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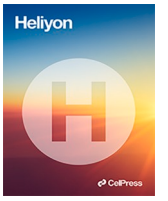

Research article

In vivo and *in silico* antihypertensive, anti-inflammatory, and analgesic activities of *Vernonia amygdalina* Del. leaf extracts

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ABSTRACT

Vernonia amygdalina (VA) leaves contain many potential active ingredients and exhibit diverse pharmacological activities. The antihypertensive, anti-inflammatory, and analgesic effects of VA crude and fraction extracts were carried out using Swiss albino mice models. VAE is considered safe to be administered due to LD_{50} being greater than 10,000 mg/kg body weight. A dose-dependent increase in antihypertensive, anti-inflammatory, and analgesic activities was observed in both VAE and fractions, similar to the reference drugs. The antihypertensive effect of the VAE 2.0 (2000 mg/kg, SBP: ↓26.05 %, DBP: ↓34.51 %) was nearly equivalent to Captopril (100 mg/kg, SBP: ↓30.28 %, DBP: ↓40.71 %) with no statistically significant difference ($p > 0.05$). The VAE 1.0 (1000 mg/kg), and EA 30 (30 mg/kg) showed potent anti-inflammatory activity when reducing the total edematous paw volume significantly ($p < 0.01$) by ↓65.58 %, and ↓69.34 %, respectively, similar to Ibuprofen (7.5 mg/kg, ↓67.03 %). Besides, VAE (>500 mg/kg), and W 400 (water, 400 mg/kg) fraction extracts showed a potent analgesic effect equivalent to Para 50 (paracetamol 50 mg/kg) for the highest protection (>65 %) against the acetic acid-induced writhing after 35 min. Moreover, cepharanthine, cynaroside, and vernoniosides of VA leaf extract exhibited the highest affinity (>10 kcal/mol) in anti-inflammatory and analgesic targets (iNOS and COX-2) and antihypertensive targets (ACE and β_1 adrenoreceptor). Therefore, the crude and fraction extracts of VA leaves from the percolation method and bioactive metabolites are a potential source that can be developed into antihypertensive, anti-inflammatory, and analgesic agents in herbal medicine.

1. Introduction

Herbal plants remain a crucial source of drugs with diverse pharmacological activities used to maintain health, aid in the treatment and prevention of disease, and search for active molecules, such as phytochemicals, to fight various diseases such as hypertension, pain, and inflammation. Many disease treatments are carried out using herbal plants because there are few side effects when used long-term [1–3]. Therefore, the demand for herbal plants is increasing due to increasing scientific evidence confirming the health benefits of

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extracts and bioactive compounds (phenolics, flavonoids, alkaloids, cardiac glycosides, saponins, terpenoids, steroids, and tannins) isolated from plants. Herbal plants or natural products contribute to the provision of primary health care for approximately 80 % of the global population, especially being used as an important source of pharmaceuticals [4,5]. However, the need for more scientific evidence on the effectiveness, appropriate dosage, optimal extracts, and safety of herbal plants by *in vivo* testing is still a huge challenge.

Inflammation is a type of self-protective immune response of the body to infections, damaged cells, irritants, and injuries to destroy pathogens and promote wound healing [6]. However, uncontrolled inflammation leads to tissue damage and necrosis [7]. Pain, edema, heat, and redness are the four main signs associated with acute inflammation [8]. Pain is one of the common manifestations of inflammatory disorders and occurs as a result of nociceptive, inflammatory, or neuropathic conditions [9]. Currently, most anti-inflammatory and analgesic drugs are mainly synthetic chemical compounds, often causing side effects for patients such as gastrointestinal bleeding and cardiovascular effects. Therefore, finding natural products with anti-inflammatory and analgesic effects would be a safe and effective alternative because their pharmacological effects can be equivalent to synthetic compounds with minimal side effects.

On the other hand, hypertension is the largest factor of disease burden, impaired quality of life, and mortality globally by causing coronary artery disease, stroke, heart failure, and chronic kidney disease [10]. Hypertension is estimated to affect approximately 25 % of adults worldwide according to WHO and adults with hypertension will reach 1.56 billion by 2025 [11]. Unfortunately, approximately 90 % of hypertensive patients are classified as primary hypertension and the underlying mechanism remains unclear, especially as disease control remains unsatisfactory with many side effects when using currently available antihypertensive drugs. Therefore, natural antihypertensive products from herbal plants need to be researched to develop new treatment methods to effectively prevent cardiovascular complications.

Bitter leaf (*Vernonia amygdalina*, family Asteraceae) is a small shrub widely distributed in tropical Africa and Asia. It has a characteristic bitter taste and is commonly used as a medicinal plant [12,13]. *Vernonia amygdalina* (VA) leaves are part of the plant that accumulates the highest chemical and nutritional components such as fats, proteins, fibers, minerals, amino acids, carbohydrates, and vitamins, so VA leaves have become an important part of the human diet [14,15]. VA is used in traditional medicine to treat a variety of diseases including inflammation-associated diseases (such as malaria, dysentery, yellow fever, joint pain, and hepatitis), headaches, stomach pain, digestive disorders, urinary tract infections, high blood pressure, diabetes, and constipation as well as topical use for wound healing [16–18]. Besides, many bioactive components or phytochemicals have been identified and isolated in VA leaves such as steroid glycosides (vernoniosides, vernoniamyosides, vernoniamyosides, vernoamyosides, and veramyosides) [19,20], flavonoids (apigenin, luteolin, luteolin 7-O-glycoside, and luteolin 7-O-glucuronide) [15,21], sesquiterpene lactones (vernolide, vernodalol, vernoamygdalin, and vernolepin) [22,23] and bisabolane-type sesquiterpenoids (amygdanoids A-G) [24]. Furthermore, extracts and phytochemicals of VA have been reported to have diverse and potential pharmacological activities including anti-inflammatory [25–27], analgesic [28–30], antipyretic [31], treatment of cough, constipation, and hypertension [32], anti-allergic [33], antimalarial [34,35], hepatoprotective [36,37], nephroprotective [38,39], neuroprotective [40], antimicrobial [41,42], antioxidant [43], treat digestive disorders [44], hypolipidaemic [45], antidiabetic [46,47], anxiolytic and sedative [48], antimutagenicity [49], anti-leishmanial [50], hemolytic [51], spermatogenic [52], attenuations of dietary-induced obesity [38], treating typhoid [53], treating sexually transmitted diseases and intestinal infections urology [53] and anti-cancer [54,55].

On the other hand, up to now, there has been no report on the *in vivo* antihypertensive, anti-inflammatory, and analgesic activities of crude extract using the percolation method and fraction extracts of different polarities of solvents of VA leaves. Therefore, the present work aimed to conduct qualitative and quantitative analysis of phytochemicals (phenolics and flavonoids), phytocompound isolation, determination of acute toxicity, evaluation of antihypertensive, anti-inflammatory, and analgesic activities of VA leaf extracts by *in vivo* mice models, and *in silico* molecular docking studies for the screen the chemical constituents in VA leaf extract that are responsible for these biological activities.

2. Material and methods

2.1. Materials

Carrageenan, Ibuprofen, Aspirin, Paracetamol, Diclofenac, Captopril, Atropine sulfate, Xylometazoline hydrochloride, acetic acid, and solvents (hexane, ethyl acetate, chloroform, butanol, ethanol, and water) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All chemicals and reagents used in the present study were of analytical grade.

Vernonia amygdalina was collected from Dong Nai province, and identified by the Lac Hong University, Vietnam. A voucher specimen (LDVA.01.19) was deposited in the Faculty of Pharmacy, Lac Hong University. The freshly collected plant leaves were washed thoroughly in tap water, shaded at room temperature for 48 h, and then dried by drying oven Memmert (Germany) at 70 °C for 8 h. The dried leaves were ground into powder with a size of less than 2 mm and used for solvent extraction.

2.2. Experimental animals

Healthy adult male Swiss albino mice weighing 20–25 g were provided by Nha Trang Pasteur Institute (Vietnam). Mice were housed in polypropylene cages lined with husk to acclimatize for one week under the following conditions: 12 h light/dark cycle, temperature of 25 ± 2 °C, and relative humidity of 55 ± 10 %. Mice were fed on a standard pellet diet and had free access to water. All experiments were performed in compliance with the relevant laws and institutional guidelines (Animal Research Ethics Committee of Lac Hong University, No. LHU-01.2023 and Vietnam Animal Law, 45/2017/TT-BYT).

2.3. Extract preparation

The crude extract of *Vernonia amygdalina* was obtained by extracting the dried leaf powder (5 Kg) with 70 % ethanol (70 L) in the percolator apparatus. The extracted liquid was filtered and evaporated under reduced pressure using a rotary vacuum evaporator (Hei-VAP Gold 3, Heidolph, Germany) at 50 °C. Then, the extract was dried in a vacuum oven (VO29, Memmert, Germany) to obtain a concentrated leaf extract of *Vernonia amygdalina* (VAE) with humidity in the range of 10–12 %. VAE was shaken with different solvents (such as *n*-hexane - Hex, chloroform - CF, ethyl acetate - EA, n-butanol - Bu, and water - W) and evaporated to obtain the corresponding fractions for *in vivo* biological activity studies.

2.4. Phytochemical screening

The preliminary identification of the chemical constituents (polyphenols, alkaloids, tannins, saponins, anthraquinones, cardiac glycosides, coumarin, and flavonoids) from *Vernonia amygdalina* extracts was carried out using standard methods to detect the plant secondary metabolites [56]. Tentative identification of phytocompounds of potential fractions and extracts was performed using the LC-MS method.

2.5. Phytocompound isolation

The ethyl acetate fraction extract of *Vernonia amygdalina* (EA) was chromatographed on a silica gel column (Silica gel 60 - Merck - Germany, 0.040–0.063 mm). Solvent mixtures of increasing polarity composed of ethyl acetate and chloroform were used for elution, and the fractions were collected. The purity of all the fractions collected was determined by thin-layer chromatography on silica gel with methanol and petroleum ether (90:10 v/v) as the mobile phase. Spots were visualized by spraying the plates with a 254 nm UV lamp and 5 % $FeCl_3$ reagent in ethanol [3]. The column fractions that showed a single spot of the same Rf value were pooled. The structures of the isolated compounds were determined using MS spectra on the Agilent 1100 MC-LSD mass spectrometer and NMR spectra on the Bruker AM 500 FT-NMR spectrometer.

2.6. Total phenolic content

The total phenolic content (TPC) of the *Vernonia amygdalina* extracts was determined using the Folin-Ciocalteu spectrophotometric method in which gallic acid was used as a standard phenol [57]. To briefly illustrate, the reaction mixture consisted of 250 µl of Folin-Ciocalteu reagent (Merck), 250 µl of distilled water, and 250 µl of extract (1000 µg/ml) was mixed well in a test tube. Then, 250 µl Na_2CO_3 10 % solution was added, mixed well, and incubated for 30 min at 40 °C in a thermostatic bath. The spectral absorbance of the reaction mixture was measured at 765 nm. TPC results are presented as mg gallic acid equivalents (GAE) per g dry mass (mg GAE/g). TPC is calculated using the following formula: $C = C_1 \times V/m$, where C = total phenolic content (mg GAE (gallic acid equivalent)/g), C_1 = gallic acid concentration (mg/mL) calculated using the standard curve equation: $y = 0.0365x + 0.1083$ ($R^2 = 0.9902$), V = extract volume (ml) and m = extract weight (g). The measurements were performed in triplicate.

2.7. Total flavonoid content

Total flavonoid content (TFC) was determined by the aluminum chloride ($AlCl_3$) colorimetric method in which quercetin was used as a reference compound [58]. The reaction mixture consisted of 40 µl of $NaNO_2$ 5 % solution in 200 µl distilled water and 200 µl of extract (500 µg/ml) was incubated at room temperature for 5 min in a test tube. Then, 40 µl of $AlCl_3$ 10 % solution was added, mixed well, and let stand for 6 min. Finally, 400 µl of NaOH 1M solution and distilled water were added to make 1 ml. The absorbance of the mixture was measured at 510 nm using a UV–Vis spectrophotometer. TFC was calculated using the standard curve equation of quercetin ($y = 0.0041x + 0.0063$, $R^2 = 0.9913$). The results of TFC were expressed as milligrams of quercetin equivalent (QE) per gram of dry mass (mg QE/g). All measurements were carried out in triplicate.

2.8. Acute toxicity

Acute oral toxicity was performed in male and female Swiss albino mice according to OECD test guideline 423 [2,59]. Mice were randomly allocated into four groups of 12 mice each (6 males and 6 females). Mice were fasted overnight before the administration of test drugs in the toxicity study. The control mice group received only distilled water. Meanwhile, the other three mice groups were orally administered VAE at single doses of 1000 mg/kg, 5000 mg/kg, and 10000 mg/kg body weight. Animals were observed closely for mortality and clinical symptoms (such as increased motor activity, salivation, convulsion, coma, and death) of toxicity during the first 6 h after dosing. Subsequent observations were made at regular intervals for 24 h and the aminals were under more investigation for the period of 14 days. The number of mice that died and body weight within the study period were noted. LD_{50} (lethal dose in 50 %) was determined by the guideline principle if any from the geometric mean of the maximum dose survived by all mice and the minimum dose that caused a fatality in all mice in the experiment.

