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Pharmacological evidence of *Vitex thyriflora*, *Entandrophragma cylindricum*, and *Anonidium mannii* used for the management of inflammation in Cameroon

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Abstract:

Background: Inflammation is the most common health problem faced in life relating to a vast number of diseases. The present study evaluated the pharmacological effect of three plants (*Vitex thyriflora*, *Entandrophragma cylindricum*, and *Anonidium mannii*) commonly used in the Cameroon pharmacopeia for the management of inflammatory response.

Methods: The pharmacological effect was characterized by the antioxidant capacity, anti-inflammatory, analgesic, and antipyretic properties of the ethanol extracts of the three plants. Antioxidant capacity was determined using total phenolic content, total flavonoid content, hydrogen peroxide, ferric reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) radical scavenging assays. Anti-inflammatory activity was evaluated *in vitro* by protein denaturation and hypotonic-induced hemolysis methods and *in vivo* by carrageenan paw edema method. Analgesic and antipyretic activities were studied *in vivo* using acetic acid-induced writhing and brewer's yeast-induced hyperpyrexia models.

Results: All selected extracts showed high phenolic (15.93–64.45 mgCAE/g) and flavonoid (336.03–1053.48 mgCAE/g) contents and high ferric reducing power (288.75–364.91 mgCAE/g). These extracts exhibited good DPPH (IC₅₀ = 0.30–1.65 µg/mL), ABTS (IC₅₀ = 0.52–1.90 µg/mL), and H₂O₂ (IC₅₀ = 1.40–3.55 µg/mL) radical scavenging activities. All extracts inhibited protein denaturation (6.79–82.27%) and protected the erythrocyte membrane from lysis induced by hypotonic solution (18.90–88.00%). The extracts significantly reduced dose-dependent paw edema (p < 0.05), fever, and abdominal writhing (p < 0.001) especially at 400 mg/kg.

Conclusions: All extracts exhibited interesting antioxidant properties, as well as significant anti-inflammatory, analgesic, and antipyretic effects.

Keywords: analgesic activity, anti-inflammatory activity, antipyretic activity, ethanol extract, medicinal plants,

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Introduction

Scientists began taking interest in inflammatory disorders at the beginning of the 17th century, and since then, many studies were focused on the inflammatory reactions and etiology [1]. This is important because, in many diseases, a large part of tissue damage is due to the inflammatory responses, which, in itself, is responsible for

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the discomfort induced by the disease. Inflammation is a normal physiological defense response or an adaptation to aggression, which may be caused by a microorganism or any substance foreign to the organism [2]. It is one of the most important mechanisms of the body's defenses and requires fine regulation. Generally, in the case of a microorganism, inflammation leads to the elimination of the pathogen and the return to homeostasis of the injured tissue [3]. However, an inadequate response may lead to immunodeficiency that can degenerate to secondary infection or cancer [4]. Exacerbated on the contrary, inflammation increases morbidity and mortality in diseases such as rheumatoid arthritis, Crohn's disease, diabetes, cardiovascular diseases, Alzheimer's disease, and arteriosclerosis [5]. If poorly controlled, inflammation can spread to the rest of the body through the bloodstream [6]. It can then lead to irreversible local or generalized tissue damage, sometimes to septic shock leading, in the most severe cases, to death [6].

The inflammatory response involves different phases in which a large number of cells, particularly neutrophils, phagocytic cells, and macrophages, play a preponderant role in inflammatory mechanisms, as they are the first line of defense against infectious agents. Accumulation of these different cells at the inflammatory site will induce the release and increased accumulation of reactive oxygen species (ROS) [7]. If ROS production is too high and natural antioxidant systems are not efficient, cells are subjected to oxidative stress, which maintains the inflammatory state [8]. Therefore, ROS neutralization by antioxidants and radical scavengers could attenuate inflammation. In addition, inflammatory reaction leads to pain, heat, edema, and redness. Depending on the inflammatory severity, a fever characterized by an increase in temperature may appear [9].

