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Phytochemical Screening and *In Vitro* Anti-Inflammatory Activity through Protein Denaturation Inhibition of Tigarun (*Crataeva nurvala* Buch. -Ham.) Stem Bark

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ABSTRACT

Crataeva nurvala Buch. -Ham. is a medicinal plant commonly found in South Kalimantan, Indonesia. This study aimed to identify the phytochemical constituents and evaluate the *in vitro* anti-inflammatory activity of the stem bark extract of *C. nurvala*. The stem bark was extracted by maceration using ethanol and then fractionated by liquid-liquid partition with *n*-hexane, ethyl acetate, and *n*-butanol. Phytochemical screening was conducted using standard tube tests. The *in vitro* anti-inflammatory activity was assessed by measuring the inhibition of protein denaturation using Bovine Serum Albumin (BSA), and the IC_{50} values were calculated. Phytochemical analysis revealed that the ethanol extract and the ethyl acetate fraction contained alkaloids, flavonoids, triterpenoids, phenols, and tannins. The *n*-hexane fraction showed the presence of triterpenoids, while the *n*-butanol fraction contained flavonoids. The IC_{50} values of the ethanol extract, *n*-hexane, ethyl acetate, and *n*-butanol fractions were 122.07 ppm, 55.72 ppm, 96.01 ppm, and 155.88 ppm, respectively. Diclofenac sodium, used as a reference, showed an IC_{50} of 59.79 ppm. These results indicate that the *n*-hexane fraction of *C. nurvala* stem bark possesses the strongest *in vitro* anti-inflammatory activity among the tested samples.

INTRODUCTION

Inflammatory diseases are among the most common health problems affecting the elderly population in Indonesia. Inflammation is commonly treated with steroidal or non-steroidal anti-inflammatory drugs (NSAIDs); however, prolonged use of these agents may cause adverse effects, including gastrointestinal disorders (Novika *et al*., 2021). One medicinal plant known for its anti-inflammatory potential is *Crataeva nurvala* (*C. nurvala)* Buch. -Ham., locally known as Tigarun. Lupeol, a triterpenoid isolated from *C. nurvala*, has demonstrated anti-inflammatory activity by inhibiting the arachidonic acid pathway, thereby suppressing COX-2 enzyme production and preventing inflammation (Thirumalaisamy *et al*., 2020).

The protein denaturation inhibition assay is a simple and effective method for evaluating anti-inflammatory activity, since denatured proteins can trigger inflammatory responses by acting as autoantigens. Heat is one of the main factors that causes protein denaturation by disrupting hydrophobic interactions and hydrogen bonds, which leads to the breakdown of the secondary, tertiary, or quaternary structures of proteins (Minarti *et al*., 2021). Accordingly, compounds capable of preventing protein denaturation are considered potential anti-inflammatory agents (Yesmin *et al*., 2020; Dharmadheva *et al*., 2018; Heendeniya *et al*., 2018; Farida *et al*., 2018).

In addition to triterpenoids, the stem bark extract of *C. nurvala* has also been reported to contain other secondary metabolites such as flavonoids, alkaloids, and saponins (Kaushik *et al*., 2021). The composition of secondary metabolites in plants can be influenced by various environmental factors, including soil pH, temperature, light intensity, humidity, climate,

and seasonal variations (Akasia *et al*., 2021; Ratiu *et al*., 2021; Li *et al*., 2020). Since the biological activity of plant extracts is often determined by specific secondary metabolites, proper identification through phytochemical screening is crucial (Akasia *et al*., 2021). Therefore, this study aimed to investigate the phytochemical profile of the ethanol extract and ethyl acetate fraction of *C. nurvala* Buch. -Ham. stem bark and to evaluate their *in vitro* anti-inflammatory activity. Compared to other medicinal plants with anti-inflammatory properties, *C. nurvala* is relatively underexplored, particularly in terms of fraction-based evaluation of its bioactive compounds. While several studies have reported its pharmacological potential, comparative studies on different solvent fractions and their contribution to anti-inflammatory activity remain limited. Thus, this study aims to provide a more comprehensive evaluation of the phytochemical profile and fraction-specific anti-inflammatory activity of *C. nurvala* stem bark.

METHODS

The equipment used in this study included Ultraviolet-Visible (UV–Vis) spectrophotometer (PerkinElmer Lambda 356) analytical balance (Ohaus), filter paper, hot plate (Stuart), stopwatch, pH meter (ATC), separating funnel (Pyrex), incubator (Memmert), 50 mesh sieve (Retsch), glass funnel and rotary evaporator (IKA® RV 10), water bath (Memmert), thin layer chromatography (TLC) plate, chamber, and UV lamp.