2.9. Antihypertensive activity

The Swiss albino mice with a diastolic blood pressure of 100–120 mmHg were randomly assigned to seven groups (8 mice each/n = 8), including a control group (daily gavaged with NaCl 0.9 % solution), a model group (without treatment and daily gavaged with NaCl 0.9 % solution), a Captopril treated group (daily gavaged with 100 mg/kg captopril), and four VAE treated groups with different doses (500 mg/kg - VAE 0.5, 1000 mg/kg - VAE 1, 1500 mg/kg - VAE 1.5, and 2000 mg/kg - VAE 2). The VAE 2, VAE 1.5, VAE 1, and VAE 0.5 groups were denoted as treated groups with *Vernonia amygdalina* crude extract. All doses were given by referring to the dosage in preliminary survey studies and other related reports [32,60]. After 30 min, the model group, Captopril group, and VAE groups were induced to increase blood pressure with atropine 1 mg/kg (SC - subcutaneous injection) combined with xylometazoline 1 mg/kg (IP - intraperitoneal injection). Immediately after injection, the blood pressure (systolic blood pressure - SBP and diastolic blood pressure - DBP) and heart rate (HR) were measured for 15 cycles (20 min) using the CODA high throughput system 21088 (noninvasive blood pressure measurement system, Kent Scientific Corporation, USA).

2.10. Anti-inflammatory activity

The anti-inflammatory evaluation was carried out using the carrageenan-induced paw edema method [25,26]. Before induction of inflammation, baseline recordings were performed by measuring the volume of the uninflamed left hind paws of mice. Paw edema was induced by injecting 25 μl of 1 % freshly prepared carrageenan into the subplantar surface of the left hind paw of mice, whereas the control group (n = 8) received an intraplantar injection of vehicle (25 μl of normal saline). The volume of the inflamed left hind paw was measured 3 h later using the Ugo Basile plethysmometer 37140 (Italy) to assess the effect of carrageenan on paw size. Mice whose paw volume increased by more than 50 % compared to the baseline were selected with 8 mice each (n = 8) for the model group (without treatment and daily gavaged with distilled water), Ibu 7.5 group (daily gavaged with 7.5 mg/kg ibuprofen), and treatment groups (daily gavaged with VAE and fraction extracts). The volume of the left hind paw of mice was measured after administering the drugs and extracts for 30 min each day for 6 days, to ascertain the effect of VAE (500, 1000, 1500, and 2000 mg/kg) and fraction (30 mg/kg - EA 30, Hex 30, CF 30; 80 mg/kg - Bu 80; 800 mg/kg - W 800) extracts on the inflamed left paw. The percentage of change in paw edema was calculated according to the formula:

$$\text{Paw edema } (\%) = \frac{V_t - V_0}{V_0}\, x\, 100$$

Where V_0 is paw volume of normal mice (ml), V_t is paw volume of inflamed mice after administering the drugs and extracts (ml).

2.11. Analgesic activity

The analgesic evaluation was performed using the acetic acid-induced writhing test to detect the peripheral analgesic activity [28–30]. Mice were randomly divided into twelve groups (8 mice each, n = 8) including a model group (without treatment and daily gavaged with distilled water), a Para 50 treated group (daily gavaged with 50 mg/kg paracetamol), four VAE treated groups with different doses (100 mg/kg - VAE 0.1, 500 mg/kg - VAE 0.5, 1000 mg/kg - VAE 1.0, and 2000 mg/kg - VAE 2), and six treated groups with fraction extracts (15 mg/kg - Hex 15, CF 15, EA 15; 20 mg/kg - Bu 20; 40 mg/kg - Bu 40, 400 mg/kg - W 400). After orally administering the drug and extracts for 30 min, mice were administered with IP injection of acetic acid solution (0.7 %, 0.1 ml/10 g body weight). The number of writhing movements (the contractions of abdominal muscles together with stretching of the hind limbs) was recorded for each mouse at 3 times (5–10, 20–25, and 35–40 min) to assess the analgesic activity of extracts. The pain inhibition (%) was calculated as follows:

$$\text{Pain inhibition } (\%) = \frac{A_0 - A_1}{A_0}\, x\, 100$$

Where A_0 is the number of writhing movements in the model group, A_1 is the number of writhing movements in tested groups.

Table 1
Phytochemical analysis of crude and fraction extracts of *Vernonia amygdalina* leaves.

	VAE	W	Bu	CF	EA	Hex
Phenolics	+	+	+	+	+	+
Alkaloids	+	+	+	+	+	+
Saponins	+	+	+	+	+	–
Tannins	+	+	+	+	+	+
Anthraquinones	+	+	–	–	–	–
Steroids	+	+	+	+	+	–
Flavonoids	+	+	+	+	+	+
Cardiac glycosides	+	+	–	–	–	–

(+) – present, (−) – absent, VAE – crude extract of Vernonia amygdalina leaf, fraction extracts include W – water extract, Bu -butanol extract, CF – chloroform extract, EA - ethyl acetate extract, Hex – hexane extract.

Table 2
Total phenol content (TPC) and total flavonoid content (TFC) of *Vernonia amygdalina* leaf extracts.

	TPC (mg GAE/g)	TFC (mg QE/g)
VAE	273.25 ± 1.87*	112.57 ± 1.46*
W	184.71 ± 1.26	92.50 ± 0.93
Bu	**295.01 ± 2.13**$^{*,\#}$	**132.38 ± 1.88**$^{*,\#}$
CF	99.16 ± 1.32	68.65 ± 0.97
EA	**284.34 ± 1.95**$^{*,\#}$	**162.38 ± 1.90**$^{*,\#}$
Hex	35.68 ± 1.02	27.79 ± 1.14

VAE – crude extract of Vernonia amygdalina leaf, fraction extracts include W – water extract, Bu -butanol extract, CF – chloroform extract, EA - ethyl acetate extract, Hex – hexane extract, * - $p < 0.05$ compared to W, # - $p < 0.05$ compared to VAE.

Table 3
Tentative identification of chemical constituents of ethyl acetate fraction using LC-MS.

Entry	Retention Time (min)	Relative Area (%)	Relative Height (%)	*m/z*		Predicted phytocompounds
1	5.64	7.04	8.69	288.24	+	Cyanopindolol
2	6.78	**16.55**	16.93	289.36	+	**Luteolin/Luteolin glucoside**
3	7.85	6.69	8.98	291.81	+	10-Geranilanyl-O-D-yloside
4	8.75	2.80	3.33	664.48	–	Vernonioside D
5	10.63	8.30	8.67	285.16	–	**Luteolin/Luteolin glucoside**
6	11.56	2.39	2.12	482.81	+	Hydroxyursdienoic acid olide
7	12.35	2.58	2.16	269.40	+	Isocryptolepine
8	12.88	3.70	4.21	267.94	–	**Apigenin**
9	13.02	0.00	0.00	–		–
10	13.41	2.39	3.18	606.89	–	Cepharanthine
11	14.19	3.72	3.95	379.03	+	Deoxyvernodalol/Hydroxyvernolide/Vernodalinol
12	14.84	4.73	4.28	233.32	+	Neocryptolepine/Cryptolepine
13	15.51	**8.84**	5.23	449.08	+	Cynaroside
14	16.25	4.47	4.43	377.15	–	Deoxyvernodalol/Hydroxyvernolide/Vernodalinol
15	18.27	**21.79**	20.11	647.56	–	Vernonioside B1
16	19.50	4.00	3.73	394.25	–	Vernodalol

2.12. In silico molecular docking studies

The structure of phytocompounds and the standard drug were drawn in ChemBioDraw Ultra 18.0. The energy of each molecule was minimized using ChemBio3D Ultra 18.0. The ligand molecules were then used as input for AutoDock Vina, in order to carry out the docking simulation [61,62]. Protein structures of nitric oxide synthase (iNOS, PDB: 2NSI), cyclooxygenase-2 (COX-2, PDB: 6COX), angiotensin-converting enzyme (ACE, PDB: 1O8A), and β1 adrenoreceptor (PDB: 4BVN) were retrieved from the protein data bank. The water molecules in the receptors were removed and polar hydrogen and Kollman charges were added. The Graphical User Interface program BMGL Tools was used to set the grid box for docking simulations. Auto Dock Vina was compiled and run under Windows 10.0 Professional operating system. Discovery Studio 2020 was used to deduce the pictorial representation of the interaction between the ligands and the target protein [63–66].

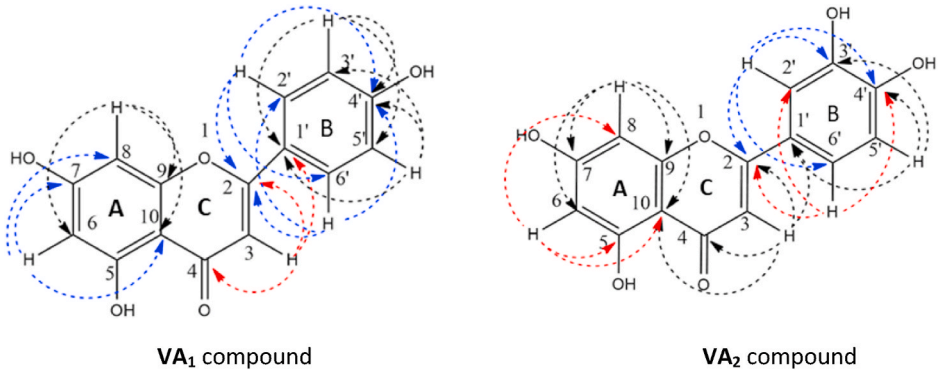


Fig. 1. Chemical shift value and summary HMBC correlation for VA_1 and VA_2 compounds.
(VA_1 - identified as apigenin, VA_2 - identified as luteolin).

Table 4
Tabulation of chemical shift data from ^{1}H NMR,^{13}C NMR, and HMBC spectra of bioactive isolate VA_1 versus reference.

Position		VA_1 (500 MHz, DMSO-d_6)			Apigenin [72] (500 MHz, DMSO-d_6)	
		δ13C	δ1H	HMBC (H-C)	δ13C	δ1H
2	>C =	163.7			163.7	
3	-CH =	102.8	6.77 (*s*, 1H)	1′, 2, 4	102.8	6.75 (*s*, 1H)
4	>C =	181.7			181.7	
5	>C =	161.4			161.3	
6	-CH =	98.8	6.19 (*d*, 1H, *J* = 2.0 Hz)	7, 8, 10	98.9	6.15 (*d*, 1H, *J* = 1.95 Hz)
7	>C =	164.4			164.6	
8	-CH =	93.9	6.48 (*d*, 1H, *J* = 2.0 Hz)	6, 9, 10	94.1	6.44 (*d*, 1H, *J* = 1.95 Hz)
9	>C =	157.3			157.4	
10	>C =	103.6			103.6	
1′	>C =	121.1			121.2	
2′, 6′	-CH =	128.4	7.92 (*d*, 2H, *J* = 8.5 Hz)	2, 4′, 6’/2′	128.5	7.91 (*d*, 2H, *J* = 9.05 Hz)
3′, 5′	-CH =	115.9	6.92 (*d*, 2H, *J* = 9.0 Hz)	1′, 4′, 5’/3′	116.0	6.90 (*d*, 2H, *J* = 9.05 Hz)
4′	>C =	161.1			161.5	
5-OH			12.95 *s*			

Table 5
Tabulation of chemical shift data from ^{1}H NMR,^{13}C NMR, and HMBC spectra of bioactive isolate VA_2 versus reference.

Position		VA_2 (500 MHz, DMSO-d_6)			Luteolin [73] (500 MHz, DMSO-d_6)	
		δ13C	δ1H	HMBC (H-C)	δ13C	δ1H
2	>C =	163.9			163.92	
3	-CH =	102.8	6.65 (*s*, 1H)	1′, 2, 4, 10	102.91	6.67 *s*
4	>C =	181.6			181.70	
5	>C =	161.4			161.52	
6	-CH =	98.8	6.17 (*d*, 1H, *J* = 2.0 Hz)	5, 8, 10	98.87	6.19 (*d*, 1H, *J* = 2.0 Hz)
7	>C =	164.2			164.16	
8	-CH =	93.8	6.43 (*d*, 1H, *J* = 2.0 Hz)	6, 7, 9, 10	93.88	6.45 (*d*, 1H, *J* = 2.0 Hz)
9	>C =	157.3			157.32	
10	>C =	103.6			103.74	
1′	>C =	121.4			121.56	
2′	-CH =	113.3	7.38 (*d*, 1H, *J* = 2.0 Hz)	2, 3′, 4′, 6′	113.4	7.42 (*m*, 1H)
3′	>C =	145.7			145.77	
4′	>C =	149.8			149.73	
5′	-CH =	116.0	6.88 (*d*, 1H, *J* = 8.5 Hz)	1′, 3′, 4′	116.05	6.90 (*d*, 1H, *J* = 8.0 Hz)
6′	-CH =	118.9	7.40 (*dd*, 1H, *J* = 2.0, 8.0 Hz)	2, 2′, 4′	119.02	7.42 (*m*, 1H)
5-OH			12.96 *s*			

2.13. Statistical analysis

All values are expressed in Mean ± SEM (Standard Error of Mean) from three independent experiments. The difference of groups was analyzed by Mann-Whitney analysis using Minitab 18.0 software. The results were considered statistically significant if $p < 0.05$, more significant if $p < 0.01$, and highly significant if $p < 0.001$ as compared with the background group. The chart is drawn using Microsoft Excel 2022 software.

