Anti-Inflammatory Activity of an Indole Alkaloid Isolated from Bebuas (*Premna serratifolia*)

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ABSTRACT

*Premna serratifolia* known as bebuas has long been used by Malay community for both food and traditional medicine. The most notable advantage of this plant is to heal woman after childbirth and to raise the notion that contains anti-inflammatory bioactive compounds. The purpose of this research was to study the anti-inflammatory activity of *Premna serratifolia* leaves ethanolic extract and indole alkaloid isolated. This study was conducted by extraction of *P. serratifolia* leaves using hexane and ethanol and isolation of indole alkaloid isolated. The extract and isolate were tested anti-inflammatory activity on rat. In this study, the hexane extract of *P. serratifolia* leaves was only contained steroids. Meanwhile the ethanol extract contained various secondary metabolites such as phenolics, alkaloids, flavonoids, steroids and saponins. The ethanol extract was further separated since the targeted compound isolate was an alkaloid. The isolate obtained was a yellow crystal which transformed to yellow oil after being exposed to air. The anti-inflammatory evaluation of the compound gave a result with a strong activity with ED50 = 4.06 mg/kgBW. Characterization by UV-Vis, FT-IR and compared with references showed that the isolate had similarities with bufotenine. It revealed that the isolate shared the same basic skeleton with the bufotenine, especially the indole skeleton. Its physical form is the same with bufotenine. These evidences strengthened the assumption that the isolate was an indole alkaloid.

Keywords: *Premna serratifolia*, anti-inflammatory, indole, alkaloid, bufotenine

INTRODUCTION

*Premna serratifolia* (bebuas) is a medicinal plant, widely distributed in the tropical and subtropical regions and commonly used in traditional medicine. *P. serratifolia* is a scandent, erect shrub or small tree, more or less thorny on the trunk and large branches. Leaves are opposite or whorled and entire or serrate. *P. serratifolia* have tremendous medicinal value and already studied. Previous pharmacological studies include reports of diseases such as colds, treating intestinal worms, eliminating bad breath, lung infections, diarrhea, rheumatism, headaches, febrifuge, hypolipidemic, anti-inflammatory, antidiabetic, CNS depressant, antitumor activity and can help to restore women’s health after childbirth [1], [2]. Previous phytochemical investigations have revealed the presence of several glycoside including iridoid glycosides and phenylethanoids like premethanoside A and B, some xanthones, steroids and saponins, flavonoids, triterpenoids and diterpenoids (including premnones A and C) in *Premna serratifolia* leaves. Investigations on *Premna serratifolia*...
flower buds have revealed the presence of volatile oil comprising mainly 1-octen-3-ol, (Z)-n-hexanol, 2-phenyl ethyl alcohol, (E,Z)-2,4-nonadienal and linalool [3], [4].

The genus *Premna* is mainly characterized by its compounds. One study has identified phenolics. Phenolic acids were reported in *P. fulva* and *P. hainanensis* and several aldehydes were isolated from *P. integrifolia* and *P. tomentosa*. One indole carboxylic acid was also isolated from *P. microphylla*. Some alkaloids were only identified in *P. integrifolia*. Lignans, a phenylpropanoid derivatives, were identified within 6 species of *Premna* and commonly found as furan lignans and furofuran lignans in the genus *Premna*. Meanwhile, the occurrence of flavonoids was reported from 13 species. Most of the flavonoids were flavonols and flavones, although quite a number were flavanones, isoflavones and one flavan-3-ol. A few flavonoid glycosides were also reported. In addition, two xanthones were isolated from *P microphylla* and four chalcones were reported in *P. yunnanensis* W.W.Sm [3], [4].

*Premna* species are known to have high-antioxidant capacity, such as *P. cordifolia* Roxb., *P. esculenta* Roxb., *P. integrifolia*, *P. microphylla* and *P. serratifolia*. The wide distribution of flavonoids and phenolics within this genus seems to contribute to this activity. Secondary metabolites such as flavonoids, xanthones, chalcone and other phenolic compounds with high-hydroxyl group substitution are hypothetically contributing to the high antioxidant activity of the plant. The activity of a compound as an antioxidant is related to being anti-inflammatory [3], [4].