The conventional treatment of inflammation is based on the provision of nonsteroidal (NSAIDs) and steroidal (SAIDs) anti-inflammatory drugs. Anti-inflammatory drugs affect the initiating or enhancing effects of inflammation (migration of inflammatory cells, release of prostaglandins and leukotrienes, ROS). However, the use of synthetic anti-inflammatory molecules essentially is not without harmful effects for the body. SAIDs have hormonal activity, mainly related to metabolic regulation, and exert a braking effect on the hypothalamic-pituitary-adrenal axis [10]. All NSAIDs, regardless of their administration route, are at risk for gastrointestinal tract disorders, kidney and skin toxicity. The risk is even greater when dosage is high and treatment is prolonged [11]. Therefore, search for active molecules with low side effects is necessary. Such molecules are present in plants that are sometimes the only source of treatment for the local poor people. Indeed, there is evidence that natural products modulate various inflammatory mediators and have an effect on the expression of the pro-inflammatory molecules (cyclooxygenase 2, inducible nitric oxide synthase, interleukin 1 β , tumor necrosis factor α and interleukin 10), which are the key to inflammation [12]. Many traditional medicines have a promising potential in the management of the pathologies currently afflicting humans, including inflammatory disorders. However, although increasingly used, many remain untested and used empirically. As a result, scientific knowledge of their efficacy and potential side effects is limited. This makes the identification of therapies and the promotion of their rational use more difficult.

This study aimed to show the therapeutic effects of the ethanol extracts of three medicinal plants, *Vitex thyrsoiflora* (EtOHVT), *Entandrophragma cylindricum* (EtOHEC), and *Anonidium mannii* (EtOHAM) used in the management of inflammation.

Materials and methods

Plant material

The different plant parts used (leaves of *V. thyrsoiflora*, stem barks of *E. cylindricum* and *A. mannii*) were collected from the Littoral and Centre Regions of Cameroon. Plants were identified at the National Herbarium (Yaoundé, Cameroon), where a voucher specimen was deposited. Detailed information on each medicinal plant is given in Table 1.

Table 1: General information and reports on evidence of biological activities and chemistry of the studied plants.

Species (family); voucher number ^a ; vernacular name	Traditional uses	Parts used; collection site (% yield) ^b	Bioactive or potentially bioactive components	Biological activities
<i>Vitex thyrsoiflora</i> Baker (Verbenaceae) 34861/HNC Ndombi	Treatment of orchitis, stomach pains, sterility and parasitic infections [13]	Leaves, Melong, Cameroon (15%)	Not reported	Antihyperglycemic and antioxidant potential of ethanol extract of <i>Vitex</i> <i>thyrsoiflora</i> leaves [14]

<i>Entandrophragma cylindricum</i> (Sprague) Sprague (Meliaceae) 54965/SFRCAM Asseng-Assie	Treatment of rheumatism, bronchitis, lung complaints, colds, edema, malaria, yellow fever, typhoid fever, diarrhea and stomachache [15]	Stem barks, Mount Kalla, Cameroon (14.05%)	Sapelenins G-J, (+)-7',7'-dimethyl-S-hydroxy-2R,3S-trans-pubeschin, sapelenins A-D, sapelenins E-F [16], [17], ekeberin D2 [18], (+)-catechin, epicatechin and anderolide G [19]	Anti-inflammatory activities of the sapelenins G-J by suppressing the secretion of IL-17 by phytohemagglutinin-stimulated human peripheral blood mononuclear cells [20]. Antisickling, antihemolytic and radical scavenging activities of essential oil, methanol, and aqueous extracts [21]. Antiplasmodial activity of methanol, ethyl acetate and aqueous against chloroquino-sensitive 3D7 and chloroquino-resistant INDO strains of <i>Plasmodium falciparum</i> ; antioxidant activity of these extracts [15]
<i>Anonidium mannii</i> (Oliv.) Engl. & Diels (Annonaceae) 1918/SRFGK Ebon	Spider and snake bites, bronchitis, dysentery, gastroenteritis, syphilis [22], diarrhea, malaria, Cancer Diabetes and arterial hypertension [23]	Stem barks, Babimbi II, Cameroon (9.26%)	Prenylated bisindole alkaloids (Annonidine A-E) [24]	Not reported