3. Results and discussion

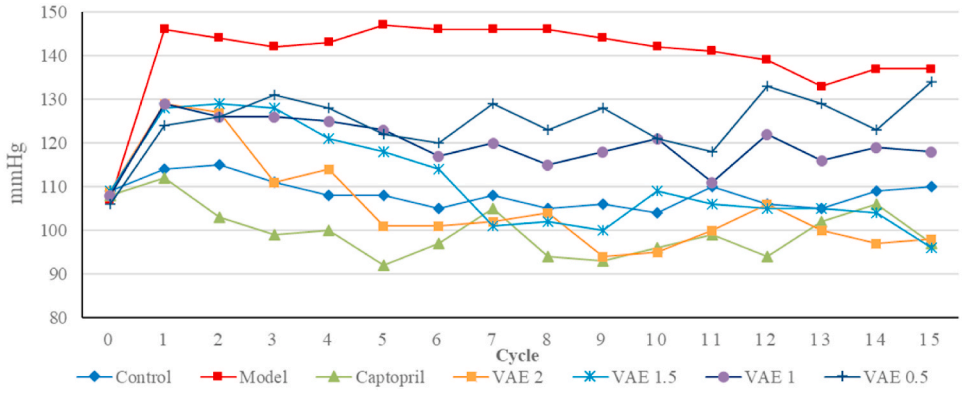
3.1. Phytochemical screening

Phytochemicals are naturally occurring bioactive compounds known for their health benefits. The results of the preliminary qualitative phytochemical screening on the six types of extracts are shown in Table 1. Phytochemical analysis of crude extracts (VAE) and fractions (W, Bu, CF, EA, Hex) of *Vernonia amygdalina* leaf revealed the presence of phenolics, alkaloids, tannins, and flavonoids as described previously [13,22,27,67,68]. Besides, saponins and steroids were present in VAE, W, Bu, CF, and EA, but these compounds were absent in Hex. On the other hand, tannins were detected in VAE and W but were absent in Bu, CF, EA, and Hex (Table 1).

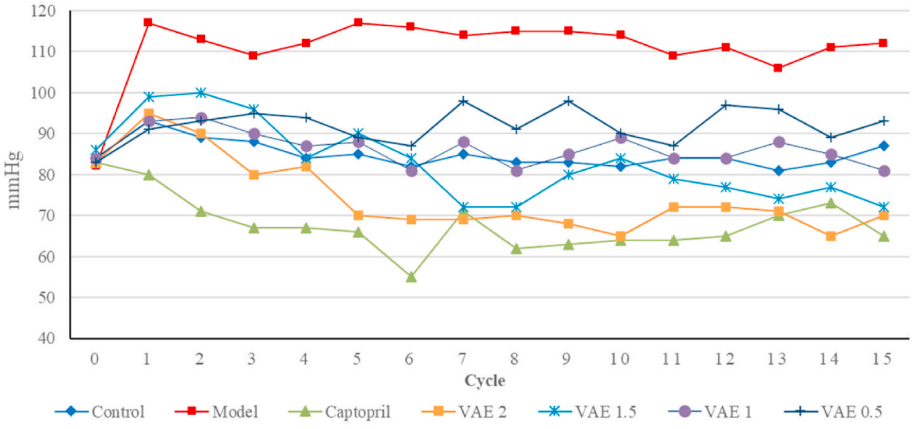
3.2. Total phenol content (TPC) and total flavonoid content (TFC)

Phenolics and flavonoids are secondary metabolites that are commonly found in plants. Table 2 shows the TPC and TFC of *Vernonia amygdalina* extracts. The high TPC and TFC were in VAE, Bu, and EA extracts and the low TPC and TFC were in W, CF, and Hex extracts. The highest TPC was in Bu extract (TPC = 295.01 mg GAE/g) and the highest TFC was in EA extract (TFC = 162.38 mg QE/g). The

Systolic blood pressure (SBP)



Diastolic blood pressure (DBP)



Heart rate (HR)

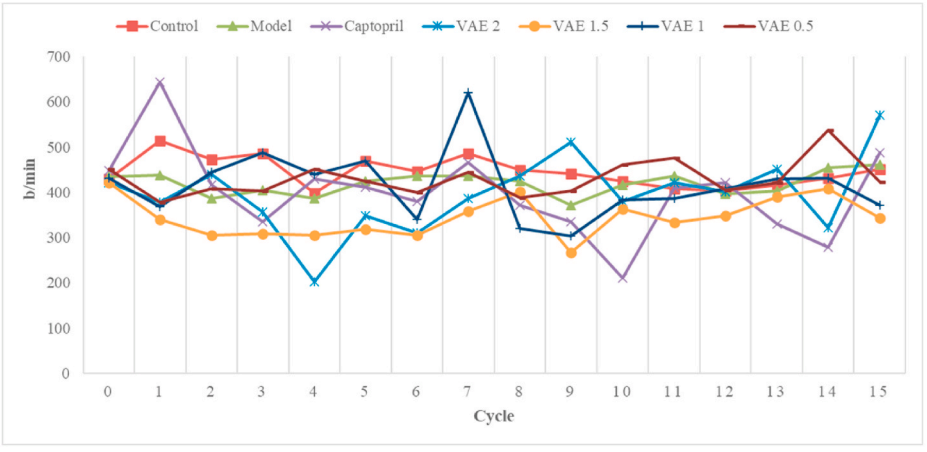


Fig. 2. Heart rate (HR), systolic blood pressure (SBP), and diastolic blood pressure (DBP) of mice among the tested groups during fifteen cycle. **VAE** represents *Vernonia amygdalina* leaf extract with different doses (500 mg/kg - VAE 0.5, 1000 mg/kg - VAE 1, 1500 mg/kg - VAE 1.5, and 2000 mg/kg - VAE 2).

lowest TPC and TFC were in Hex extract (TPC = 35.68 mg GAE/g, TFC = 27.79 mg QE/g). The phenolic and flavonoid levels in crude extract VAE were 273.25 mg GAE/g and 112.57 mg QE/g, respectively. Meanwhile, fraction extracts including Bu (TPC = 295.01 mg GAE/g, TFC = 132.38 mg QE/g) and EA (TPC = 284.34 mg GAE/g, TFC = 162.38 mg QE/g) demonstrated significantly higher TPC and TFC compared to crude extract VAE ($p < 0.05$). In addition, VAE, Bu, and EA extracts contained significantly ($p < 0.05$) higher polyphenol and flavonoid contents compared to the W extract (TPC = 184.71 mg GAE/g, TFC = 92.50 mg QE/g).

The highest TPC and TFC from VA leaves collected in Malaysia with ethanolic microwave-assisted extraction are 113.76 mg GAE/g and TFC 94.08 mg QE/g, respectively using response surface methodology as an optimization tool [69]. TPC (108.25 mg/g Catechin equivalent) and TFC (189.52 mg/g GAE) were found in methanol extract from VA leaves collected in Nigeria with an extraction time of 72 h using 90 % methanol solvent [40]. The ethanol extract of VA leaves from Indonesia was found to contain TPC (54.61 mg GAE/g) and TFC (22.53 mg QE/g) using ethanol 100 % solvent with the maceration method [70]. TPC (33.54–281.58 mg GAE/g) and TFC (1.59–3.79 mg QE/g) in VA leaf extracts were also determined when extracted with different solvents such as ethanol (TPC = 281.58 mg GAE/g, TFC = 3.79 mg QE/g), ethyl acetate (TPC = 61.15 mg GAE/g, TFC = 3.65 mg QE/g), and hexane (TPC = 33.54 mg GAE/g, TFC = 1.59 mg QE/g) for three days with the maceration method at a room temperature [71]. Also with this method, 96 % ethanol extract of VA leaves in Indonesia showed TPC and TFC of 21.47 mg GAE/g and 25.62 mg QE/g, respectively. The difference in TPC and TFC levels in the studies may be due to the extraction method, types of solvent, solvent concentration, and some physical factors as well as the growing conditions of the plant herbs [3]. Generally, the study results showed that TPC and TFC of crude extract and fractions of VA leaves collected in Vietnam were both at high levels with the percolation method compared to published studies [40,69–71]. The Bu, EA, and VAE extracts exhibited similar TPC (273.25–295.01 mg GAE/g) but about 30–43 times higher TFC compared to Syahputra et al. [71]. In contrast, these extracts showed similar TFC (112.57–162.38 mg QE/g) but about 2.5–2.7 times higher TPC compared to Oladele et al. [40].

3.3. Phytocompound isolation

The present study comprehensively determined the components of the ethyl acetate fraction of *Vernonia amygdalina* using LC-MS analysis (Table 3). The phytocompounds including vernonioside B1, cynaroside, and luteolin/luteolin glucoside showed the highest relative peak area percentage in the EA fraction. Besides, the two isolated compounds **VA_1** and **VA_2** from *Vernonia amygdalina* ethyl acetate fraction were identified as known flavonoids apigenin and luteolin, respectively (Fig. 1). These structures of flavonoids were confirmed by ^{1}H NMR, ^{13}C NMR, HMBC, HSQC, COSY, and MS spectroscopy (Tables 4 and 5). The presence of apigenin and luteolin in VA extract has been reported [15,21]. However, these flavonoids were first isolated and structurally determined in the ethyl acetate fraction of *Vernonia amygdalina* leaves.

VA_1 compound: yellow powder, a single spot-on the TLC plat over a silica gel 60 F254, blue color with $FeCl_3$ reagent, UV (MeOH) λ_{max} (log ε) 268, 333 nm. ^{1}H NMR (500 MHz, DMSO-d_6, δ ppm), ^{13}C NMR (125 MHz, MeOD, δ ppm), DEPT, and HMBC spectroscopic data were shown in Table 4. The HSQC correlation includes δ_H 7.92 with δ_C 128.4; δ_H 6.92 with δ_C 115.9; δ_H 6.77 with δ_C 102.8; δ_H 6.48 with δ_C 93.9; δ_H 6.19 with δ_C 98.8. The COSY correlation includes δ_H 7.92 with δ_H 6.92; δ_H 6.48 with δ_H 6.19. ESI-MS (m/z) $[M - H]^-$ calcd for $C_{15}H_9O_5$ 269.0455, found 269.29.

The ESI-MS of isolated compound VA_1 was performed in negative ion mode and molecular ion peak was observed at m/z 269.29 $[M - H]^-$. The ^{1}H NMR spectra of compound VA_1 in DMSO-d_6 exhibited five peaks resonating between δ_H 6.19 and 7.92 ppm integrated to seven protons and confirming the presence of seven aromatic protons. These peaks are assignable to (i) one isolated proton at δ_H 6.77 (s, 1H, H-3), (ii) six spin-spin coupling protons at δ_H 6.19 (d, 1H, $J = 2.0$ Hz, H-6), 6.48 (d, 1H, $J = 2.0$ Hz, H-8), 7.92 (d, 2H, $J = 8.5$ Hz, H-2′,6′), and 6.92 (d, 2H, $J = 9.0$ Hz, H-3′,5′). In addition, the ^{13}C NMR spectra of compound VA_1 exhibited 13 sp^2-hybridized carbon signals between δ_C 93.9 and 181.7 ppm. Seven carbons were identified as methine carbons while the remaining were quaternary ones according to the DEPT spectra. Furthermore, the HSQC, HMBC, and COSY spectra showed the correlation of H-C and H-H, which were in complete agreement with the structure of apigenin.

VA_2 compound: yellow powder, a single spot-on the TLC plat over a silica gel 60 F254, blue color with $FeCl_3$ reagent, UV (MeOH) λ_{max} (log ε) 255, 350 nm. ^{1}H NMR (500 MHz, DMSO-d_6, δ ppm), ^{13}C NMR (125 MHz, MeOD, δ ppm), DEPT, and HMBC spectroscopic data were shown in Table 5. The HSQC correlation includes δ_H 7.40 with δ_C 118.9; δ_H 7.38 with δ_C 113.4; δ_H 6.88 with δ_C 116.0; δ_H 6.65

Table 6
Blood pressure and heart rate of mice among the tested groups of *Vernonia amygdalina* leaf extract.

Group	Physiological index			Average index over 15 cycles		
	SBP *(mmHg)*	DPB *(mmHg)*	HR *(b/min)*	SBP *(mmHg)*	DPB *(mmHg)*	HR *(b/min)*
Control	109 ± 5	84 ± 4	429 ± 33	108 ± 3	85 ± 3	447 ± 34
Model	107 ± 3	82 ± 3	434 ± 24	142 ± 4$^{###}$	113 ± 3$^{###}$	419 ± 26
Captopril	108 ± 3	83 ± 2	448 ± 21	99 ± 5***	67 ± 6***	395 ± 99
VAE 2	109 ± 3	83 ± 2	422 ± 31	**105 ± 11*****	**74 ± 9*****	394 ± 87
VAE 1.5	109 ± 3	86 ± 3	422 ± 26	**111 ± 11*****	**83 ± 10****	340 ± 40
VAE 1	108 ± 3	84 ± 3	431 ± 16	120 ± 5	**87 ± 14****	414 ± 78
VAE 0.5	106 ± 4	83 ± 2	452 ± 44	126 ± 5	92 ± 4	428 ± 41

SBP - systolic blood pressure, DBP - diastolic blood pressure, HR - heart rate, b/min - beats per minute, ** - $p < 0.01$; *** - $p < 0.001$ compared to model group (group of hypertensive mice), $^{###}$ - $p < 0.001$ compared to control group.

with δ_C 102.8; δ_H 6.43 with δ_C 93.8; δ_H 6.17 with δ_C 98.8. The COSY correlation includes δ_H 7.40 with δ_H 6.88; δ_H 6.43 with δ_H 6.17. ESI-MS (m/z) $[M - H]^-$ calcd for $C_{15}H_9O_6$ 285.0405, found 285.18.