Inflammatory reaction occurs due to pathogen invasion into the body or other types of body injury which can cause injury to the tissues or cells as well. At macroscopic level, inflammation is indicated by reddened, swollen, hot, pain, and loss of function of the inflamed area. The loss of function is usually referring to simple loss of mobility in a joint due to pain or edema, or the replacement of functional tissue by the scar tissue. This inflammatory event usually will be followed by the release of mediators from the cells or plasma which modify and regulate the immune response (innate/nonspecific and specific immunological response). Hence, several studies have been conducted to evaluate the anti-inflammatory effect of the extracts of *Premna* species [3], [4]. In addition, an extensive study identified several compounds from *P. obtusifolia* roots that exhibited potent anti-inflammation activity. Of 20 isolated compounds, four diterpenes showed potent *in vitro* lipopolysaccharide (LPS) induced NO inhibitor (IC50 6.1, 7.8, 1.7 and 6.2 μM) that were comparable to positive control, caffeic acid phenylester (IC50 5.6 μM) [3], [4]. Meanwhile, megastigmane only showed weak anti-inflammatory activity. Further structure-activity relationship analysis suggested that the presence of a hydroxyl group in an ortho-naphtoquinone skeleton provided stronger anti-inflammation activity. It was postulated that these active compounds might be responsible for the strong NO inhibitor activity of the hexane and dichloromethane extracts (IC50 4.3 and 6.1 μg/mL, respectively). Another species, *P. integrifolia*, also showed significant *in vivo* anti-inflammatory activity in both acute and chronic inflammation models; further *in vitro* study suggested inhibition of prostaglandin synthase and stabilization of plasma erythrocyte membrane might play role in the *in vivo* activity [3], [4].

The secondary metabolite compounds that are active as anti-inflammatory such as alkaloid, phenolic, and steroid groups [5] have been confirmed to present in ethanol extract of *P. serratifolia* [6]. This idea is also supported by some scientific reports that some plants within the same genus showed an anti-inflammatory activity [7]. Since chemotaxonomically, a plant in the same genus, contains the same chemical compounds [8], it can become a strong evidence that *P. serratifolia* has the same activity. Besides, from the reports of plant with the same genus, ethnobotanically, the notion emphasizes that bebauas leaves contain active anti-
inflammatory compounds supported by the fact that they can reduce fever and restore women's health after childbirth.

Naturally, the body response to injury or infection will cause an inflammation [9]. Although it is a natural process, inflammation should be addressed immediately since a prolonged inflammation can cause various harmful conditions such as atherosclerosis, fever, rheumatoid arthritis, and ischemic heart disease [10]. In Indonesia, the ischemic heart disease caused by inflammation is one of the top 50 causes of death. The death was reached by 10,408 people in 2017 [11]. Therefore, an effective anti-inflammatory drug is needed.

Anti-inflammatory drugs are commercially available today. They are compounds to inhibit and treat symptoms caused by inflammation. They are generally 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. However, these anti-inflammatory drugs still cause some side effects if consumed for a long term such as stomach ulcers and muscle development disorders [12]. Therefore, the search for better anti-inflammatory compounds is being continued.

**EXPERIMENT**

**Plant materials**

The bebasus (*P. serratifolia*) leaves were collected from Kuala Dendang village, Dendang district, Tanjung Jabung Timur Regency, Jambi Province, Indonesia. The specimen was taxonomically identified and confirmed by a taxonomist from the Research Center for Plant Conservation and Botanic Gardens, Indonesian Institute of Sciences. A voucher specimen (No. B-338/IPH.3/KS/II/2020) was deposited.

**Chemicals and reagents**

The chemicals used were ethyl acetate, ethanol, n-hexane, silica gel, methanol, chloroform, ammonia, sulphuric acid, Mayer's reagent, hydrochloric acid, anhydrous acetic acid, Mg, ferric chloride solution, carrageenan, Na. diclofenac and Na. CMC were purchased from Sigma-Aldrich, Jakarta, Indonesia.

**Extraction**

Dried *P. serratifolia* leaves (500 g) were extracted three times with 2 L of hexane, (24 h each) by maceration technique. After the extraction by hexane was finish, the sample (*P. serratifolia* leaves) were extracted three times with 2 L of ethanol, (24 h each). The macerate was then concentrated, evaporated and dried in a vacuum at 60°C using a rotary evaporator (buchi rotavapor R-205). The yield value was as much as 1.024% (w/w) of hexane extract and 6.062% (w/w) of ethanol extract. The dry extracts was stored in refrigerator at 4°C until when it will be used.

**Phytochemical screening**

Phytochemical screenings of the extracts and isolate were performed to estimate the presence of its chemical constituents such as alkaloid, flavonoid, saponin, triterpenoid, steroid, tanins, glycosides and phenolic.