Materials

The materials used in this study were the bark of the *C. nurvala* Buch. Ham plant, distilled water (OneMed), 70% ethanol (OneMed), n-hexane (Bratachem), n-butanol (Polylab), ethyl acetate (Brataco), tris base (Merck), Bovine Serum Albumin (Himedia), glacial acetic acid (Merck), NaCl (Merck), Mayer's reagent, Wagner's reagent, Dragendorff's reagent, $FeCl_3$ (Sigma-Aldrich), Mg powder (Merck), HCl (Merck), n-hexane (Teknis), H_2SO_4 (Merck), and sodium diclofenac (Aarti Drugs Limited).

Sample Preparation

The stem bark of *Crataeva nurvala* Buch. -Ham. was collected from Astambul, Banjar Regency. The plant material was taxonomically identified and authenticated at the Basic Laboratory of the Faculty of Mathematics and Natural Sciences, Lambung Mangkurat University, Banjarbaru, and a voucher specimen was deposited under reference number (026a/LB.LABDASAR/II/2022). The samples were wet-sorted and washed to remove impurities. The sample were cleaned, cut into small pieces and air-dried to reduce moisture content (Fitriana *et al*., 2020). After drying, a final sorting was performed to obtain high-quality crude simplicia. The dried material was ground using a blender and sieved to obtain fine powder, which was stored in an airtight container (Pratini & Florentina, 2017).

Extraction of *C. nurvala* Stem Bark

A total of 400 g of powdered stem bark was extracted by maceration using 70% ethanol. The powder was fully submerged in solvent with a minimum solvent depth of 1 cm (Niah & Helda, 2016; Heliawati, 2018). Maceration was done for 24 hours, repeated three times with intermittent stirring. After each 24-hour period, the solvent was replaced. The combined filtrates were filtered to separate the residue (Nurdin *et al.,* 2017) and concentrated in a water bath at 50°C to obtain a viscous extract (Milanda *et al*., 2021). The yield was calculated as a percentage based on the dry weight of the powder. The crude extract was then subjected to phytochemical screening and anti-inflammatory assays.

Fractionation of *C. nurvala* Stem Bark Extract

Ten grams of the concentrated ethanol extract was dissolved in water for liquid-liquid partitioning. Partitioning was performed three times for each solvent. The solution was transferred to a separatory funnel and partitioned sequentially with n-hexane at a ratio of 1:3 (extract:solvent) (Husni *et al*., 2020). The funnel was shaken thoroughly with intermittent venting to release gas. After phase separation, both layers were collected. The aqueous layer was re-extracted with fresh n-hexane until the n-hexane layer became clear and no spots appeared on the TLC. The same procedure was repeated sequentially with ethyl acetate and then n-butanol, yielding three fractions: n-hexane, ethyl acetate, and n-butanol. The n-hexane and n-butanol fractions were concentrated by evaporation to obtain viscous fractions. The fractions were concentrated until a constant weight was achieved to ensure minimal solvent residue, as recommended for extract standardization (Sumiwi *et al*., 2016).

Phytochemical Screening

Test solutions were prepared by dissolving 10 mg of each extract or fraction in 10 mL ethanol (Palgunadi *et al*., 2021). The following qualitative phytochemical tests were performed:

Alkaloid Test: One milliliter of test solution was divided equally into three test tubes (approximately 0.33 mL each). To each tube, 10 drops of H_2SO_4 were added and shaken vigorously. Dragendorff's, Wagner's, and Mayer's reagents were added respectively. Alkaloids presence was indicated by reddish (Dragendorff's), brown (Wagner's), or white precipitates (Mayer's).
Saponin Test: One milliliter of the test solution was added to five milliliters of water and heated for 5 minutes. After cooling, the mixture was shaken vigorously and left to stand for 10 minutes. Persistent froth with 1–10 cm height indicated positive saponins. Foam stability was confirmed by adding one drop of 2N HCl.
Phenolic Test: One milliliter of test solution mixed with 3–4 drops of ferric chloride resulted in green to dark blue-black color if phenolics were present.
Steroid and Triterpenoid Test: Test solution was mixed with 0.5 mL glacial acetic acid and carefully layered with 1–2 mL concentrated H_2SO_4. A bluish-green ring indicated steroids, while a brown ring indicated triterpenoids.
Flavonoid Test: Test solution mixed with 0.1 g magnesium powder and 1 mL concentrated HCl produced red, yellow, or orange colors if flavonoids were present .
Tannin Test: One milliliter of test solution with 5 drops of ferric chloride showed greenish-brown to blackish-blue color if tannins were present .