The ESI-MS of isolated compound VA_2 was performed in negative ion mode and molecular ion peak was observed at m/z 285.18 $[M - H]^-$. The ^1H NMR spectra of compound VA_2 in DMSO-d_6 exhibited five peaks resonating between δ_H 6.17 and 7.40 ppm integrated to six protons and confirming the presence of six aromatic protons. These peaks are assignable to (i) one isolated proton at δ_H 6.65 (s, 1H, H-3), (ii) five spin-spin coupling protons at δ_H 6.17 (d, 1H, $J = 2.0$ Hz, H-6), 6.43 (d, 1H, $J = 2.0$ Hz, H-8), 7.38 (d, 1H, $J = 2.0$ Hz, H-2′), 6.88 (d, 1H, $J = 8.5$ Hz, H-5′), and 7.40 (dd, 1H, $J = 2.0$, 8.0 Hz, H-6'). In addition, the ^{13}C NMR spectra of compound VA_2 exhibited 15 sp^2-hybridized carbon signals between δ_C 93.8 and 181.6 ppm. Six carbons were identified as methine carbons while the remaining were quaternary ones according to the DEPT spectra. Furthermore, the HSQC, HMBC, and COSY spectra showed the correlation of H-C and H-H, which were in complete agreement with the structure of luteolin.

3.4. Acute toxicity

The acute oral toxicity of VAE was determined to demonstrate safety for pharmaceutical applications. The maximum dose of VAE extract that can be given orally to mice is 10,000 mg/kg body weight. All mice in the group given the 1,000, 5,000, and 10,000 mg/kg doses did not die during the 14-day observation period. There was no significant difference in body weight and no toxic signs observed in clinical parameters in the tested groups compared to the control group. Moreover, gross dissection of the dead animal did not reveal any obvious damage to tissues or organs. Therefore, the no-observed adverse effect level (NOAEL) was determined in the maximum dose of 10,000 mg/kg body weight and LD_{50} can be found at doses greater than 10,000 mg/kg body weight.

In similar studies, LD_{50} of acetone and 80 % methanol extracts of VA leaves could be 1600 mg/kg and greater than 2000 mg/kg body weight, respectively using the maceration method [27,74]. In particular, VA ethanol and aqueous extract are considered safe to be administered due to LD_{50} being greater than 5000 mg/kg body weight [28,75]. Therefore, the LD_{50} result of this study can be considered similar to Tijjani et al. and Zakaria et al. [28,75], and it would be easy to assume that this plant is safe.

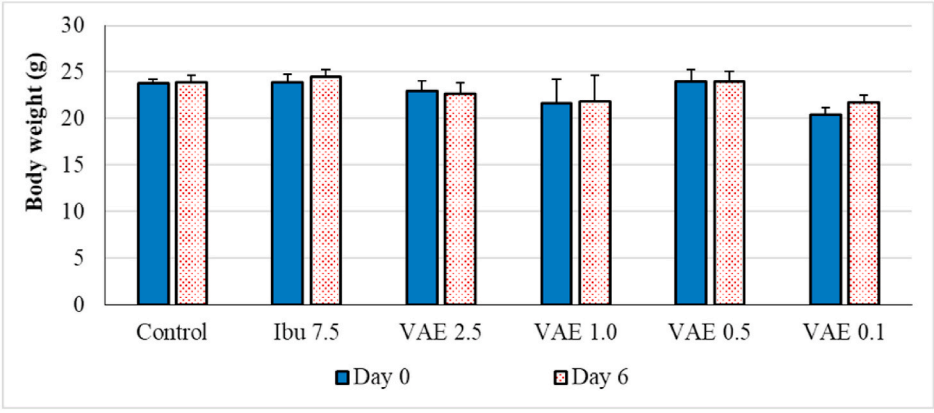
3.5. Antihypertensive activity

No mice died during the entire experiment. The slight down-regulation of HR (heart rate) was observed among all of the tested groups. After treatment, the average HR of mice in the model, VAE 1, and VAE 0.5 treated groups (>400 b/min) were higher the that of other groups in 15 cycles. The average HR of mice in the VAE 0.5 treated group (low-dose group) was equivalent to that of the control group meanwhile the average HR of mice in the VAE 2.0 treated group (high-dose group) was equivalent to that of the Captopril group. Low-dose VAE-treated group showed a better effect on HR variation than high-dose VAE-treated groups, suggesting that dose affects HR. However, the average HR did not show a significant difference between the tested groups and the control group.

The blood pressure of the control group (average SBP (systolic blood pressure) = 108 ± 3 mmHg, average DBP (diastolic blood pressure) = 85 ± 3 mmHg) demonstrated stabilization with systolic blood pressure in the range of 104–115 mmHg and diastolic blood pressure in the range of 81–93 mmHg. On the contrary, the blood pressure of the model group (SBP = 142 ± 4 mmHg, DBP = 113 ± 3 mmHg) showed a clear increasing trend compared to the control group. The difference in SBP and DBP of these two groups is statistically significant ($p < 0.05$). The blood pressure of the Captopril group (SBP = 99 ± 5 mmHg, DBP = 67 ± 6 mmHg) showed an obvious decreasing trend compared to the model group. This showed the effectiveness of Captopril in lowering systolic and diastolic blood pressure at a dose of 100 mg/kg.

The effects of different *Vernonia amygdalina* extracts with different doses on SBP and DBP in mice revealed similar trends in the range between cycle 1 and cycle 15 (about 20 min) after VAE administration (Fig. 2). Simultaneously, mice's average SBP and DBP descended in *Vernonia amygdalina* extract-treated groups compared to the model group. The high-dose treated groups displayed a more significant impact on blood pressure reduction compared to the low-dose treated group, implying dose of VAE domain a critical role in lowering blood pressure. In particular, the strongest decrease in blood pressure was shown in groups VAE 2 (SBP: 93–126 mmHg, DBP: 65–91 mmHg) and VAE 1.5 (SBP: 96–126 mmHg, DBP: 65–91 mmHg). In addition, SBP and DBP of groups VAE 2 (SBP: ↓26.05 % (37 mmHg), DBP: ↓34.51 % (34 mmHg)), VAE 1.5 (SBP: ↓21.83 % (31 mmHg), DBP: ↓26.55 % (30 mmHg)) and Captopril group (SBP: ↓30.28 % (43 mmHg), DBP: ↓40.71 % (46 mmHg)) showed a good decreasing trend compared to the model group (Table 6). Meanwhile, SBP and DBP of groups VAE 1 (SBP: ↓15.49 % (22 mmHg), DBP: ↓23.00 % (26 mmHg)) and VAE 0.5 (SBP: ↓11.27 % (16 mmHg), DBP: ↓18.58 % (21 mmHg)) showed a less than twofold decreasing trend compared to the Captopril group. Moreover, the average SBP and DBP of the VAE 2.0 and VAE 1.5 groups were not statistically significant compared to the control group ($p > 0.05$). The Captopril and VAE 2.0 groups demonstrated the best efficiency in lowering blood pressure among the tested groups. The antihypertensive effect of the VAE 2.0 group was nearly equivalent to the reference drug Captopril with no statistically significant difference (SBP: $p > 0.05$, DBP: $p > 0.01$). This is a potential dose for the treatment direction of *Vernonia amygdalina* extract in supporting blood pressure lowering.

The aqueous leaf extract of *Vernonia amygdalina* showed a good antihypertensive effect using intravenous administration on rats and cats. The high doses of VA aqueous extract (50 and 100 mg/kg) from the Soxhlet method caused a more significant reduction in mean arterial pressure in rats than the lower doses, the initial temporary elevation in blood pressure was not observed [76]. On the other hand, VA leaf extracts significantly reduced blood pressure and showed a more effective response to an intravenous dose of 1 mg/mL but had a lower antihypertensive effect compared to the standard drug acetylcholine [77]. In particular, VA methanol extract from the maceration method for 5 days significantly ($p < 0.05$) reduced blood pressure, heart rate, and blood volume after 4 weeks with oral doses of 200 mg/kg and 400 mg/kg body weight compared to reference drug lisinopril (30 mg/kg) in a dose-dependent



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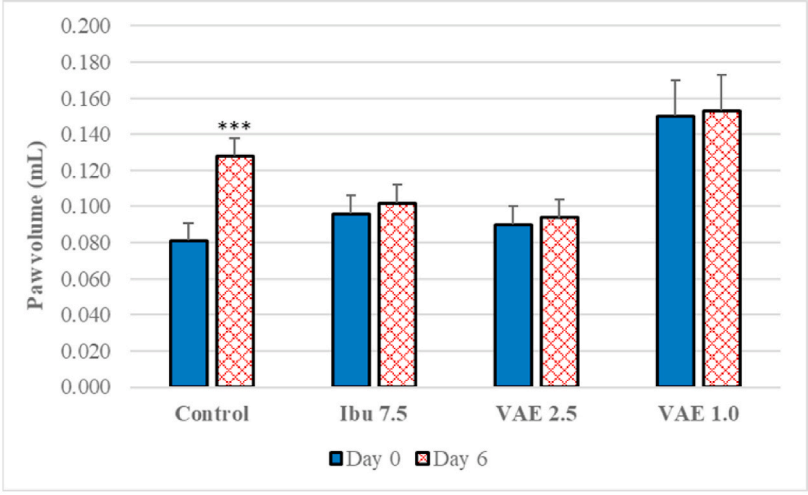

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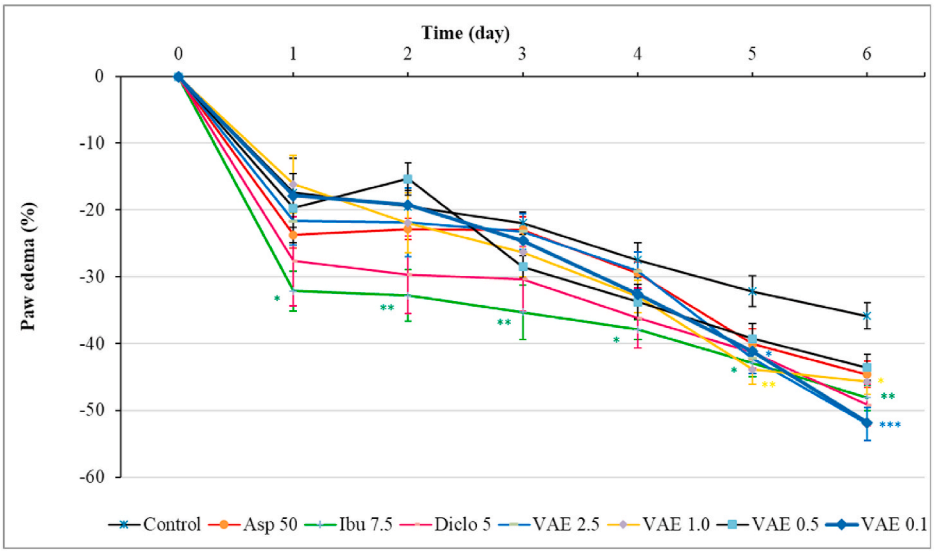

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Fig. 3. Anti-inflammatory activity of crude extract of *Vernonia amygdalina* leaf on carrageenan-induced edema of mice.
A - Body weight of mice after 6 days of treatment, **B** - Paw volume (mL/cm^3) of mice after 6 days of treatment (*** - $p < 0.001$ compared to day 0), **C** - Reduction of paw edema (%) over time (* - $p < 0.05$, ** - $p < 0.01$; ***, $p < 0.001$ compared to control group; Asp 50 - aspirin 50 mg/kg; Ibu 7.5 - ibuprofen 7.5 mg/kg; Diclo 5 - diclofenac 5 mg/kg; **VAE** represents *Vernonia amygdalina* leaf extract with different doses (100 mg/kg - VAE 0.1, 500 mg/kg - VAE 0.5, 1000 mg/kg - VAE 1.0, and 2500 mg/kg - VAE 2.5)).

manner [60]. This study also showed that the VA ethanol extract from the percolation method exhibited a dose-dependent antihypertensive effect. High dose VAE 2.0 (2000 mg/kg body weight) showed the best antihypertensive effect with efficacy equivalent to the reference drug Captopril with blood pressure returning to normal after 15 measurement cycles (about 20 min). The blood-lowering effect is faster and stronger the higher the dose of VAE extract. These results are an indication that VAE 2.0 can be a potential candidate for the treatment of acute hypertension.

Currently, in addition to lifestyle changes, it is also necessary to use pharmaceutical drugs daily and lifelong to control blood pressure. However, pharmaceutical drugs often have many unwanted effects. Therefore, drugs derived from medicinal herbs are an alternative with fewer side effects [78]. Besides, maintaining normal blood pressure depends on the balance between cardiac output and peripheral vascular resistance as well as the regulation of blood pressure through the autonomic nervous system and the renin-angiotensin-aldosterone system [79]. Therefore, the blood pressure-lowering mechanism of VA extract may act through one or a combination of blood pressure-regulating factors, so more research needs to be done to clarify.

3.6. Anti-inflammatory activity

3.6.1. Crude extract VAE

The tested groups (Ibu 7.5, VAE 2.5, and VAE 1.0) and the control group (no treatment) showed no significant difference in body weight before and after treatment ($p > 0.05$) (Fig. 3A). This showed that body weight did not change significantly and did not affect the paw volume of mice during the test.

Fig. 3B showed the inhibitory effect of the crude extracts (2500 and 1000 mg/kg) and reference drug (Ibuprofen 7.5 mg/kg) on carrageenan-induced paw edema in mice. The control group (no treatment) showed a statistically significant difference in paw volume on day 6 compared to day 0 with a p-value <0.05 ($p = 0.0009$). In contrast, the paw volume of mice on day 6 exhibited no statistically significant difference compared to day 0 in groups Ibu 7.5, VAE 2.5, and VAE 1.0 with p-value >0.05. The study results showed that high-dose VAE extract reduced inflammation through a significant reduction of paw edema to normal after 6 days of treatment similar to the reference drug Ibuprofen. The inhibitory effect of VAE on the increase in paw edema is shown in Fig. 3C. The reduction in paw volumes in animals treated with VAE and reference drugs (Ibuprofen, Aspirin, Diclofenac) for 6 days was significantly higher ($p < 0.05$) than in untreated animals (control group). VAE (2500 and 1000 mg/kg) showed dose-dependent inhibition of edema (↓52.02 %, $p < 0.001$ and ↓45.72 %, $p < 0.05$) at day 6 post carrageenan injection. At the same time, Ibuprofen (7.5 mg/kg) also showed significant inhibition (↓48.08 %, $p < 0.01$) on the increase in paw volume at day 6.