**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; Dragendorff reagent, Meyer reagent and Wagner reagent. The test results were stated positive if Dragendorff's reagent with a red to
orange precipitate was formed, Meyer reagent with a yellowish-white precipitate was formed and Wagner's reagent with a brown precipitate was formed [13].

**Flavonoid Test.** A number of samples were added a few drops of concentrated HCl and then added Mg powder. The positive results were shown by the formation of foam and the color change of the solution to orange [13].

**Saponin Test.** Saponins was detected by a foam test in hot water. A stable foam can last a long time and did not disappear when adding 1 drop of 2N HCl to indicate the presence of saponins [13].

**Tannin Test.** A number of samples were added with FeCl₃, then the mixture was homogenized. A positive reaction was indicated by the formation of a greenish black color in the mixture [13].

**Steroid and Triterpenoid Test.** A number of samples were added with anhydrous acetic acid and concentrated sulfuric acid (Liebermann-Burchad reagent). Formation of a blue or green color indicated the presence of steroids. Meanwhile formation of a purple or orange color indicated triterpenoids [13].

**Isolation and purification**

**Alkaloid Extraction.** The concentrated ethanol extract were separated by using an acid-based liquid extraction method. It was dissolved in 500 mL of water, and then acidified with 2 M HCl to make the pH became 3. The acidic solution was partitioned using 100 mL hexane for 5 repetitions. The two layers were separated; the acidic water layer below and the hexane layer above. Then the acidic water layer was re-partitioned using 100 mL ethyl acetate for 5 repetitions. The partition resulted in two layers; 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 amonia solution until the pH of the solution became 9. The basic water extract then extracted again using 100 mL of ethyl acetate to separate the non-water-dissolve compounds. The extraction was conducted for 5 repetitions. The ethyl acetate layer obtained was combined and concentrated by using a rotary evaporator to obtain the crude alkaloid extract. This extract was then weighed and further separated using Liquid vacuum chromatography (VLC) [14].

**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. The eluent was made by comparing the organic solvent with multilevel polarity. The extract was dotted at the lower boundary of the plate with a capillary tube, and then eluted. The elution process was stopped after the eluent moved to the upper limit. The shape of the stain was watched directly under a 254 nm and a 395 nm UV lamps (Shimadzu, Japan). All fractions obtained from the column chromatography were also subjected to TLC test to see the stain pattern. The fractions having the same spots were combined and re-analyzed by TLC.

**Column Chromatography.** Liquid vacuum chromatography (VLC) was performed by using the silica gel as the stationary phase with a sample ratio:silica gel (1:20). The concentrated 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 gradual ratios. The eluates obtained were collected in vial bottles and collected based on each band. The eluates were performed TLC again. Eluates were combined based on the Rₓ value on the chromatogram and stain pattern similarities. The fraction that still had many spots was then further separated by using gravity column chromatography (GCC). The eluate 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 tests, characterization and anti-inflammatory activity evaluation were performed. The alkaloid structure was confirmed with UV and IR Spectrophotometer (Shimadzu, Japan). All data compared with literature and previous data.

**Animal**

The test for anti-inflammatory activity used in this research was rats (*Rattus norvegicus*) galur Wistar from Department of Biology, Faculty of Science and Technology, Universitas Jambi. In vivo study of anti-inflammatory activity was conducted using adult female rats weighed 200 to 250 g at age 2 – 3 months.

**Anti-Inflammatory Assay**

**Preparation of Extract Suspensions.** Each extracts, both ethanol and hexane were suspended with Na CMC in distilled water. Na CMC was sprinkled over hot water in the mortar by 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 filled with distilled water to 10 mL [15].

**Anti-inflammatory Activity Evaluation.** The experimental procedures relating to the animals were authorized by Ethical committee, No. 589/UN21.8/PT.01.04/2021 from Faculty of Medicine and Health Sciences, University of Jambi. Anti-inflammatory activity evaluation was carried out on positive control (Na diclofenac), negative control (Na CMC), ethanol extract, hexane extract, VLC fraction, and the isolate. Before testing, the rats were fasted for 18 hours (without eating but still drinking). Each group consisted of four rats. The categories of each group were; C0 = negative control (-) for Na CMC 1%; C1 = control (+) for 10 mg/KgBW of 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 to the five VLC fractions with codes F.1; F.2; F.3; F.4; and F.5 and a test dose of 10 mg/KgBW. Meanwhile, the anti-inflammatory activity of the isolate was evaluated at doses of 5, 10, and 20 mg/KgBW.