Anti-Inflammatory Activity Assay
Preparation of Tris Buffer Saline (TBS)

Tris Buffer Saline (TBS) was prepared by dissolving 0.87 g of NaCl in distilled water. Subsequently, 0.121 g of Tris base was added, and the pH was adjusted to 6.2–6.5 using glacial acetic acid. The volume was then adjusted to 100 mL with distilled water (Reynaldi & Yani, 2021).

Preparation of 0.2% Bovine Serum Albumin (BSA)

A 0.2% BSA solution was prepared by dissolving 0.2 g of Bovine Serum Albumin (BSA) in a 100 mL volumetric flask, then diluted to volume with TBS (Reynaldi & Yani, 2021).

Preparation of Negative Control Solution

The negative control was prepared by mixing 500 µL of ethanol or ethyl acetate with 0.2% BSA solution in a 5 mL volumetric flask, then diluted to volume (Farida *et al*., 2018).

Preparation of Test Solutions and Positive Control (Diclofenac Sodium)

Stock solutions of diclofenac sodium, extracts, and fractions were prepared by dissolving 50 mg of each sample in 25 mL of ethanol or ethyl acetate to obtain a concentration of 2,000 ppm. Serial dilutions were then made to yield concentrations of 1,000, 500, 250, and 125 ppm (Kanjikar *et al*., 2017).

Determination of Anti-Inflammatory Activity

A volume of 500 µL from each concentration was mixed with 0.2% BSA in a 5 mL volumetric flask, resulting in final concentrations of 12.5, 25, 50, 100, and 200 ppm. The samples, along with negative and positive controls, were incubated at 37°C for 15 minutes and subsequently heated at 70°C for 5 minutes. After cooling to room temperature, the solutions were shaken vigorously, and absorbance was measured at 660 nm using a UV-Vis spectrophotometer (Kanjikar *et al*., 2017).

Calculation of Percentage Inhibition

The percentage inhibition of protein denaturation was calculated using the formula:

$$\%\text{inhibition} = \frac{ANC - ATS}{ANC} \times 100\%$$

Description:
ANC = Negative Control Absorbance, ATS = Absorbance of Test Solution.

Blank correction was not applied in this study because the solvent used showed negligible absorbance at 660 nm, and preliminary measurements confirmed that its contribution to total absorbance was insignificant.

IC_{50} Determination

IC_{50} values were determined by plotting percentage inhibition against concentrations (12.5, 25, 50, 100, and 200 ppm) and calculating the value from the linear regression curve. IC_{50} was determined for diclofenac sodium, ethanol extract, and the n-hexane, ethyl acetate, and n-butanol fractions of *C. nurvala* stem bark (Kanjikar *et al*., 2017; Novika *et al*., 2021).

RESULTS AND DISCUSSION

The stem bark of *Crataeva nurvala* (*C. nurvala*) Buch. -Ham. was collected, peeled, and washed under running water to remove impurities. Approximately 1 kg of stem bark was air-dried to produce dried simplicia. The dried material was ground and passed through a 50-mesh sieve to increase surface area, enhancing solvent penetration and extraction efficiency (Pratini & Florentina, 2017).

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Table 1. Phytochemical profile of *C. nurvala* Buch.-Ham. stem bark

Compound Class	Ethanol Extract	n-Hexane Fraction	Ethyl Acetate Fraction	n-Butanol Fraction
Alkaloids	+	-	+	-
Saponins	-	-	-	-
Phenolics	+	-	+	-
Steroids	-	-	-	-
Triterpenoids	+	+	+	-
Flavonoids	+	-	+	+
Tannins	+	-	+	-

Table 2. Anti-inflammatory activity of Sodium Diclofenac

Concentration (ppm)	% Inhibition			Mean % Inhibition	SD	RSD (%)
	Rep I	Rep II	Rep III			
12.5	9.607	9.234	8.502	9.114	0.002	0.222
25	42.980	42.821	42.711	42.837	0.001	0.175
50	67.066	67.209	67.087	67.121	0.002	0.480
100	77.044	77.205	77.136	77.128	0.002	0.660
200	96.594	96.469	96.592	96.552	0.001	2.135

RSD, relative standard deviation; SD, standard deviation.