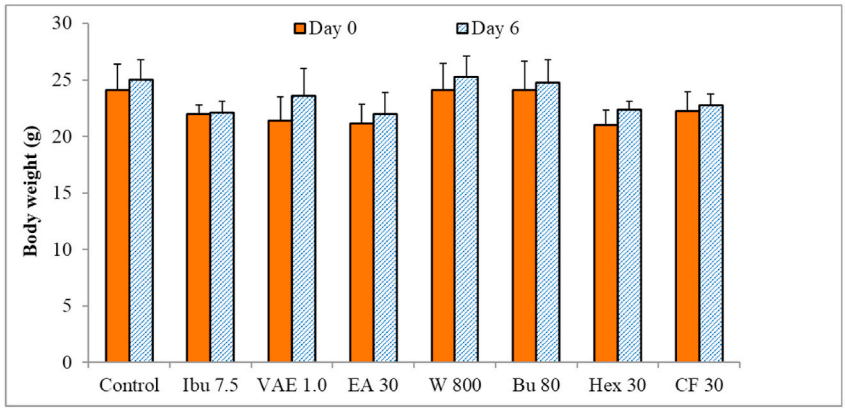
3.6.2. Fraction extracts

The body weight of mice in all groups at pre- and post-test showed no statistically significant differences. This demonstrated that body weight did not change significantly or affect the paw volume of mice during the anti-inflammatory test (Fig. 4A).

Edema was observed 2 h post-injection on day 0 by carrageenan in the left hind paw. An average increase in paw volume of 75.16 % (73.22–77.68 %) was observed in the control and tested groups as shown in Fig. 4C. Treatment with the fraction extracts showed a significant dose-dependent reduction ($p < 0.05$) in edema similar to that of the VAE crude extracts. Based on preliminary testing, this study was conducted to select the optimal dose for each fractional extract (W 800, EA30, Bu 80, Hex 30, and CF 30).

In the paw edema test, the ethyl acetate leaf extract of *Vernonia amygdalina* (30 mg/kg, EA 30) caused a significant decrease in paw edema induced by carrageenan compared to the control group. More specifically, the paw volume on day 6 showed a significant difference ($p < 0.01$) compared to day 0 (before carrageenan injection) in the control, W 800, Bu 80, Hex 30, and CF 30 groups. This showed that the paw volume did not recover to normal without treatment or treatment for 6 days with W 800, Bu 80, Hex 30, and CF 30. The paw edema (%) of these groups tended to decrease but was not statistically significant ($p > 0.05$) when compared to the control group (Fig. 4C). Therefore, these groups exhibited weak anti-inflammatory effects under experimental conditions. In contrast, the paw volume on day 6 showed no significant difference ($p > 0.05$) compared to day 0 (before carrageenan injection) in the two groups Ibu 7.5 ($p = 0.270$) and EA 30 ($p = 0.092$) groups (Fig. 4B). This proved that the paw volume has recovered to normal after treatment with Ibu 7.5 and EA 30. Besides, treatment with Ibuprofen (7.5 mg/kg; reference anti-inflammatory drug), VAE 1.0, and EA 30 reduced the total edematous paw volume significantly ($p < 0.01$) by ↓67.03 %, ↓65.58 %, and ↓69.34 %, respectively (Fig. 4C). These results demonstrated that VAE 1.0 and EA 30 extracts showed good anti-inflammatory activities, especially the anti-inflammatory effect of EA 30 was greater relative to the reference drug (Ibu 7.5).

The VA leaf extracts have shown potential anti-inflammatory activity in *in vitro* and *in vivo* tests when extracted with different solvents and methods [25–27]. However, the *in vivo* anti-inflammatory activity of the crude and fraction extracts from VA leaves using the percolation method was evaluated for the first time in this study. The acetone leaf extract of VA caused a significant reduction ($p < 0.05$) in the carrageenan-induced rat paw edema at 1, 2, and 3 h compared to the control rat group. The anti-inflammatory effect of acetone leaf extract (200 mg/kg) using the maceration method is most pronounced at 3 h compared to the reference drug indomethacin (10 mg/kg) [27]. Similarly, the methanol leaf extract of VA (50–200 mg/kg) significantly inhibited the carrageenan-induced



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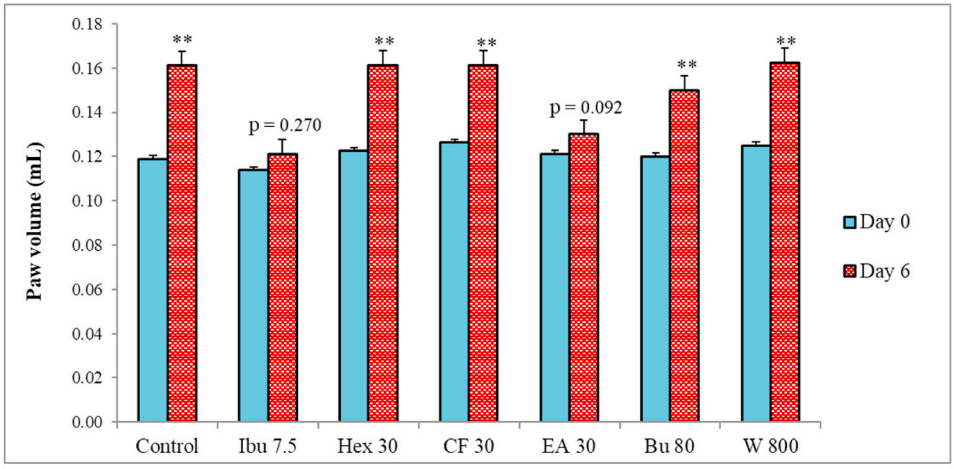

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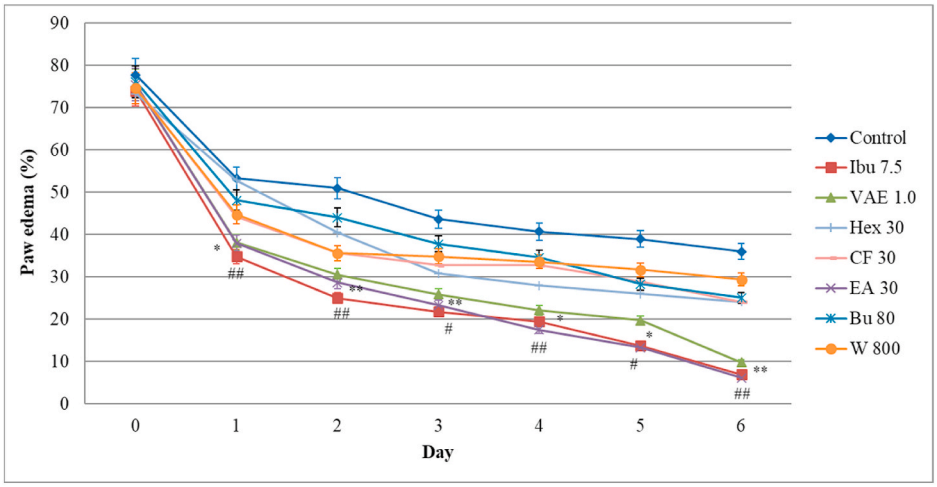

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Fig. 4. Anti-inflammatory activity of fraction extract of *Vernonia amygdalina* leaf on carrageenan-induced edema of mice.
A - Body weight of mice after 6 days of treatment, **B** - Paw volume (mL/cm^3) of mice after 6 days of treatment (** - $p < 0.01$ compared to day 0), **C** - Reduction of paw edema (%) over time (* - $p < 0.05$, ** - $p < 0.01$: EA 30 group compared to control group; # - $p < 0.05$, ## - $p < 0.01$ Ibu 7.5 group compared to Ibuprofen group; Ibu 7.5 - ibuprofen 7.5 mg/kg; **VAE** represents *Vernonia amygdalina* crude extract (1000 mg/kg - VAE 1.0), fraction extracts include EA 30 (ethyl acetate, 30 mg/kg), W 800 (water, 800 mg/kg), Bu 80 (butanol, 80 mg/kg), Hex 30 (hexane, 30 mg/kg), CF 30 (chloroform, 30 mg/kg).

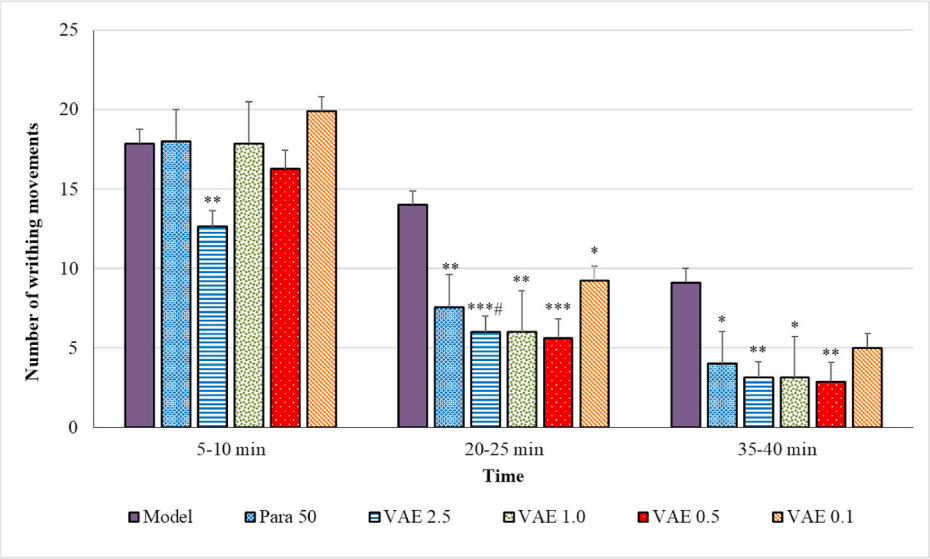


Fig. 5. The analgesic effect of *Vernonia amygdalina* crude extracts in acetic acid-induced writhing test in mice.
* - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$ compared to model group; # - $p < 0.05$, compared to Para 50 group (paracetamol, 50 mg/kg), **VAE** represents water extracts of *Vernonia amygdalina* leaf with different doses (100 mg/kg - VAE 0.1, 500 mg/kg - VAE 0.5, 1000 mg/kg - VAE 1.0, and 2500 mg/kg - VAE 2.5).

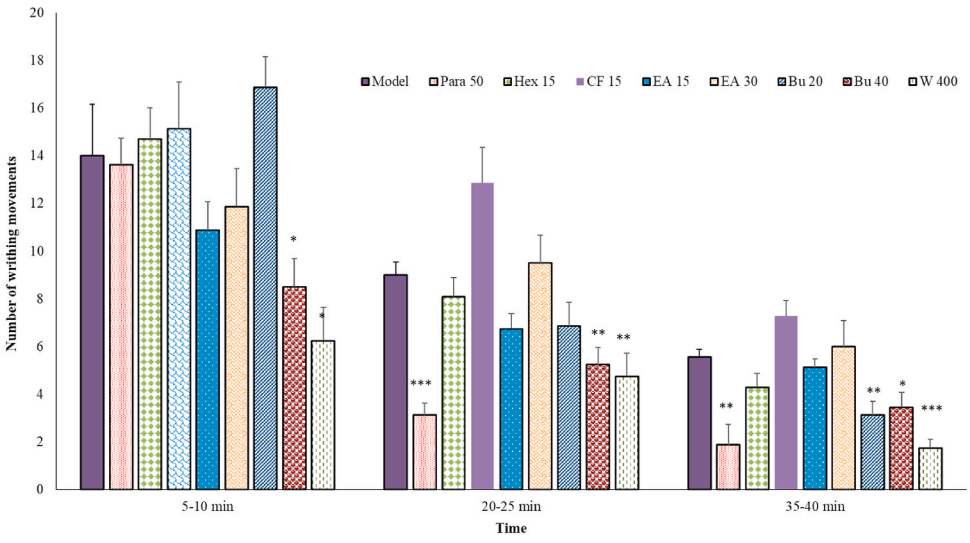


Fig. 6. The analgesic effect of *Vernonia amygdalina* fraction extracts in acetic acid-induced writhing test in mice.
* - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$ compared to model group, fraction extracts include Hex 15 (hexane, 15 mg/kg), CF 15 (chloroform, 15 mg/kg), EA 15 (ethyl acetate, 15 mg/kg), Bu 20 (butanol, 20 mg/kg)), Bu 40 (butanol, 40 mg/kg), W 400 (water, 400 mg/kg).

rat paw edema at the 4 h post carrageenan injection when rats were pre-treated with this extract for 3 days before the experiment [26]. These reported results are an indication that acetone and methanol extracts of VA leaves can be effective in acute inflammatory disorders. However, inflammation can become chronic, leading to prolonged inflammation and negative health impacts. There are many causes of chronic inflammation such as stress, diet, or certain diseases [80]. Many studies have shown that chronic inflammation contributes to many diseases, from heart disease and arthritis to diabetes and cancer. In this study, EA 30 fraction extract (30 mg/g) and VAE 1.0 crude extract (1000 mg/kg) were shown to be potential anti-inflammatory agents for supporting chronic inflammation.