At the time of testing each rat was weighed and marked on the tail. After that, the rat's left paw volume was measured with a plethysmometer. The volume was recorded as the initial volume (\(V_0\)). Each rat’s paw was after that injected subplantarly with 0.1 ml carrageenan 1% solution. After thirty minutes, the paw volume measurements were conducted 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 [16]. After the measurement, the volume of edema and AUC (Area Under the Curve) was calculated from the average edema against time curve and the percentage of inflammation inhibition [17].

Calculation of volume of rat paw edema, AUC from the time-average edema curve, and the percent anti-inflammatory effect [17] are as follows:

\[
V_u = V_t - V_0
\]

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%

\[
AUC_{t_{n-1}}^t = \frac{V_{u_{n-1}} + V_{u_n}}{2} (t_n - t_{n-1})
\]
Where:
- $V_{\text{un}-1}$: volume average edema at $t_{n-1}$
- $V_{\text{un}}$: volume average edema at $t_n$

\[
\% \text{ the percentage of inflammation} = \frac{AUC_k - AUC_p}{AUC_k} \times 100\%
\]

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 [18].

**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 its logarithmic form. The probit of inhibition was then plotted against the logarithm value of the dose. The linear equation of the graph was determined by using the software of Microsoft Excel 2010. From the linear equation, the ED$_{50}$ value was calculated by entering 5 as $y$ and $x$ as the calculated variable. The ED$_{50}$ then calculated as the anti-algorithmic value of $x$ obtained [19].

**RESULT AND DISCUSSION**

**Extraction**

Producing number of extracts in this research carried out experimentally. Total of 500 g of dried powder of bebuas ($P. serratifolia$) leaves was macerated with hexane and then with ethanol. The results of maceration obtained concentrated hexane extract had a yellowish green color and sticky-tough character like a sap. Furthermore, the leaves was then extracted using ethanol. Meanwhile, the dry ethanol extract had a resin-like sticky character and was blackish green in color. The results showed that in the percent of yield, ethanol extract was higher than the hexane. This could happen because ethanol has an ability to dissolve almost all organic compounds in bebuas ($P. serratifolia$) leaves [20], [21], [22]. Mass and yield of the dried extracts are presented in Table 1.

| Extract   | Mass (g) | Yield (%) |
|-----------|----------|-----------|
| Hexane    | 5.12     | 1.024     |
| Ethanol   | 30.31    | 6.062     |

**Phytochemical Screening**

Producing number of extracts in this research carried out experimentally. Dried powder of bebuas ($P. serratifolia$) leaves was macerated with hexane and then with ethanol. The results of maceration obtained concentrated hexane extract and ethanolic extract.

The phytochemical screening of hexane and ethanolic extract of $P. serratifolia$ leaves revealed the presence of some secondary metabolites such as alkaloids, flavonoids, phenolics, tannins, saponins and steroids (Table 2). The secondary metabolites in $P. serratifolia$ leaves are known to be responsible for some medicinal activity which in this present study is
anti-inflammatory activity. The screening for the potential anti-inflammatory properties of the phytochemicals exist in ethanolic extract of *P. serratifolia* will become a basis to the next assay on determination of anti-inflammatory activity.

Phytochemical screening was carried out on both dry extracts obtained. The results of the phytochemical screening (Table 2) gave the same results as the results obtained by Oktaviani et al. [23].

| Table 2. Phytochemical screening of Bebuas leaf extract |
|---------------------------------------------------------|
| Hexane Extract | Ethanol Extract |
|---------------|----------------|
| Alkaloids      | -              |
| Meyer         | -              |
| Dragendorff   | -              |
| Flavonoids    | -              |
| Phenolics/Tanins | -    |
| Saponins      | -              |
| Steroids      | +              |
| Terpenoids    | -              |
|               | +              |
| Note: - : negative, + : positive                        |

Phytochemical screening attested that the compounds extracted in ethanol solvent were more diverse than those extracted in hexane, because hexane tends to only extract nonpolar compound groups such as steroids.

Based on the phytochemical screening, the ethanol extract positive contained phenolic and flavonoid compounds. This result agreed with the typical compound groups contained in the genus of *premna*. 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, glycero-glycolipids, and ceramides [4], [24]. Phytochemical screening confirmed that the extract contained alkaloids, flavonoids, and steroids which are classes of compounds that have anti-inflammatory activity. This scientific evidence supports the fact that bebuas leaf can be used as anti-inflammatory drugs, as is often used to cure various diseases associated with inflammation by the eastern Sumatra Malay community.

**Separation and Purification of the Compound**

The isolation of alkaloid from extract was conducted to the ethanol extract. The targeted compound isolated was an alkaloid since most of the active anti-inflammatory NSAID compounds come from that group [25], and also in accordance that phytochemical screening showed ethanol extract contained alkaloids. Therefore, a specific method to isolate alkaloid compounds was used. The dried ethanol extract was dissolved in distilled water.