^a(HNC), Cameroon National Herbarium; (SRFCAM), Société des Réserves Forestières du Cameroun. ^bYield calculated as the ratio of the mass of the obtained ethanol extract/mass of the plant powder.

Preparation of the crude extracts

The dried leaves of *V. thyrsoiflora* (2 kg), and stem barks of *E. cylindricum* (2 kg) and *A. mannii* (2 kg) were macerated at room temperature in ethanol (5 L, 72 h) to obtain the crude extracts (300, 281, 185.1 g, respectively) after evaporation of ethanol under vacuum using a rotary evaporator (Büchi R200). The yields of the extraction were 15%, 14.05%, and 9.26% (w/w) respectively.

Phytochemical screening

All extracts were subjected to phytochemical analysis for the identification of plant bioactive constituents (alkaloids, flavonoids, glycosides, carbohydrates, tannins, resins, steroids, proteins, and amino acids) using standard methods as earlier described by Khandelwal [25].

Experimental animals

Nulliparous and non-pregnant Wistar rats (180–200 g) used in this study were housed in plastic cages under standard laboratory conditions (12 h light/dark cycle: 25 ± 2 °C) for 7 days prior to the commencement of the experiments. All animals were given food and water *ad libitum*.

Ethical guidelines

Experimental animals were handled in accordance with the prescription of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) issued by the Ministry of Environment and Forests, Government of India. The animal study was carried out at VNS Group of Institutions, Faculty of Pharmacy, Bhopal (Madhya Pradesh) with the permission of the Institutional Animal Ethics Committee (Registration No. 778/PO/a/03/CPCSEA; 03.09).

Chemicals and drugs

Chemicals used included carrageenan, acetic acid, egg albumin, hydrogen peroxide, and brewer's yeast. Other chemicals were Dragendorff's reagent, Mayer's reagent, Benedict's reagent, Folin-Ciocalteu, catechin, ascorbic acid, 2,21-azinobis-(3-ethylbenthialozine)-6-sulfonic acid (ABTS), and 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-tri[2-pyridyl]-s-triazine (TPTZ). The standard drugs used were diclofenac sodium, indometacin, and aspirin. All chemicals and drugs used were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA).

Acute oral toxicity

Acute oral toxicity was carried out as earlier described [26]. Three groups of six animals each were administered as follows: the control group received water (1 mL/100 g bw), while the two test groups received plant extracts (1000 and 2000 mg/kg bw). Animals were then observed for toxic manifestations for the next 5 h and subsequently observed intermittently for signs of morbidity and mortality for 7 days.

Determination of total phenolic content

TPC was estimated by the Folin-Ciocalteu method [27]. Twenty microliters of extract (0.625–10 mg/mL) were added to 0.4 N Folin-Ciocalteu reagent (980 μ L). Catechin (10–80 μ M) was used as a standard for the calibration curve ($y = 0.0066x + 0.0030$; $r^2 = 0.9978$). Absorbance at 760 nm was measured after 10 min of incubation at room temperature.

Determination of total flavonoid content

TFC was determined by the colorimetric method [28]. Of the extract, 0.1 mL (0.625–10 mg/mL) was mixed with 5% (w/v) sodium nitrite (0.2 mL). After 5 min, 10% (w/v) aluminum chloride (0.2 mL) and 1 M NaOH (2 mL) were added. Catechin (50–600 μ M) was used as a standard for the calibration curve ($y = 0.0002x - 0.0033$; $r^2 = 0.9828$). Absorbance was measured at 510 nm.