A total of 400 g of powder was used for extraction. Extraction was performed by maceration, chosen for its simplicity and absence of heat application. Powdered stem bark (400 g) was macerated with 70% ethanol at a solvent-to-sample ratio of 2.5:1. Ethanol 70% was selected for its relatively low boiling point, ease of evaporation, low toxicity, and ability to extract both polar and non-polar compounds effectively (Syarif et al., 2016). The sample was soaked for 24 hours in a tightly sealed container, with occasional stirring and solvent replacement every 24 hours. Stirring maximizes contact between solvent and plant material, facilitating extraction of bioactive compounds (Sambodo *et al*., 2022). After each 24-hour maceration, the mixture was filtered to separate residue. This process was repeated three times with fresh solvent. Combined filtrates were concentrated to obtain a viscous extract, yielding 47.11 g (11.78%). This yield differs from 4.16% reported using methanol solvent (Bhandari *et al*., 2021), likely due to differences in solvent type, solvent ratio, extraction duration, and sample size (Harborne, 1998).

The ethanol extract was fractionated based on solvent polarity and specific gravity, aiming to isolate secondary metabolites and reduce interference (Adrian *et al*., 2021). Solvents used were n-hexane, ethyl acetate, and n-butanol, which effectively separate compounds according to polarity. Fraction yields were 2.99 g (9.97%) for n-hexane, 1.34 g (4.46%) for ethyl acetate, and 1.90 g (6.33%) for n-butanol fractions. Adjgaba (2017) reported fraction yields from *C. adansonii* leaves as 0.44%, 1.34%, and 6.26% for cyclohexane, ethyl acetate, and n-butanol, respectively. Differences may be attributed to species and plant part variation. Previous *C. nurvala* studies reported yields of 13.93% (chloroform) and 32.88% (ethyl acetate) fractions (Raut & Gaikwad, 2014), likely due to solvent type, ratio, extraction conditions, and duration differences (Harborne, 1998).

Phytochemical screening was conducted to identify the secondary metabolites present in the ethanol extract and ethyl acetate fraction of *C. nurvala* stem bark. Tests included alkaloids, phenolics, saponins, flavonoids, tannins, steroids, and triterpenoids. The results are summarized in Table 1.

Based on Table 1, both the ethanol extract and ethyl acetate fraction contained multiple bioactive compounds. Bhattacharjee *et al*. (2015) similarly reported triterpenoids, phenolics, flavonoids, alkaloids, tannins, and saponins in ethanol extracts, and alkaloids, phenolics, flavonoids, triterpenoids, and tannins in ethyl acetate fractions of *C. nurvala* stem bark. The presence of specific phytochemicals can be influenced by internal factors such as genetics, and external factors including temperature, light intensity, soil pH, humidity, altitude, and soil nutrients (Sholekah, 2017).

The anti-inflammatory activity was evaluated using the protein denaturation inhibition assay with bovine serum albumin (BSA) as the model protein. BSA denatures upon heat exposure, as temperature and extreme pH values can disrupt protein structures (Yuliana, 2018). To maintain pH stability during the assay, Tris Buffer Saline (TBS) at pH 6.2–6.5 was employed (Umeti *et al*., 2019).

Test samples—including sodium diclofenac, and the n-hexane, ethyl acetate, and n-butanol fractions of *C. nurvala* stem bark—were prepared at concentrations of 12.5, 25, 50, 100, and 200 ppm. Absorbance at 660 nm was measured by UV-Vis spectrophotometry. Percentage inhibition of protein denaturation was calculated, and results are summarized in Table 2.

Table 2 shows increasing percentage inhibition with rising concentrations. At 12.5 ppm, inhibition was limited (9.11%), but concentrations ≥25 ppm demonstrated >20% inhibition, indicating significant anti-inflammatory activity. Diclofenac, a widely used NSAID, inhibits cyclooxygenase (COX) enzymes and binds to albumin at tryptophan residues, stabilizing the protein against thermal denaturation (Novika *et al*., 2021; Hossain *et al*., 2016; Czub *et al*., 2020). As shown in Table 3, the mean IC_{50} was 55.10 ± 0.46 ppm, consistent with literature values, confirming its potency. The ethanol extracts inhibited protein denaturation above 50 ppm, reaching 65% inhibition at 200 ppm (Table 4). Regression analysis yielded IC_{50} = 122.07 ppm, comparable to methanol extracts of *C. adansonii* leaves (Umeti *et al*., 2019). It should be noted that the referenced study used a similar protein denaturation inhibition method, although slight variations in experimental conditions may influence the IC_{50} values.