In a basic water, alkaloids returned to their neutral molecular form and their solubility in ethyl acetate increased. From the partition, a yellow ethyl acetate layer (above) and a reddish black layer of basic water were obtained. The partition was carried out several times until the ethyl acetate appeared colorless, indicating that there were no compound can be extracted any further. The ethyl acetate fraction of basic water (EaBW) was then concentrated. It was obtained 3.513 g of concentrated EaBW 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 their phytochemical content (Table 3). The
screening of the three fractions showed that the HAW extract was merely positive to steroids. This is because steroids have a nonpolar property, just similar as hexane. Steroids also tended to be non-ionic either in acidic or basic conditions. Therefore, steroids remained in their neutral state and observed in the HAW.

Table 3. Phytochemical Screening of Fractions

|                | HAW | EaAW | EaBW |
|----------------|-----|------|------|
| Alkaloids      | -   | -    | +    |
| (Dragendorff)  |     |      |      |
| Flavonoids     | -   | +    | -    |
| Fenolics/Tannins| -  | +    | +    |
| Saponins       | -   | -    | -    |
| Steroids       | +   | -    | -    |
| Terpenoids     | -   | -    | -    |

Flavonoids and phenolics were observed to present in EaAW. This is because in acidic conditions, these both of groups are not charged. The aromatic –OH groups in flavonoids and phenolics gave semipolar properties and made them to have a large solubility in semipolar solvents such as ethyl acetate [3], [8]. Meanwhile, the EaBW was positive contained alkaloids and phenolics. In a basic condition, the alkaloids return to a neutral charge and make their solubility in semipolar solvents such as ethyl acetate also increases [3], [8]. In contrast, alkaloids were not extracted by EaAW since the alkaloids are in the form of charged salts in acidic conditions, so they tend to be more soluble in water. However, phenolics were extracted both in EaAW and EaBW. This was possible because there might compounds that contain both aromatic -OH (phenolic) and cyclic nitrogen (alkaloids) functional groups to give positive results on both [3], [8].

The isolation was continued on EaBW by using vacuum liquid chromatography (VLC). Gradient elution was performed by using 100% ethyl acetate to 100% methanol as eluent. Both of those solvents were used since the TLC test showed that the EaBW could only be eluted by those two. A 395 nm UV lamp was used to monitor the elution process (Figure 1). This monitoring aimed to see the separation band clearer by the fluorescence of the compound.

At first when the VLC was carried out, it was observed that ethyl acetate cannot elute the second band and the band was retained on silica. Therefore a gradient elution was performed to increase the separation resolution of complex mixtures such as the sample.
After the eluent polarity was increased by the addition of methanol, the band can be eluted. From the VLC, eleven vials were obtained. After the solvent was evaporated, a TLC test was carried out with 100% ethyl acetate as eluent. The vials that had the same stain pattern were combined. From the TLC test, five fractions were obtained with label of F.1; F.2; F.3; F.4; and F.5 according to their elution time sequence. The fractions mass are summarized in Table 4.

Table 2. Fractions mass obtained from VLC

| Fraction | Mass (mg) |
|----------|-----------|
| F.1      | 35.35     |
| F.2      | 164.85    |
| F.3      | 18.30     |
| F.4      | 859.95    |
| F.5      | 137.48    |

Phytochemical screening (Table 5) showed that all fractions were positive contained alkaloids. Meanwhile, in addition to alkaloids, F.1 was also positive for steroids and F.2 and F.3 were positive for phenolics. This can happen because those fractions probably was a mixture of different compounds or the compounds in them had more than one functional group, thus giving positive results to more than two groups of compounds.

Table 5. Phytochemical Screening of Fraction

|                   | F.1 | F.2 | F.3 | F.4 | F.5 |
|-------------------|-----|-----|-----|-----|-----|
| Alkaloids (Dragendorff) | +   | +   | +   | +   | +   |
| Flavonoids        | -   | -   | -   | -   | -   |
| Fenolics/Tannins  | -   | +   | -   | +   | -   |
| Saponins          | -   | -   | -   | -   | -   |
| Steroids          | +   | -   | -   | -   | -   |
| Terpenoids        | -   | -   | -   | -   | -   |

