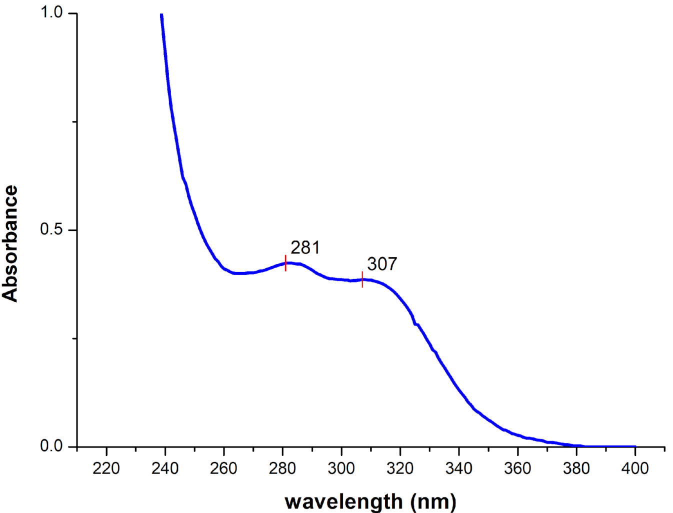


Fig. 9. UV-Vis spectrum of the isolates, λ = 281 nm and 307 nm

FT-IR characterization was carried out to determine the functional groups contained in the isolate (Fig. 10). Based on the comparison with literature [26,27], the FT-IR spectrum pattern of the isolates is similar to the IR spectrum of ciprofloxacin. This strengthens the notion that the isolate is a compound that has a structure similar with ciprofloxacin.

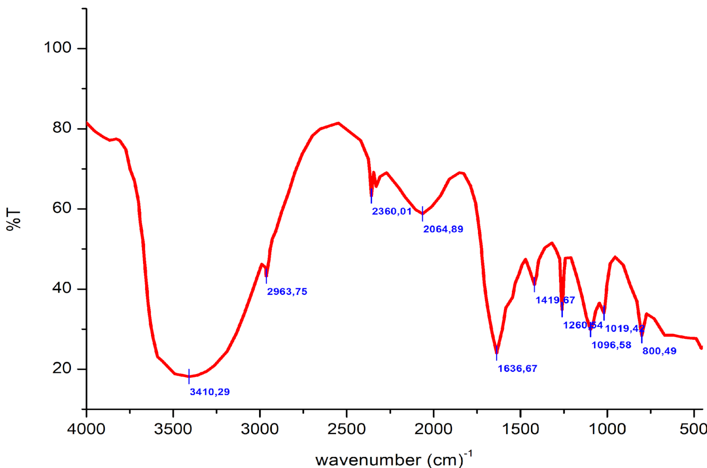


Fig. 10. FT-IR spectrum of the isolate

Ciprofloxacin is an alkaloid compound derived from quinolone which has been widely used as an antibiotic. The quinolone skeleton in the isolate can be seen clearly based on the UV-Vis and FT-IR spectra. In FT-IR, the presence of absorption at wave number 1260.54 indicated the presence of aromatic C-N bonds, which is a characteristic of the basic quinolone skeleton.

The interpretation of functional groups based on the FT-IR spectrum is presented in Table 7. An alkaloid compound that has the same FT-IR spectrum pattern with this compound is ciprofloxacin (Fig. 11). The functional groups presented in the FT-IR spectrum of the isolate corresponded to the characteristic of functional groups presented in ciprofloxacin.

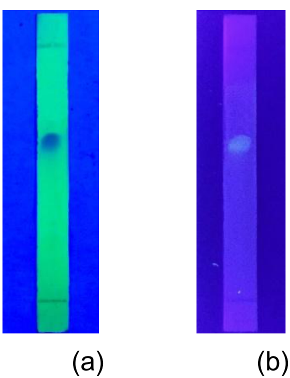
Fig. 11. Structure of Ciprofloxacin

The assumption that the isolate was a compound with characters like ciprofloxacin is also supported by the compatibility of their physical forms. Ciprofloxacin is known to be in the form of yellowish crystals which are hygroscopic. This is in accordance with the physical form of the isolate in the form of yellowish crystals when it was freshly dried from the solvent, but after being exposed for a long time by air, the isolate changed into a liquid like oil due to absorbing water vapor from the air.

Table 7. Interpretation and Comparison of IR Spectra

Interpretation	Adsorption (cm^{-1})		
	Isolate	Ciprofloxacin [26]	Ciprofloxacin [27]
O-H	3410,29	3342.64	3308.70
C-H Alkane/aromatic	2963,75	-	2974.33
C=N, C=C	1636,67	1635.64	1577.8
O-H Bending	1419,67	-	1413.87
C-N aromatic	1260,54	1204.56	1288.49
C-H aromatic	800,49	-	-

The unique characteristic that was also observed from the isolate was that they could fluoresce under a 395 nm UV lamp, which emitted a blue light as can be seen in Fig.12.



(a) (b)

Fig. 12. TLC plate resulted from the isolate elution when observed under a UV light (a) 254 nm (b) 395 nm

Ciprofloxacin has also been reported to provide fluorescence [19,28]. The fluorescence of the ciprofloxacin compound occurs due to the quinolone skeleton in its structure. Ciprofloxacin has a maximum absorption at a wavelength of 280 nm and 331 nm [29]. Meanwhile, the wavelength emitted by ciprofloxacin when it glows is 445 nm. The wavelength corresponding to the range of visible light blue is in the range of 435-500 nm [30]. This shows that the nature of the isolate which could glow blue under a UV lamp of 395 nm further strengthens the notion that the isolate was a compound with a basic skeleton similar to ciprofloxacin.

CONCLUSION

The study showed that bebuas leaves contain compounds that are active as anti-inflammatory agents. The ED_{50} values of hexane extract, ethanol extract, and F.3 (isolate), as anti-inflammatory agents were 1,162.30 mg/kgbw, 571.56 mg/kgbw, and 5.45 mg/kgbw, respectively. Compounds that are active as anti-inflammatory agents in bebuas leaves are best extracted with ethanol. The anti-inflammatory active compound that was isolated from the ethanolic extract was an alkaloid. The isolate had similar characters with ciprofloxacin, namely yellow color, hygroscopic crystal, and containing quinolone scaffold as interpretated from UV-Vis and FT-IR spectra.

Further characterization of the isolate using LC-MS/MS and NMR instrument to ensure the purity as well as to determine the exact structure of the isolate might be needed for future research.

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all authors have equal responsibility in the research and preparation of the manuscript.

FUNDING INFORMATION

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DATA AVAILABILITY STATEMENT

The utilized data to contribute to this investigation are available from the corresponding author on reasonable request.

DISCLOSURE STATEMENT

The views and opinions expressed in this article are those of the authors and do not necessarily reflect the official policy or position of any affiliated agency of the authors. The data is the result of the author's research and has never been published in other journals.

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