This fraction exhibited >20% inhibition at all concentrations, with a maximum of 69.48% at 200 ppm (Table 4). The IC_{50} of 96.01 ppm (y = 0.1982x + 30.968) was slightly higher than reported values for *C. adansonii* ethyl acetate leaf fraction (40 ppm) (Umeti *et al*., 2019). Lower IC_{50} values indicate stronger inhibitory effects (Farida *et al*., 2018). The n-butanol fraction showed moderate inhibition, exceeding 20% only at concentrations >25 ppm, with an IC_{50} of 155.88 ppm, higher than *C. adansonii* leaf fraction (120 ppm) (Umeti *et al*., 2019). A similar method was used in the cited study, but variations in experimental conditions may explain the differences in IC_{50} values. Containing flavonoids, it likely inhibits COX and lipoxygenase pathways contributing to anti-inflammatory activity (Octavian, 2022).

Table 3. IC_{50} value of Sodium Diclofenac

IC_{50}	$\bar{x}IC_{50}$ ± SD (ppm)	RSD (%)
54.725 54.958 55.612	55.098 ± 0,460	0.834

RSD, relative standard deviation; SD, standard deviation.

Table 4. % inhibition data of *C. nurvala* stem bark

Concentration (ppm)	Ethanol extract (Mean ± SD)	n-hexane fraction (Mean ± SD)	Ethyl acetate fraction (Mean ± SD)	n-butanol fraction (Mean ± SD)
12.5	10.18 ± 0.0024	36.529 ± 0.001	29.86 ± 0.0075	17.594 ± 0.003
25	15.93±0.0058	37.541 ± 0.004	34.39 ±0.0056	29.011 ± 0.003
50	46.89 ± 0.0027	52.51± 0.002	47.18 ±0.0125	29.522 ±0.004
100	51.3 ± 0.0068	65.256 ±0.001	50.72 ± 0.0076	35.62 ± 0.002
200	65 ± 0.0066	91.407 ±0.008	69.48 ± 0.0011	60.187 ± 0.002

SD, standard deviation.

Table 5. Mean IC_{50} values of *C. nurvala* stem bark fractions

Sample	Mean IC_{50} ± SD (ppm)
Ethanol extract	122.07 ± 0.50
n-Hexane fraction	55.720 ± 1.059
Ethyl acetate fraction	96.01 ± 0.74
n-Butanol fraction	155.883 ± 0.881

SD, standard deviation.

Table 5 ranks sample potency as: sodium diclofenac (55.10 ppm) ≈ n-hexane fraction (55.72 ppm) > ethyl acetate fraction (96.01 ppm) > ethanol extract (122.07 ppm) > n-butanol fraction (155.88 ppm). Fractions showed higher activity than crude extract but were slightly less potent than the standard drug. Fractionation enriches active compounds by removing interfering substances, enhancing bioactivity (Susilowati *et al*., 2016). The enriched fractions contain fewer compounds at higher relative concentrations, explaining increased anti-inflammatory effects. Antagonistic interactions present in crude extracts may also be absent in fractions (Hakim & Sugijanto, 2014).

Phytochemical analysis confirmed alkaloids, flavonoids, triterpenoids, phenolics, and tannins in ethanol extract and ethyl acetate fraction. Triterpenoids and flavonoids contribute to protein stabilization through hydrogen bonding involving hydroxyl groups and conjugated double bonds with BSA amino acids (Firdiyani *et al*., 2015; Zinellu *et al*., 2015; Minarti *et al*., 2021). Lupeol inhibits arachidonic acid metabolism, preventing COX-2 expression and inflammation (Thirumalaisamy *et al*., 2020), while flavonoids inhibit COX and lipoxygenase, suppressing prostaglandin and leukotriene synthesis (Riansyah *et al*., 2015).

CONCLUSIONS

In conclusion, the ethanol extract and ethyl acetate fraction of *Crataeva nurvala* Buch. - Ham. stem bark contains several bioactive compounds, including alkaloids, flavonoids, triterpenoids, phenolics, and tannins. The n-hexane fraction was rich in triterpenoids, while the n-butanol fraction primarily contained flavonoids. Anti-inflammatory activity assessment via protein denaturation inhibition revealed IC_{50} values of 122.07 ppm, 55.72 ppm, 96.01 ppm, and 155.88 ppm for the ethanol extract, n-hexane, ethyl acetate, and n-butanol fractions, respectively. The positive control, sodium diclofenac, exhibited an IC_{50} value of 59.79 ppm. These results indicate that the n-hexane fraction of *C. nurvala* stem bark possesses the strongest anti-inflammatory activity among the tested samples, suggesting its potential as a natural anti-inflammatory agent.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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