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 2. From the test, it can be seen that F.5 has the highest activity as an anti-inflammatory. Therefore, the isolation was continued to F.5. F.5 has a paste-like appearance, was greenish yellow in color, and did not dissolve in either nonpolar or semipolar solvents. F.5 was then washed with ethyl acetate and hexane to remove impurities that may still be mixed up. After washing, F.5 was then tested using TLC. Based on the TLC test, methanol and ethyl acetate eluent could not elute its stain. This showed that the compound in F.5 was polar so that it can interact strongly with the silica. This is what causes the compound to be retained on silica and cannot be eluted, even with polar organic solvents such as methanol. Therefore, eluent polarity was increased by using 1:1 methanol:water. The stains obtained from the using of this eluent are summerized in Table 6.
Table 3. Stain spots observed from F.5

| Sample | Rf value observed under UV lamp |
|--------|---------------------------------|
|        | 395 nm | 294 nm |
| F.5    | 0.05   | 0.05   |
|        | -      | 0.6    |
|        | 0.75   | 0.75   |

This observation showed that F.5 was still a mixture of compounds (Figure 2). Therefore, the separation was continued by using GCC.

![TLC test results of F.5 with methanol:water 1:1 under UV lamp](image)

Figure 2. TLC test results of F.5 with methanol:water 1:1 under (a) 294 nm UV and (b) 395 nm UV

From the further separation by GCC, two fractions were obtained and coded F.5.1 and F.5.2. The physical character of F.5.1 was yellow with small crystal grains. Meanwhile F.5.2 was a yellow crystal which turned to a liquid like oil form with a relatively high viscosity after exposed by air.

The two fractions obtained were then tested by TLC using 100% methanol as eluent. From the TLC test (Figure 3), it can be seen that F.5.1 gave two imperfectly separated spots. Meanwhile, F.5.2 gave a single spot. Their Rf values are summarized in Table 4.

Table 4. Rf value of fractions obtained from further separation to F.5

| Rf Value of Fractions |
|-----------------------|
| F.5.1     | F.5.2     |
| 0.7       | -         |
| 0.95      | 0.98      |
Even though it gave a single stain, the results of the TLC test of F.5.2 with 100% methanol gave a high $R_f$ value. Therefore, the TLC test was continued to F.5.2 by reducing the polarity of the eluent used. F.5.2 was then eluted on TLC by using eluents with different polarity (Table 8).

**Table 5.** $R_f$ values of F.5.2 with various eluent

| Sample | Eluent Used                     | $R_f$ Value |
|--------|---------------------------------|-------------|
| F.5.2  | Ethyl acetate                   | 0.14        |
|        | Ethyl acetate:methanol 1:1      | 0.5         |
|        | Ethyl acetate:methanol 1:3      | 0.55        |

TLC test showed that F.5.2 consistently gives a single stain (Figure 4). A single stain on the TLC indicates that a sample most likely a single compound or a mixture of several compounds with very close polarity. Because of its relatively simple stain pattern, F.5.2 was designated as the isolate and evaluated for its anti-inflammatory activity test.

**Figure 4.** TLC test of F.5.2 (a) ethyl acetate eluent (b) ethyl acetate:methanol 1:1 eluent (c) ethyl acetate:methanol 1:3 eluent

**Anti-Inflammatory Activity Evaluation**

The anti-inflammatory activity evaluation was carried out on on male albino rats (*Rattus novergicus*). These rats were used as test animals because many human condition symptoms can be replicated on them and their genome is similar to human genome (90%) [4], [26]. Before being tested, the rats were not fed for 18 hours in order to avoid the influence of...
other substances on the test. Tests were carried out for crude extract of hexane and ethanol of the bebuas left. The results can be seen in Figure 5.

![Graph of Edema volume against time](image)

**Figure 5.** Graph of Edema volume against time

From the data in Figure 5, it can be seen that ethanol extract at a dose of 250, 500, 1000 mg/KgBW was able to reduce the rats’ paw swelling at first 60 minutes after induction. In contrast, the hexane extract that had a significant effect at first 60 minutes only observed with dose of 1000 mg/KgBW. Meanwhile, at doses of 250 and 500 mg/KgBW they were only able to significantly reduce the swelling in first 120th minute.

Based on the data in Figure 5, the average percent inhibition of C1, C2, C3, C4, C6, and C7 respectively were 79.30%; 8.37%; 22.90%; 43.83%; 25.35%; 45.42%; and 68.35%. Meanwhile, C0 used was Na CMC 1% and the inhibition was designated as 0% as it is the negative control. These showed that both hexane and ethanol extract have strong anti-inflammatory activity (percent inhibition is above 40%). However, the anti-inflammatory activity of the hexane extract was weaker than that of the ethanol extract. This can be seen from the percentage of inhibition above 40% was only achieved by hexane extract at a dose of 1000 mg/KgBW. Meanwhile, the ethanol extract was able to reach above 40% at a dose of 500 mg/KgBW.