Determination of ferric reducing antioxidant power (FRAP)

FRAP was performed by the method of Benzie and Strain [29]. FRAP reagent was prepared with 300 mM acetate buffer pH $\pm$ 3.6 (100 mL), 10 mM 2,4,6-tripyridyl-s-triazine (10 mL), and 20 mM FeCl₃, H₂O (10 mL). Seventy-five microliters of the extract (0.625–10 mg/mL) were added to the FRAP reagent (2 mL), and the mixture was incubated at room temperature for 15 min. Catechin (50–600 μ M) was used as a standard for the calibration curve ($y = 0.0021x - 0.0154$; $r^2 = 0.9984$). Absorbance was measured at 593 nm.

Determination of DPPH free radical scavenging activity

Free radical scavenging activity of the extracts on the stable radical DPPH was estimated by the method of Mensor et al. [30]. One hundred microliters of the extract (0.25–4 mg/mL) were mixed with 1900 μ L of DPPH methanol solution (30 mg/L) and kept for 30 min at room temperature in a dark cupboard. The decrease in the solution absorbance, due to proton donating of substances was measured at 517 nm. L-Ascorbic acid (15.62–250

$\mu\text{g/mL}$) was used as positive control. The percentage of DPPH radical scavenging activity was calculated using the following formula:

$$\text{DPPH radical scavenging activity (\%)} = [(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \times 100$$

Determination of hydrogen peroxide radical scavenging activity

Hydrogen peroxide scavenging activity of the extracts was determined using the method described by Rutch et al. [31]. A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). Hydrogen peroxide solution (0.6 mL) was mixed with 1.4 mL of extract (0.625–10 mg/mL). The absorbance of hydrogen peroxide at 230 nm was determined after 10 min of incubation against a blank solution containing phosphate buffer without hydrogen peroxide. L-Ascorbic acid (10–80 $\mu\text{g/mL}$) was used as the reference standard. The percentage of H_2O_2 scavenging activity was calculated using the following formula:

$$\text{H}_2\text{O}_2 \text{ scavenging activity (\%)} = [(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \times 100$$

Determination of ABTS radical scavenging activity

2,2'-Azinobis(3-ethylbenzothiazoline 6-sulfonic acid) (ABTS^+) scavenging effect of the extracts was analyzed in accordance with the method of Re et al. [32]. ABTS radical was generated by mixing equal volumes of 7 mM of ABTS and 4.9 mM of potassium permanganate (KMnO_4) and kept in the dark room for 24 h. Eight milliliters of the ABTS generated radical solution was diluted further in 72 mL of distilled water. One milliliter of the diluted radical solution was added to 20 μL of plant extract (0.625–10 mg/mL), and the optical density read at 734 nm after 12 min of incubation. L-Ascorbic acid (15.62–250 $\mu\text{g/mL}$) was used as positive control.

$$\text{ABTS scavenging activity (\%)} = [(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \times 100$$

Anti-inflammatory activity

Protein denaturation

The protein denaturation method earlier described by Padmanabhan [33] was used for the determination of anti-inflammatory activity of the extracts. A standard NSAID, diclofenac sodium was used as the control drug. The reaction mixture contained 2 mL of extract or standard at varying concentrations (50–1000 $\mu\text{g/mL}$), phosphate buffer saline pH 6.4 (2.8 mL), and 5% egg albumin (2 mL). This was incubated at 27 °C for 15 min after which denaturation was induced by raising the reaction temperature to 70 °C in a water bath for 10 min. The reaction mixture was then allowed to cool to room temperature and the absorbance measured at 660 nm using double distilled water as blank. Each extract was analyzed in triplicate. The formula below was used to calculate the percentage inhibition of protein denaturation.