The paw edema is a suitable experimental animal model to evaluate the anti-edema effects of natural products. Carrageenan-induced paw edema is increasingly being used to test new anti-inflammatory drugs as well as to study the mechanisms involved in inflammation [26]. The first phase (1 h) of this model involves the release of serotonin and histamine. Meanwhile, the second phase (over 1 h) is mediated by prostaglandins and cyclooxygenase products. Edema depends on the engagement of kinin and polymorphonuclear leukocytes with their proinflammatory factors, including prostaglandins [27]. VA extract has been shown to inhibit the biosynthetic pathway of prostaglandins (E2 and D2) and protect animals against carrageenan-induced lipid peroxidation through decreased NO radical production [26,27]. This may be because VA extracts have high levels of flavonoids, phenolics, saponins, and steroids with many strong anti-inflammatory substances. Based on the high TPC and TFC results of extracts, the anti-inflammatory effects of the VA crude and fraction extracts in this study may be due to the inhibition of cyclooxygenase-1, thereby inhibiting the release of prostaglandin release and other mediators.

3.7. Analgesic activity

3.7.1. Crude extract VAE

To the analgesic effect, as it relates to the acetic acid writhing test, VAE crude extracts caused a significant decrease in the number of writhes at doses 2500 mg/kg (VAE 2.5), 1000 mg/kg (VAE 1.0), and 500 mg/kg (VAE 0.5) compared to the model group (Fig. 5). The reference drug paracetamol (50 mg/kg, Para 50) also caused a significant reduction ($p < 0.05$, ↓45.93 % at 20–25 min and ↓56.16 % at 35–40 min) in the number of writhes when compared to the model group after 20–40 min. However, VAE 2.5, VAE 1.0, and VAE 0.5 were found to have better pain inhibition percentages than Para 50 in the middle (20–25 min: ↓57.14–59.82 %) and late (35–40 min: ↓65.75–68.49 %) phases. In particular, VAE (>500 mg/kg including VAE 2.5, VAE 1.0, and VAE 0.5) showed the highest protection against the acetic acid-induced writhing (>65 %) after 35 min, while Para 50 showed 56.16 % reduction in the writhes count. In addition, only high-dose VAE (VAE 2.5) significantly reduced ($p < 0.05$, ↓29.37 %) the number of writhes in the early phase (after 5–10 min) when compared with the other tested groups and Para 50. No significant differences were obtained between VAE (2.5, 1.0, and 0.5) and paracetamol drug in the reduction of writhes count, except for VAE 2.5 at 20–25 min (↓57.24 %). High-dose VAE revealed greater protection than Para 50, medium-dose, and low-dose VAE in three phases. The acetic acid-induced writhing response serves as an indication of the antinociceptive activities of VAE. Therefore, the VAE (2.5, 1.0, and 0.5) and paracetamol exhibited a significant antinociceptive power in the middle and late phases. The analgesic effect is more pronounced in the middle and late phases than in the early phase.

In the early phase (5–10 min), the two groups Bu 40 (butanol extract, 40 mg/kg) and W 400 (water extract, 400 mg/kg) significantly reduced ($p < 0.05$) the number of writhes when compared to the model group with pain inhibition percentages of ↓39.29 % and ↓55.36 %, respectively. In the middle phase (20–25 min), the Para 50, Bu 40, and W 400 groups significantly inhibit writhes response compared to the model group by ↓65.28 %, ↓41.67 %, and ↓47.22 %, respectively. In particular, the number of writhes of Para 50, Bu 20 (butanol extract, 20 mg/kg), Bu 40 and W 400 groups decreased significantly ($p < 0.05$) at the late phase (35–40 min) as compared to the model group with pain inhibition percentages of ↓66.25 %, ↓43.75 %, ↓37.90 %, and ↓68.50 %, respectively (Fig. 6). The results showed that the Bu 40 and W 400 fraction extracts had analgesic effects equivalent to the reference drug Para 50 ($p > 0.05$) and were superior to Para 50 in the early phase when the analgesic effect was recorded at 5–10 min.

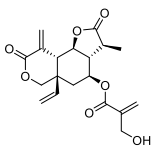
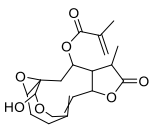
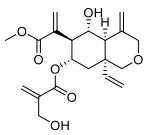
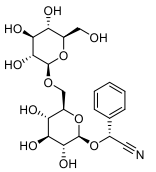
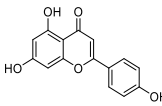
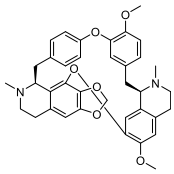
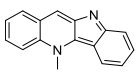
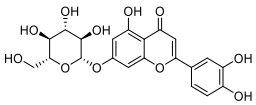
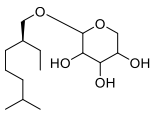
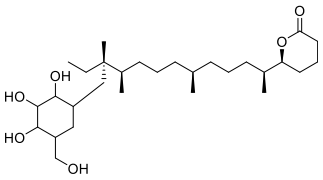
In the analgesic test, the acetone leaf extract of VA caused a significant ($p < 0.05$) decrease in the number of writhes induced by acetic acid compared to the control group. After orally administering for 60 min, the 100 and 200 mg/kg doses of these extracts showed a significant analgesic effect (↓60–80 %) for 5–20 min relative to the standard drug indomethacin [27]. Besides, the methanol leaf extract of VA (50 and 100 mg/kg) was also observed to significantly reduce the number of writhes [26]. By pretreatment with the extract for 3 days, methanol extract at doses of 50, 100, and 200 mg/kg significantly inhibited writhes response by ↓32.5 %, ↓50.9 %, and ↓63.9 %, respectively but lower than that of reference drug indomethacin (10 mg/kg once, ↓84.7 %) [26]. In this study, Bu 40 (40 mg/kg), W 400 (400 mg/kg) and VAE (500–2500 mg/kg) also showed strong and fast antinociceptive activity similar to VA acetone leaf extract (100–200 mg/kg) from the maceration method. Moreover, the VA extracts all showed dose-dependent analgesic activity.

The acetic acid-induced writhing test is considered a typical model for assessing pain due to visceral inflammation and is known to cause tissue necrosis by chemical irritants in the peritoneum. Endogenous inflammatory mediators of nociceptive neurons (bradykinin, prostaglandin, and cytokines such as TNF-α, interleukin-1β, and interleukin 8) are released upon intraperitoneal injection of acetic acid. In particular, this model allows the assessment of both central and peripheral analgesic activities because signals are sent to the central nervous system via sensory afferent C fibers entering the dorsal horn. Therefore, VA crude and fraction leaf extract may exhibit analgesic activity through both peripheral and central mechanisms due to being rich in phytochemicals with potential and diverse pharmacological activities.

3.8. Molecular docking

The iNOS and COX-2 play key roles in inflammation and inhibiting these targets reduces inflammation and pain [81–83]. Many

Table 7
Affinity and physicochemical parameters of phytochemicals of *Vernonia amygdalina* leaves.

E	Ligand	Structure	Physicochemical parameters		Affinity (Kcal/mol)			
					iNOS	COX-2	ACE	β1
1	Dihydrovernodalin		MW: 362.37 NHA: 7 NHD: 1	NRB: 5 LogP: 1.74 TPSA: 99.13	−8.9	−8.1	−8.5	−8.2
2	Dihydrovernolide		MW: 364.39 NHA: 7 NHD: 1	NRB: 3 LogP: 1.55 TPSA: 94.59	−9.2	−8.7	−9.4	−7.5
3	Deoxyvernodalol		MW: 378.42 NHA: 7 NHD: 2	NRB: 8 LogP: 1.61 TPSA: 102.29	−6.8	−7.0	−7.7	−6.9
4	Amygdalin		MW: 457.43 NHA: 12 NHD: 7	NRB: 7 LogP: −2.2 TPSA: 202.32	−9.4	−9.8	−8.9	−8.0
5	Apigenin		MW: 270.24 NHA: 5 NHD: 3	NRB: 1 LogP: 2.11 TPSA: 90.90	−9.6	−9.0	−8.5	−9.6
6	Cepharanthine		MW: 606.71 NHA: 8 NHD: 0	NRB: 2 LogP: 5.33 TPSA: 61.86	**−11.6**	**−11.6**	**−12.3**	**−10.7**
7	Cryptolepine		MW: 232.28 NHA: 1 NHD: 0	NRB: 0 LogP: 3.29 TPSA: 17.82	**−10.6**	−9.6	−8.6	−9.5
8	Cynaroside		MW: 448.38 NHA: 11 NHD: 7	NRB: 4 LogP: 0.15 TPSA: 190.28	**−10.3**	**−10.9**	−9.7	**−10.3**
9	10-Geranilanyl-O-D-yloside		MW: 290.40 NHA: 5 NHD: 3	NRB: 8 LogP: 1.66 TPSA: 79.15	−6.4	−7.1	−6.4	−7.0
10	Glucopyranosyl-1,5-olide		MW: 498.74 NHA: 6 NHD: 4	NRB: 14 LogP: 4.90 TPSA: 107.22	−7.6	−8.0	−8.3	−8.4

(*continued on next page*)

Table 7 (*continued*)

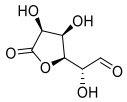
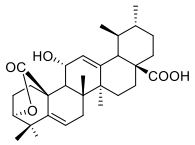
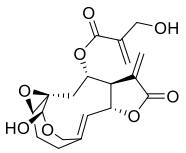
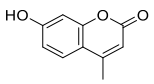
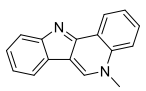
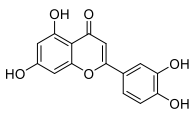
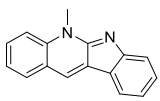
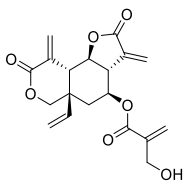
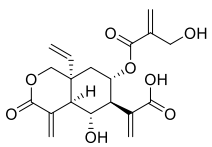
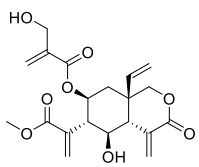
E	Ligand	Structure	Physicochemical parameters		Affinity (Kcal/mol)			
					iNOS	COX-2	ACE	β1
11	Glucurolactone		MW: 176.12 NHA: 6 NHD: 3	NRB: 2 LogP: −1.77 TPSA: 104.06	−6.4	−6.0	−5.7	−5.6
12	Hydroxyursdienoic acid olide		MW: 482.65 NHA: 5 NHD: 2	NRB: 1 LogP: 3.94 TPSA: 83.83	−8.9	**−10.0**	**−10.8**	−8.5
13	Hydroxyvernolide		MW: 378.37 NHA: 8 NHD: 2	NRB: 4 LogP: 0.73 TPSA: 114.82	−9.2	−8.0	−9.0	−7.5
14	Hymecromone		MW: 176.17 NHA: 3 NHD: 1	NRB: 0 LogP: 1.81 TPSA: 50.44	−8.3	−7.7	−7.0	−8.3
15	Isocryptolepine		MW: 268.74 NHA: 1 NHD: 0	NRB: 0 LogP: 3.10 TPSA: 17.82	−9.9	−9.7	−8.0	−9.3
16	Luteolin		MW: 286.24 NHA: 6 NHD: 4	NRB: 1 LogP: 1.73 TPSA: 111.13	−9.4	−9.1	−8.6	−9.8
17	Neocryptolepine		MW: 232.28 NHA: 1 NHD: 0	NRB: 0 LogP: 3.40 TPSA: 17.82	**−10.6**	−8.7	−8.4	−8.5
18	Vernodalin		MW: 360.36 NHA: 7 NHD: 1	NRB: 5 LogP: 1.48 TPSA: 99.13	−7.9	−8.0	−9.1	−7.7
19	Vernodalinol		MW: 378.37 NHA: 8 NHD: 3	NRB: 7 LogP: 1.00 TPSA: 130.36	−7.0	−7.8	−7.1	−6.6
20	Vernodalol		MW: 392.40 NHA: 8 NHD: 2	NRB: 8 LogP: 1.37 TPSA: 119.36	−7.4	−8.0	−7.0	−6.7

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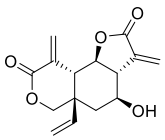
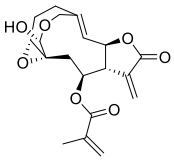
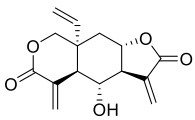
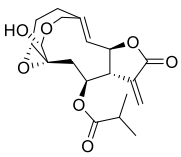
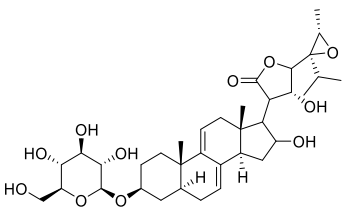
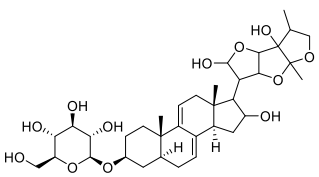
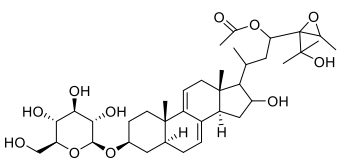
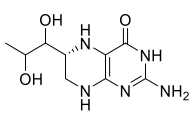
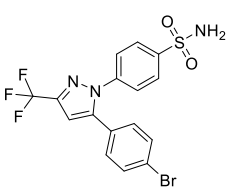
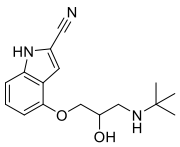
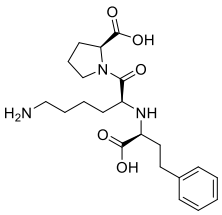
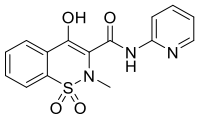
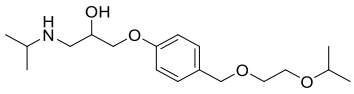
Table 7 (*continued*)