Therefore, the Post Hoc LSD test was performed to see which groups have significant differences. The tabulation table of the results of the Post Hoc LSD test is presented Table 9.

| 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 notations indicate that there is no statistically significant difference.
Post Hoc LSD test in Table 9 showed that almost all of doses statistically had significant differences in inhibition compared to the negative control. It was seen that there was no significant difference between the C6 and C4 percent of inhibition, which means the two doses had almost the same activity. However, the ethanol extract was requiring a lower dose. This showed that the ethanol extract was more active than the hexane. Compared to the negative control, all doses statistically had a significant difference in inhibition. It showed that the extracts were active for anti-inflammatory.

The percentage of inhibition of each dose was transformed into a probit form and plotted into a graph against the logarithmic value of the dose (Figure 6). From the graph, a linear equation of the probit inhibition against the logarithm of the dose was used to calculate the ED$_{50}$. The ED$_{50}$ for ethanol and hexane extract were 571.56 mg/KgBW and 1162.30 mg/KgBW, respectively. This showed that the ethanol extract had a higher anti-inflammatory activity than the hexane extract.

The active anti-inflammatory compounds in hexane extract were thought to be steroids. This was consistent with the results of the phytochemical screening which showed that the hexane extract was only positive for steroids. Meanwhile, the anti-inflammatory active compounds in ethanol extract could come from the phenolic or alkaloid groups [4], [5].

Based on the anti-inflammatory evaluation, ethanol extract had a higher percent of inhibition than hexane extract for the same dose. Therefore, the ethanol extract was continued to the isolation and the targeted compound to isolate was an alkaloid. This consideration was made since most of active NSAID compounds come from alkaloids [4],[25],[27],[28], and in accordance with the results of phytochemical screening that the ethanol extract contained alkaloids. Therefore, special extraction and isolation methods for alkaloids had been used to give the EaBW.

EaBW was further separated by VLC and each fraction was evaluated for anti-inflammatory to determine which fraction had the best activity. 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 Figure 7.
Figure 7. Fraction’s anti-inflammatory evaluation curve.

The percent of inhibition obtained from the anti-inflammatory evaluation 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%. Meanwhile, Na diclofenac as the positive control gave 79.30% inhibition. It showed that all fractions had strong anti-inflammatory activity (% inhibition > 40%) and had similar activity with the positive control. Na CMC was used as the negative control and its inhibition was designated as 0%.

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 F0.05 (6, 35) table (889.408 > 2,372) and the p value was smaller than the α value (0.000 < 0.05).

Table 10. 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      |
| 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 data on the results of the post hoc LSD test in Table 10 showed that all fractions were active as anti-inflammatory since they statistically had a significant difference with C0. It also can be seen that there was no significant difference between the percentage inhibition of F.1 and F.2, as well as F.3 and F.4. This indicated 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 can be occurred because there was the possibility of the same chemical compound in them. This was possible because the fractions were in close order according to their separation in VLC.
Figure 8. The isolate anti-inflammatory evaluation curve.

The isolate was obtained from F.5.2. The results of the isolate anti-inflammatory evaluation can be seen in Figure 8. The evaluation showed that for dose 5 mg/KgBB, 10 mg/KgBB, and 20 mg/KgBB the percentage inhibition were 53.86%, 65.88%, and 76.94% respectively. The graph of the probit of inhibition against the logarithm of dose can be seen in Figure 9. Based on the calculation of the linear equation in Figure 9, the ED$_{50}$ value of the isolate was 4.06 mg/KgBW.

Figure 9. Graph of probit vs log dose of the isolate.
Table 11. LSD Post Hoc Test Results on the Isolate.

| Substance and Dosage     | % Inhibition |
|-------------------------|--------------|
| C1                      | 79.30 a      |
| Isolate 20 mg/KgBW      | 76.94 a      |
| Isolate 10 mg/KgBW      | 65.88 b      |
| Isolate 5 mg/KgBW       | 53.86 c      |
| C0                      | 0.00 d       |

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.

One-way ANOVA statistical test showed that there was a significant difference between the groups of rats tested with the isolates. This can be seen from the calculated F value which was greater than the F_{0.05(4,13)} table (258.347 > 3.180) 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 11, it can be seen that all isolate doses were statistically significant different in inhibition compared to the negative control. It also showed that there was no statistically significant different between the positive control with the isolate 20 mg/KgBW. It meant that the isolate with dose 20 mg/KgBW had almost the same anti-inflammatory strength with the Na diclofenac 10 mg/KgBW.