$$\text{Inhibition of protein denaturation (\%)} = [(A_{\text{sample}} - A_{\text{control}})/A_{\text{control}}] \times 100$$

Erythrocyte membrane stabilization

The effect of extract on membrane-stabilizing was evaluated using hypotonic solution-induced hemolysis [34]. Whole blood collected from anesthetized rats through orbital puncture was washed until the supernatant was

clear with an isotonic solution (154 mM NaCl) through centrifugation at 3000 rpm. The erythrocyte (precipitate) was measured and reconstituted as a 40% (v/v) suspension with isotonic buffer solution (10 mM sodium phosphate buffer pH 7.4) and kept as the stock red blood cells suspension (RBCs). The reaction mixture was made up of RBCs (0.25 mL), 2.5 mL of hypotonic solution (5 mM NaCl) in 10 mM sodium phosphate-buffered saline (pH 7.4), and 0.25 mL of the extracts or indometacin at a different concentration range (50–1000 $\mu\text{g/mL}$). The control sample consisting of 0.25 mL of RBCs was mixed with hypotonic buffered saline alone. This was incubated at room temperature for 10 min and then centrifuged at 3000 rpm. The absorbance of the supernatant was measured at 540 nm. Each sample was analyzed in triplicate. The percentage inhibition of hemolysis or membrane stabilization was calculated using the following equation:

$$\text{Inhibition of hemolysis (\%)} = [1 - (A_2 - A_1)/(A_3 - A_1)] \times 100$$

where:

A_1 = test sample in isotonic solution

A_2 = test sample in hypotonic solution

A_3 = control sample in hypotonic solution

Carrageenan-induced paw edema

Paw edema was induced by intraperitoneal injection of 0.1 mL of carrageenan suspension (1% w/v) into the sub-plantar region of the right hind paw of the rats [35]. One hour prior to the carrageenan injection, five groups of six rats each were orally treated with distilled water (10 mL/kg bw), indometacin (10 mg/kg bw), and extract (200 and 400 mg/kg bw). At intervals before, and after 1, 2, 3, 4, and 5 h following the carrageenan injection, paw edema was measured by the displacement technique using a Vernier caliper to find out the circumference of paw edema. Total edema developed during this period was monitored as the area under the time-course curve (AUC). The inhibitory activity was calculated according to the formula:

$$\text{Inhibition of paw edema (\%)} = [(C_t - C_o)_{\text{control}} - (C_t - C_o)_{\text{treated}} / (C_t - C_o)_{\text{control}}] \times 100$$

where:

C_t = paw circumference at time t

C_o = paw circumference before carrageenan injection

$(C_t - C_o)$ = edema or change in paw size after time t.

Analgesic activity

The peripheral analgesic activity of the extracts was assessed in acetic acid-induced abdominal writhing in Albino rats as described by Veerappan et al. [36]. This method is characterized by abdominal constriction writhing from the intraperitoneal injection of acetic acid (10 mL/kg of 0.6% v/v glacial acetic acid solution in normal saline). Groups of six rats each were orally administered distilled water (10 mL/kg bw), diclofenac sodium (100 mg/kg bw), and extract (200 and 400 mg/kg bw). Thirty minutes after the acetic acid solution was administered, the number of writhing was counted for the next 15 min. The percentage inhibition of writhing was calculated using the formula below:

$$\text{Inhibition of writhing (\%)} = [(M_{\text{control}} - M_{\text{sample}} / M_{\text{control}})] \times 100$$

where M = mean number of writhing.

Antipyretic activity

The antipyretic activity was characterized by a fever-induced by subcutaneous injection of 10 mL/kg of 20% w/v suspension of brewer's yeast in normal saline [37]. Animals whose rectal temperature increased by at least 0.5 °C after 18 h of yeast injection were included in the study. The normal rectal temperature of each animal was measured using a digital thermometer. The animals were randomly divided into four groups of six animals and

treated as follows: distilled water (10 mL/kg bw), aspirin (100 mg/kg bw), and extract (200 and 400 mg/kg bw). The rectal temperature was again recorded at time intervals of 1, 2, 3, 4, and 5 h after extract administration. Rectal temperature reduction for each treatment was calculated in the arbitrary unit as the area under the curve (AUC).