E	Ligand	Structure	Physicochemical parameters		Affinity (Kcal/mol)			
					iNOS	COX-2	ACE	β1
21	Vernolepin		MW: 276.28 NHA: 5 NHD: 1	NRB: 1 LogP: 1.23 TPSA: 72.83	−7.9	−8.4	−8.0	−7.3
22	Vernolide		MW: 362.37 NHA: 7 NHD: 1	NRB: 3 LogP: 1.55 TPSA: 94.59	−8.6	−8.1	−9.1	−8.6
23	Vernomenin		MW: 276.28 NHA: 5 NHD: 1	NRB: 1 LogP: 1.46 TPSA: 72.83	−7.8	−8.6	−8.0	−7.4
24	Vernomygdin		MW: 364.39 NHA: 7 NHD: 1	NRB: 3 LogP: 1.62 TPSA: 94.59	−8.5	−8.9	−8.7	−8.9
25	Vernonioside B1		MW: 648.78 NHA: 11 NHD: 6	NRB: 6 LogP: 1.72 TPSA: 178.67	−9.7	**−12.0**	**−11.3**	−9.3
26	Vernonioside D		MW: 664.78 NHA: 12 NHD: 7	NRB: 4 LogP: 0.70 TPSA: 187.76	−9.5	**−11.6**	**−11.1**	−9.9
27	Vernonioside E		MW: 678.85 NHA: 11 NHD: 6	NRB: 10 LogP: 2.56 TPSA: 178.67	**−10.1**	**−10.6**	**−10.3**	−8.3
28	Biopterin[a]		MW: 241.25 NHA: 4 NHD: 6	NRB: 2 LogP: −1.32 TPSA: 136.29	−7.1	−7.3	–	–
29	Phenylpyrazole[a]		MW: 446.24 NHA: 7 NHD: 1	NRB: 4 LogP: 3.69 TPSA: 86.36	−7.9	−8.6	–	–

(*continued on next page*)

Table 7 (*continued*)

E	Ligand	Structure	Physicochemical parameters		Affinity (Kcal/mol)			
					iNOS	COX-2	ACE	β1
30	Cyanopindolol[a]		MW: 287.36 NHA: 4 NHD: 3	NRB: 6 LogP: 2.06 TPSA: 81.07	–	–	−7.6	−9.1
31	Lisinopril[a]		MW: 405.49 NHA: 7 NHD: 4	NRB: 13 LogP: 0.13 TPSA: 132.96	–	–	−7.5	−7.1
32	Piroxicam[a]		MW: 331.35 NHA: 5 NHD: 2	NRB: 3 LogP: 1.38 TPSA: 107.98	−9.5	−8.4	–	–
33	Bisoprolol[a]		MW: 325.44 NHA: 5 NHD: 2	NRB: 12 LogP: 2.59 TPSA: 59.95	–	–	−6.8	−7.1

E – entry, **MW** - molecular weight, **NHA** - number of hydrogen bond acceptors, **NHD** - number of hydrogen bond donors, **NRB** - number rotatable bonds, **TPSA** - total polar surface area (Angstroms squared), **iNOS** - nitric oxide synthase, COX-2 - cyclooxygenase-2, **ACE** - angiotensin-converting enzyme, **β1** - β1 adrenoreceptor.

[a] Co-crystallization of target/reference drug.

popular anti-inflammatory drugs (NSAIDs) have a mechanism of action through COX-2 inhibition such as ibuprofen, diclofenac, celecoxib, meloxicam, and piroxicam. Besides, a large number of drugs were developed as oral drugs to treat hypertension with potent and highly selective ACE inhibitory activity such as captopril, lisinopril, enalapril, and ramipril. On the other hand, many selective β_1-receptor antagonists used for the management of hypertension and ventricular premature beats include atenolol, bisoprolol, esmolol, acebutolol, metoprolol, and nebivolol. Therefore, the phytochemicals of VA leaves were screened for *in silico* anti-inflammatory activity on two targets including iNOS (nitric oxide synthase) and COX-2 (cyclooxygenase-2) as well as *in silico* antihypertensive activity on two targets including ACE (angiotensin-converting enzyme) and β_1 adrenoreceptor using the molecular docking method to predict the most suitable binding pose and inhibition mechanism [84,85].

Tentative identification of chemical constituents of EA fraction (Table 3), W fraction, and VAE was performed using the LC-MS method. The results of physicochemical parameters and affinity of phytochemicals of VA leaves with four targets are shown in Table 7. The interaction results of potent compounds with targets are shown in Figs. 7–10. All compounds showed physical-chemical properties following Lipinski's rules ((i) HB donor groups ≤5; (ii) HB acceptor groups ≤10; (iii) M. Wt less than 500; (iv) logP less than 5), except for amygdalin, cepharanthine, cynaroside, and vernoniosides. The absorption capacity of these compounds is predicted to be much better because the molecule achieves at least three out of four of Lipinski's rules. The protein-ligand complex is formed through the electrostatic interactions of the binding interface including hydrogen bonds (both from side chains and backbones), salt bridges, and π-π stacking. Hydrogen bonding provides stability to protein molecules and selected protein-ligand interactions, thus being one of the most important for biological macromolecule interactions. In addition, hydrogen bonds are divided into different types such as conventional, carbon, and π-donor, in which conventional hydrogen bonds are the strongest interactions [3,86–88].

The VA phytochemicals showed good interactions with four targets iNOS (−6.4 to −11.6 kcal/mol), COX-2 (−6.0 to −12.0 kcal/mol), ACE (−5.7 to −12.3 kcal/mol) and β1 (−5.6 to −10.7 kcal/mol) with high binding affinity in the range of −5.6 to −12.3 kcal/mol. For anti-inflammatory activity, cepharanthine (−11.6 kcal/mol), cynaroside (−10.3 kcal/mol), and vernonioside E (−10.1 kcal/mol) showed higher affinity than −10.0 kcal/mol and strongest interactions in the tested compounds on iNOS compared to the co-crystallization biopterin (−7.1 kcal/mol) and the reference drug piroxicam (−9.5 kcal/mol). Similarly, cepharanthine (−11.6 kcal/mol), cynaroside (−10.3 kcal/mol), vernonioside B_1 (−12.0 kcal/mol), vernonioside D (−11.6 kcal/mol) and vernonioside E (−10.6 kcal/mol) demonstrated an affinity higher than −10.0 kcal/mol and strongest interactions on COX-2 compared to the co-crystallization phenylpyrazole (−8.6 kcal/mol) and the reference drug piroxicam (−8.4 kcal/mol). These may be important compounds that create the anti-inflammatory effects of VA leaf extract. On the other hand, for antihypertensive activity, cepharanthine (−12.3 kcal/mol), vernonioside B_1 (−11.3 kcal/mol), vernonioside D (−11.1 kcal/mol) and vernonioside E (−10.3 kcal/mol) exhibited higher affinity than −10.0 kcal/mol and strongest interactions on ACE compared to the reference drug lisinopril (−7.5 kcal/

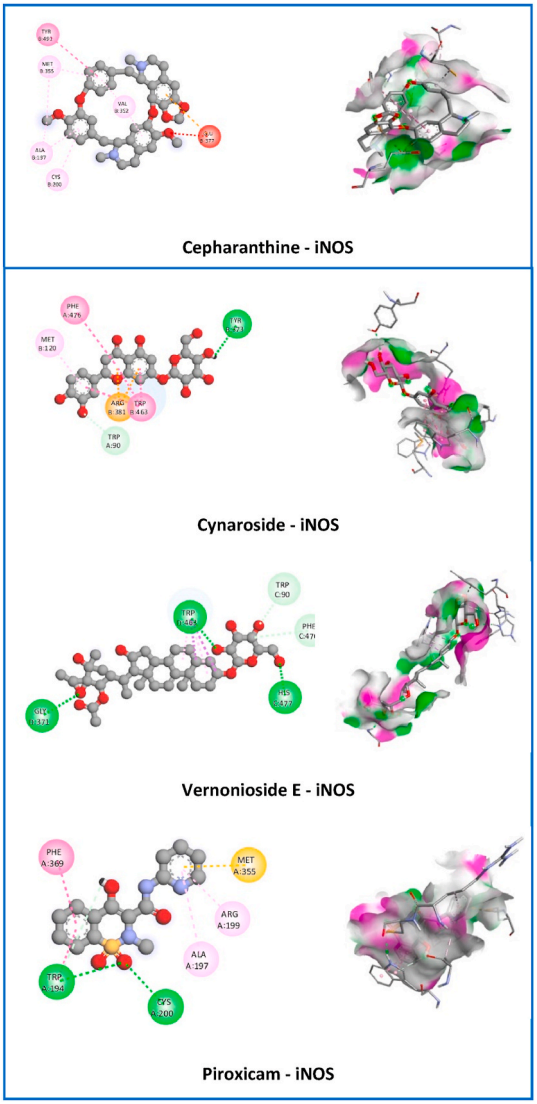


Fig. 7. 2D and 3D representation of the interaction of phytochemicals of *Vernonia amygdalina* leaves and reference drug Piroxicam with nitric oxide synthase (iNOS).

mol). Besides, cepharanthine (−10.7 kcal/mol), cynaroside (−10.3 kcal/mol), and vernonioside D (−9.9 kcal/mol) showed the highest affinity on β1 adrenoreceptor compared to co-crystallization cyanopindolol (−9.1 kcal/mol) and the reference drug bisoprolol (−7.1 kcal/mol). These may be important compounds that create the antihypertensive effect of VA leaf extract.

On the iNOS target, cepharanthine established electrostatic interaction (π-anion) with GLU377 amino acid with a bond length of 4.85 Å and hydrophobic interactions (π-π T-shaped, alkyl, π-alkyl) with TYR491, ALA197, VAL352, MET355, and CYS200 amino acids with bond lengths in the range of 3.26–5.56 Å. Cynaroside established one hydrogen bond (TYR373, 2.54 Å), one carbon-hydrogen bond (TRP90, 3.45 Å), electrostatic (π-cation, ARG381, 3.69 Å), π-cation π-donor hydrogen bond (ARG381, 3.32 Å), and hydrophobic interactions (π-π stacked, π-alkyl; PHE476, TRP463, MET120; 3.75–5.76 Å). Meanwhile, vernonioside E established three hydrogen bonds (HIS477, GLY371, TRP463; 2.11, 2.63, 2.47 Å), two carbon-hydrogen bonds (TRP90, PHE476; 2.94, 3.65 Å) and hydrophobic interactions (π-σ, π-alkyl; TRP463; 3.43–5.19 Å) (Fig. 7). Several flavonoids including quercetin have been reported to inhibit iNOS involving interaction with active site residues ILE119, THR109, SER118, TRP461, and MET480 [82,83]. Although VA phytocompounds did not interact with similar residues in these studies, cepharanthine was found to have similar interactions with the reference drug piroxicam at residues ALA197 and CYS200 in the iNOS action site (Fig. 7).

On the COX-2 target, cepharanthine established two carbon-hydrogen bonds (SER121, ARG44; 3.52, 3.55 Å), electrostatic (π-cation, ARG44, 4.51 Å and π-anion, ASP125, 4.18 Å), and hydrophobic interactions (alkyl, π-alkyl; ARG44, PRO542; 3.98–5.42 Å). Cynaroside established four hydrogen bonds (ALA156, PRO154, GLU465, GLU46; 2.64, 2.13, 2.31, 2.17 Å), electrostatic (π-cation, ARG44, 3.75 Å), and hydrophobic interactions (π-alkyl; PRO153, ARG44, LEU152; 5.10–5.49 Å). On the other hand, vernonioside E

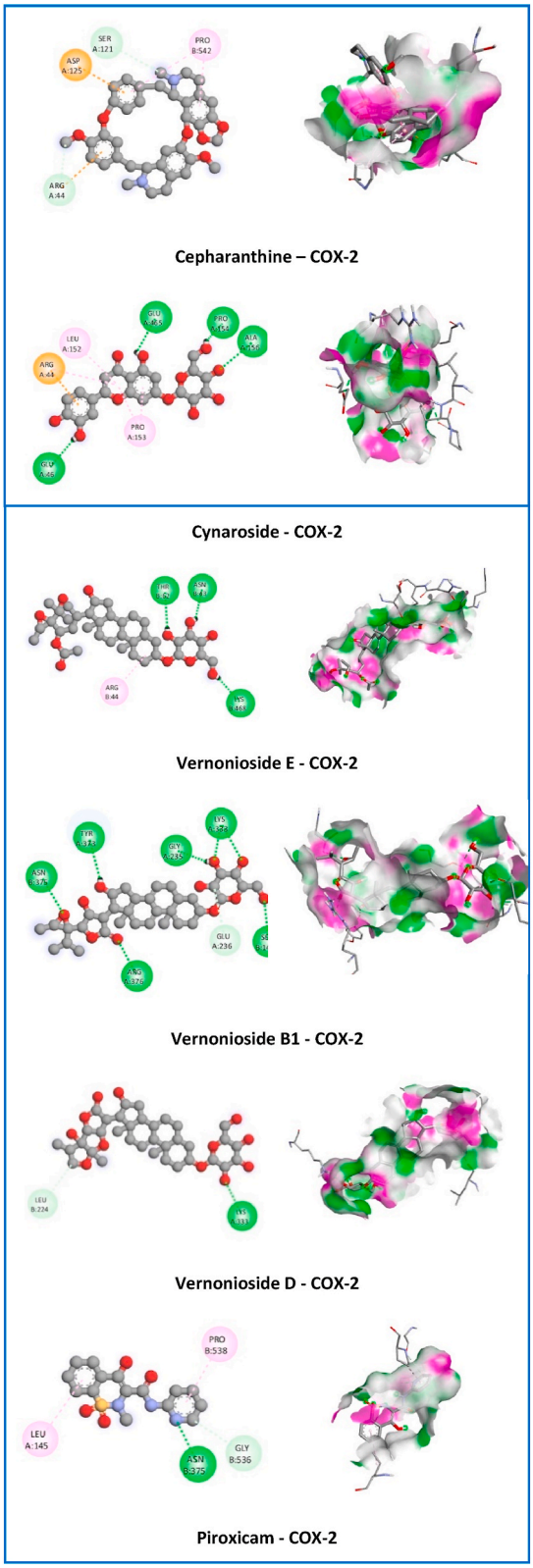


Fig. 8. 2D and 3D representation of the interaction of phytochemicals of *Vernonia amygdalina* leaves and reference drug Piroxicam with cyclooxygenase-2 (COX-2).