Characterization of the Isolate

UV-Vis and FT-IR characterization was carried out to determine the basic skeleton and functional group of the compound. The UV-Vis spectrum (Figure 10) showed that the isolate gave three absorption peaks at λ = 224 nm; 268 nm; and 321 nm, which the λ_{max} was 268 nm. These peaks showed similarities with some indoles UV-Vis spectra. The peaks around 226 nm and 229 nm were almost showed by most indole alkaloid. However, phenolic indole alkaloids could experience bathocromic shift [29]. The isolate UV-Vis spectrum in turn showed corresponding peaks with Bafotenine spectrum, which is a phenolic indole alkaloids. Bafotenin has λ_{max} at 277 nm with a shoulder at 295 nm that can shift to 322 nm in basic condition. This λ_{max} and shoulder pattern was similar to the isolate’s UV-Vis spectrum pattern, which had λ_{max} at 268 nm with a shoulder at 321 nm. This indicated that the isolate had a similar basic skeleton with bufotenine.
Figure 10. UV-Vis spectrum of the isolate, \( \lambda = 224 \text{ nm}; 268 \text{ nm}; \) and 321 nm.

The FT-IR spectrum (Figure 11) showed that functional groups contained in the isolate were –OH, N-H, C-N of aromatic amine, and C-N of amine. Based on the comparison of the literature, the FT-IR spectrum pattern of the isolates was similar to the IR spectrum of bufotenine. The comparison of both IR adsorption can be seen in Table 12. The similarities of both FT-IR spectrum strengthen the notion that the isolate was a compound that had a similar structure with bufotenine.

Figure 11. FT-IR spectrum of the isolate.

Bufotenine (Figure 12) was a tryptamine derivative related to the neurotransmitter serotonin. It naturally occurred in some species of higher plants, toads (especially the skin), and mushrooms. Bufotenine was classified as a controlled drug in some countries, since it can cause some dangerous effect if consumed by human. However, other compounds with 5-
hydroxyindole core like bufotenine, have been reported to have an anti-inflammatory activity. Even, one of compound containing 5-hydroxyindole, indomethacin had been widely used as a non-steroidal anti-inflammatory drug [30]. It showed that compounds containing 5-hydroxyindole skeleton can also act as an anti-inflammatory drug.

**Table 12. Interpretation and Comparison of IR Spectra.**

| Interpretation                      | Adsorption (cm\(^{-1}\)) |
|-------------------------------------|---------------------------|
|                                     | Isolate  | Bufotenine [27] |
| O-H stretching                      | 3421.71  | 3407           |
| N-H bending                         | 1567.21  | 1581           |
| C-H bending of alkane               | -        | 1466           |
| C-N stretching of aromatic amine    | 1345.66  | -              |
| C-N stretching of amine             | 1202.16  | 1203           |
| Finger print                        | 928.72   | 935            |
| Finger print                        | -        | 795            |

The 5-hydroxyindole 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 C-N aromatic amine bonds, as the characteristic of the 5-hydroxyindole skeleton, was a strong evidence of this supposition.

![Figure 12. Structure of Bufotenine](image)

The assumption that the isolate was a compound with characters like bufotenine was also supported by the similarities of their physical form. Bufotenine was a yellow crystals that could transform to yellow oil after oxidized by air exposure [31],[32],[33]. This was in accordance with the physical form of the isolate that was yellowish crystals when it was freshly dried from the solvent, but after being exposed for a long time by air, the isolate transformed to yellow oil due to oxidizing process [33].

On the basis of phytochemical screening results, physical form, the spectrum data of UV, IR and by the literature comparism, the main components from the isolated ethanolic leaves extract of *P. serratifolia* leaves could be inferred to be bufotenine, as seen formerly in literatures [31],[32],[33]. This further strengthens the notion that the isolate was a compound with a basic skeleton such as bufotenine.
CONCLUSION

Based on the anti-inflammatory activity of ethanolic extract of *P. serratifolia* leaves levels and percentage of inhibition obtained from the anti-inflammatory evaluation, it is observed that either ethanolic extracts of *P. serratifolia* leaves and Bufotene as alkaloid derivate isolated from the extract revealed anti-inflammatory activity. The results of this study indicated that *Premna serratifolia* (bebuas) is a potent source of natural anti-inflammatory agent.

CONFLICT OF INTEREST

No conflicts of interest is associated with this work.

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