Statistical analysis

Data were expressed as mean $\pm$ SD. Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls Multiple comparison test using SPSS 16.0 Windows software. Differences between values were considered significant at $p < 0.05$.

Results

Phytochemical screening

Phytochemical screening of the various extracts revealed the presence of bioactive constituents (Table 2). Except for proteins and amino acids, all other chemical compounds are present in all crude extracts. However, flavonoids, glycosides, and tannins were low in EtOHVT. Flavonoids and alkaloids were low in EtOHAM and EtOHEC, respectively.

Table 2: Chemical constituents of the studied plant ethanol extracts.

Phytoconstituents	Identification tests	Observations		
		<i>V. thyrsoflora</i>	<i>E. cylindricum</i>	<i>A. mannii</i>
Alkaloids	Dragendorff's test	++	+	+++
	Mayer's test	++	+	+++
Flavonoids	Lead acetate test	+	+++	+
	Shinoda's test	+	+++	+
Glycosides	Raymond's test	+	+++	++
	Killer Killani's test	+	+++	++
	Legal test	+	+++	++
Carbohydrates	Molisch's test	+++	+++	+++
	Fehling's test	+++	+++	+++
	Benedict's test	+++	+++	+++
Tannins	Vanillin-HCl test	+	+++	+++
	Gelatin test	+	+++	+++
Resins	Ferric chloride test	+++	+++	+++
	Turbidity test	+++	+++	+++
Steroids	Liebermann-Buchard's test	+++	++	+++
	Salkowski's test	+++	++	+++
	Biuret test	—	—	—
Proteins and amino acids	Precipitation test	—	—	—
	Ninhydrin test	—	—	—

—, absent; +, present at low concentration; ++, present at moderate concentration; +++, present at high concentration.

Antioxidant activity

TPC, TFC, and FRAP of studied plant extracts ranged from 15.93 to 64.45 CA mg/g, 336.03 to 1053.48 CA mg/g, and 288.75 to 364.91 CA mg/g, respectively (Table 3). The highest concentration of phenolics, flavonoids, and reducing power was found in EtOHEC (64.45 CA mg/g), EtOHAM (1053.48 CA mg/g), and EtOHVT (364.91 CA mg/g), while the lowest contents were recorded in EtOHAM (15.93 CA mg/g), EtOHVT (336.03 CA mg/g), and EtOHAM (288.75 CA mg/g), respectively. All extracts showed good DPPH, H₂O₂, and ABTS scavenging

activities (Figure 1 and Table 3) with IC_{50} values ranging from 0.30 to 1.65 $\mu\text{g/mL}$, 1.40 to 3.55 $\mu\text{g/mL}$, and 0.52 to 1.90 $\mu\text{g/mL}$, respectively, although weak when compared to those of ascorbic acid (0.05–0.63 $\mu\text{g/mL}$). A great scavenging action reflects a higher antioxidant activity and a lower IC_{50} . The IC_{50} values of extracts decreased in the following order: EtOHEC > EtOHVT > EtOHAM > ascorbic acid.

Table 3: Antioxidant activities of the studied plant ethanol extracts.