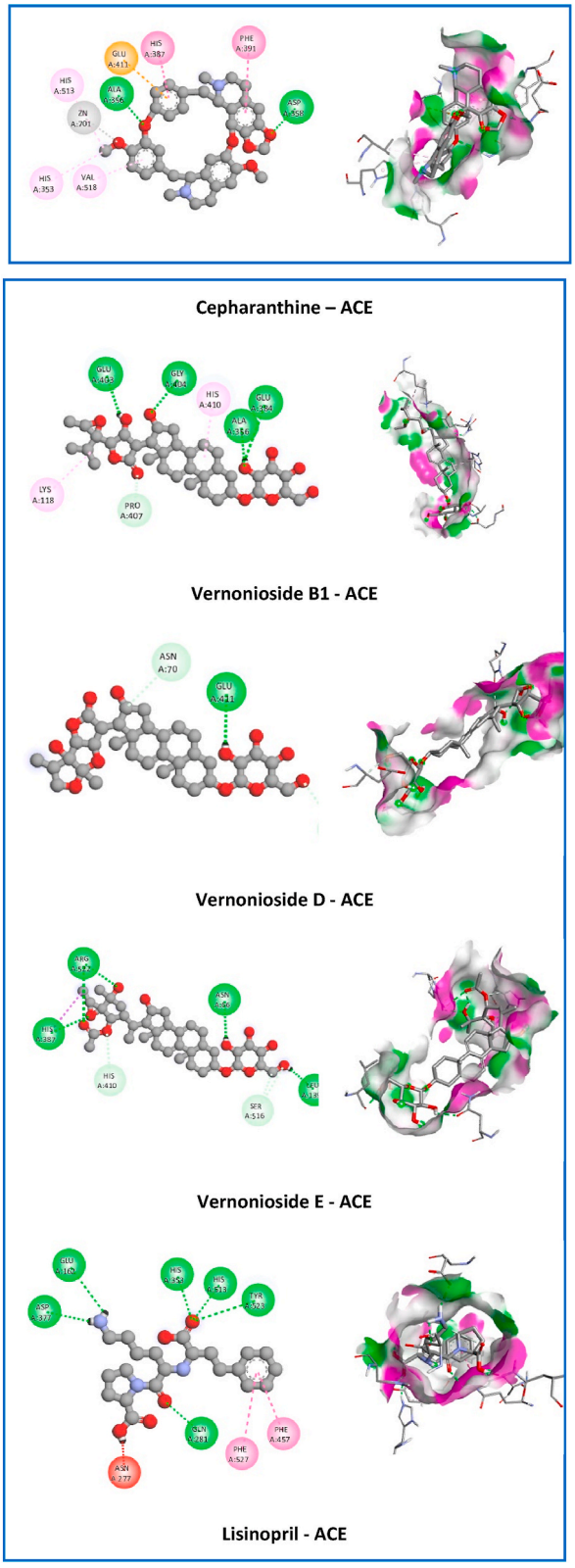


Fig. 9. 2D and 3D representation of the interaction of phytochemicals of *Vernonia amygdalina* leaves and reference drug Lisinopril with angiotensin-converting enzyme (ACE).

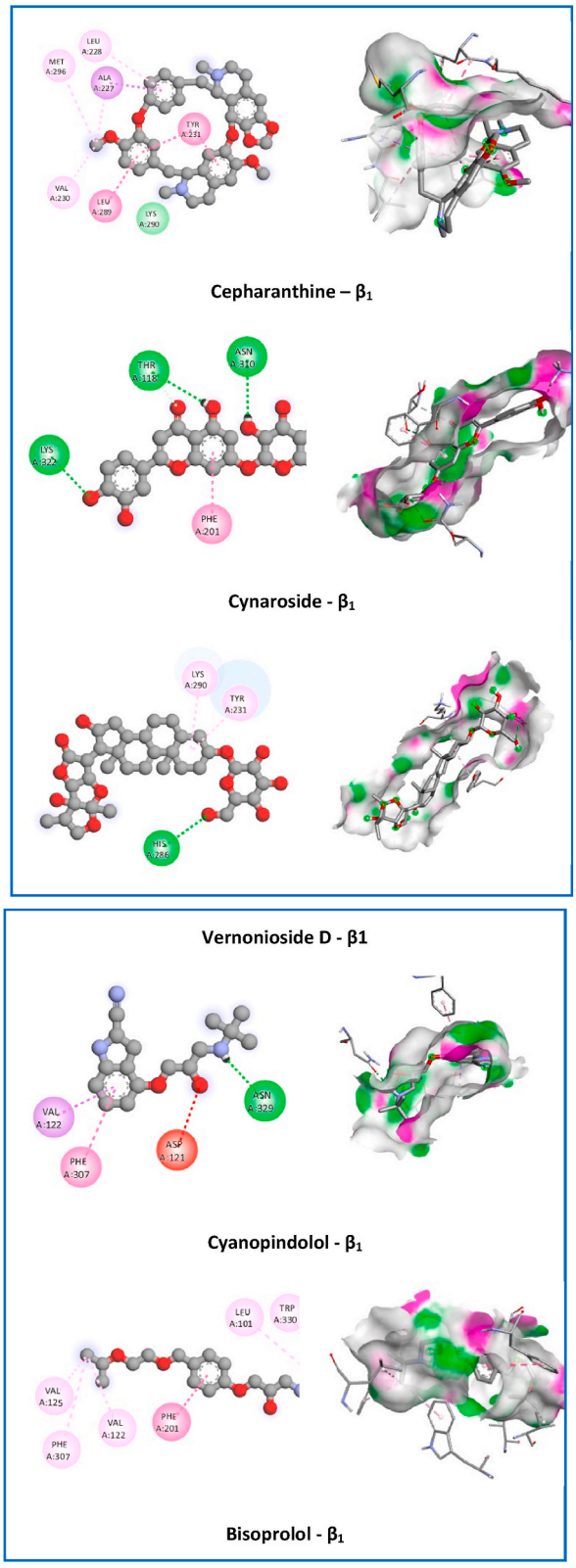


Fig. 10. 2D and 3D representation of the interaction of phytochemicals of *Vernonia amygdalina* leaves and reference drug (Cyanopindolol and Bisoprolol) with β1 adrenoreceptor.

established three hydrogen bonds (THR62, ASN43, LYS468; 2.44, 2.06, 2.16 Å), and hydrophobic interaction (alkyl, ARG44, 5.31 Å). In addition, vernonioside D established one hydrogen bond with LYS333 amino acid with a short bond length (2.36 Å) and one carbon-hydrogen bond with LEU224 amino acid with a bond length of 3.67 Å. In particular, vernonioside B_1 showed strongest affinity with eight hydrogen bond at LYS333, LYS333, ARG376, ARG376, SER143, ASN375, TYR373, and GLY235 amino acids with bond lengths of 2.47, 2.52, 2.77, 2.84, 2.60, 2.66, 2.90, 1.94 Å, respectively, and one carbon-hydrogen bond with GLU236 amino acid with a bond length of 3.41 Å (Fig. 8). Non-steroidal anti-inflammatory drugs (NSAIDs) have been strongly shown to act by blocking prostaglandin release by inhibiting COX-2. Ibuprofen and Naproxen showed the involvement of amino acid residues ARG120 and TYR355 in COX-2 inhibition [81]. Piroxicam demonstrated strong hydrogen and carbon-hydrogen interactions with amino acid residues ASN375 and GLY536 as well as hydrophobic interactions at residues LEU145 and PRO538. In this study, Vernonioside B1 showed a similar interaction with piroxicam at residue ASN375 by strong hydrogen bonding.

On the ACE target, vernonioside B_1 established four hydrogen bonds (ALA356, GLY404, GLU384, GLU403; 1.84, 2.58, 2.82, 2.27 Å), one carbon-hydrogen bond (PRO407, 3.74 Å), and hydrophobic interactions (alkyl, π-alkyl; LYS118, HIS410; 4.86–4.91 Å). Vernonioside D established one hydrogen bond (GLU411, 2.34 Å) and two carbon-hydrogen bonds (GLU403, ASN70; 3.09, 2.98 Å). Vernonioside E established the most hydrogen bonds with five hydrogen bonds at HIS387, ARG522, ARG522, ASN66, and LEU139 amino acids with bond lengths of 2.88, 2.90, 2.44, 2.34, and 1.93 Å, respectively, and three carbon-hydrogen bonds with HIS410, SER516, and SER516 amino acids with bond lengths of 3.48, 3.47, and 3.2594 Å, respectively as well as showed hydrophobic interaction (π-σ) with HIS387 amino acid (3.84 Å). Moreover, cepharanthine showed stronger interactions with the highest affinity. Cepharanthine established two hydrogen bond (ALA356, ASP358; 2.77, 1.83 Å), metal-acceptor (ZN701, 3.27 Å), electrostatic (π-anion, GLU411, 4.31 Å) and hydrophobic interactions (π-π stacked, π-π T-shaped, π-alkyl; PHE391, HIS353, HIS513, VAL518; 4.15–5.56 Å) (Fig. 9). In particular, cepharanthine demonstrated interactions with residues HIS353 and HIS513 similar to the reference drug lisinopril.

On the β1 receptor, cepharanthine exhibited hydrophobic interactions (π-σ, π-π stacked, π-π T-shaped, amide-π stacked, alkyl, π-alkyl) with ALA227, TYR231, TYR231, LEU289, LYS290, ALA227, TYR231, VAL230, MET296, LEU228, and LEU289 amino acids with bond lengths in the range of 3.74–5.56 Å. Cynaroside established three hydrogen bonds (LYS322, THR118, ASN310; 2.51, 2.68, 2.72 Å), one carbon-hydrogen bond (THR118, 3.64 Å), and hydrophobic interaction (π-π T-shaped) with PHE201 amino acid with a bond length of 4.51 Å. In addition, vernonioside D formed one hydrogen bond (HIS286, 2.71 Å) and hydrophobic interactions (alkyl, π-alkyl) with LYS290 and TYR231 amino acids with bond lengths in the range of 4.16–4.47 Å (Fig. 10). Furthermore, cynaroside showed hydrophobic interaction with residue PHE201 similar to the reference drug bisoprolol.

In summary, from the *in silico* molecular docking study results, it can be concluded that potent phytocompounds such as cepharanthine, cynaroside, and vernoniosides of VA leaf extract are considered the best dock conformation in anti-inflammatory targets (iNOS and COX-2) and antihypertensive targets (ACE and β1 adrenoreceptor).

4. Conclusion

In summary, phytochemical analysis of VA leaf extracts revealed the presence of phenolics, alkaloids, tannins, and flavonoids. The high TPC and TFC were found in VAE, Bu, and EA extracts. Two flavonoids (apigenin and luteolin) were isolated và structure determined from the ethyl acetate fraction of VA leaves. Besides, the VA crude extract would be easy to assume that this plant is safe since LD_{50} can be found at doses greater than 10,000 mg/kg body weight. The low-dose VAE-treated group showed a better effect on HR variation than the high-dose VAE-treated groups. The antihypertensive effect of the VAE 2.0 (2000 mg/kg) group was nearly equivalent to the reference drug Captopril. On the other hand, VAE 1.0 (1000 mg/kg) crude extract and EA 30 (30 mg/kg) fraction showed good anti-inflammatory activities, especially the anti-inflammatory effect of EA 30 was greater relative to the reference drug Ibuprofen (7.5 mg/kg). In analgesic activity, VAE 2.5 (2500 mg/kg), VAE 1.0 (1000 mg/kg), and VAE 0.5 (500 mg/kg) showed the highest protection against the acetic acid-induced writhing in the middle (20–25 min) and late (35–40 min) phases with reference drug paracetamol. Moreover, the molecular docking results predicted that cepharanthine, cynaroside, and vernoniosides of VA leaf extract are considered the best dock conformation in anti-inflammatory targets (iNOS and COX-2) and antihypertensive targets (ACE and β_1 adrenoreceptor). This work paved the way for isolating potential phytocompounds in VA leaf extract to research the mechanism of action as well as develop herbal products to support inflammation treatment, pain relief, and blood pressure lowering from VA leaves.

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Em Canh Pham: Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Data curation, Conceptualization. **Vien Van Doan:** Supervision, Software, Investigation, Data curation. **Tuong Vi Le Thi:** Software, Investigation. **Cuong Van Ngo:** Software, Investigation. **Lenh Vo Van:** Software, Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to

influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e38634.

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