Extracts	TPC	TFC	FRAP	DPPH	H_2O_2	ABTS
<i>V. thyrsoiflora</i>	32.97 ± 1.32^b	336.03 ± 7.49^c	364.91 ± 2.85^a	0.30 ± 0.01^b	1.40 ± 0.03^b	0.52 ± 0.03^b
<i>E. cylindricum</i>	64.45 ± 1.73^a	458.12 ± 20.17^b	325.38 ± 1.84^b	1.65 ± 0.04^d	3.55 ± 0.09^c	1.90 ± 0.05^d
<i>A. mannii</i>	15.93 ± 1.27^c	1053.48 ± 43.81^a	288.75 ± 2.96^c	0.67 ± 0.02^c	1.81 ± 0.07^b	0.84 ± 0.01^c
Ascorbic acid				0.05 ± 0.03^a	0.63 ± 0.02^a	0.05 ± 0.02^a

^{a,b,c,d}In the same column, values carrying different letters in superscript are significantly different at $p < 0.05$ (Student-Newman-Keuls test). TPC, total phenolic content; TFC, total flavonoid content; FRAP, ferric reducing antioxidant power expressed in mg catechin equivalent/g by dry weight (CA mg/g). DPPH, H_2O_2 , and ABTS expressed in inhibitory concentration 50 ($\mu\text{g/mL}$).

Anti-inflammatory activity

Protein denaturation was inhibited by all the extracts studied in a concentration-dependent manner (Table 4) with percentage inhibition between 53.94% and 82.27% (EtOHVT), 11.39 and 76.34% (EtOHEC), and 6.79 and 77.47% (EtOHAM). Diclofenac sodium had the maximum percentage of inhibition (78.22–97.53%). The protein denaturation inhibitory effects of the extracts decreased in the following order: Diclofenac sodium > EtOHVT > EtOHEC > EtOHAM.

Table 4: Effect of the studied plant ethanol extracts on protein denaturation and RBCs hemolysis.

	Concentration ($\mu\text{g/mL}$)				
	1000	500	200	100	50
Inhibition of protein denaturation (%)					
<i>V. thyrsoiflora</i>	82.27 ± 0.13^b	79.85 ± 5.45^a	64.79 ± 3.28^b	59.25 ± 1.53^b	53.94 ± 7.25^b
<i>E. cylindricum</i>	76.34 ± 6.77^b	42.31 ± 8.60^b	20.92 ± 3.50^c	14.51 ± 2.70^c	11.39 ± 1.48^c
<i>A. mannii</i>	77.47 ± 6.35^b	37.67 ± 9.40^b	19.07 ± 6.22^c	9.72 ± 2.63^{cd}	6.79 ± 5.81^c
Diclofenac sodium	94.53 ± 8.45^a	84.46 ± 3.45^a	82.43 ± 2.34^a	81.18 ± 1.28^a	78.22 ± 1.02^a
Inhibition of hemolysis (%)					
<i>V. thyrsoiflora</i>	88.00 ± 2.87^b	70.27 ± 3.55^b	50.19 ± 4.08^b	37.77 ± 5.67^b	21.18 ± 2.71^b
<i>E. cylindricum</i>	78.13 ± 1.05^c	42.76 ± 7.34^d	39.61 ± 0.37^c	28.30 ± 0.67^c	19.26 ± 1.81^b
<i>A. mannii</i>	79.35 ± 1.96^c	56.91 ± 0.97^c	37.23 ± 1.51^{cd}	24.41 ± 0.23^c	18.90 ± 0.23^b
Indometacin	95.34 ± 4.27^a	86.14 ± 9.19^a	71.66 ± 6.65^a	56.74 ± 4.83^a	44.87 ± 3.12^a

^{a,b,c,d}For the same concentration, values carrying different letters in superscript are significantly different at $p < 0.05$ (Student-Newman-Keuls test).

At the different concentrations tested, all the extracts protected the erythrocyte membrane against lysis induced by hypotonic solution (Table 4) with the inhibition percentage of hemolysis being between 21.18% and 88.00% (EtOHVT), 19.26% and 78.13% (EtOHEC), and 18.90% and 79.35% (EtOHAM). Indometacin (44.87–95.34%) exerted a higher inhibitory effect than plant extracts. Inhibitory effects of the extracts decreased in the following order: indometacin > EtOHVT > EtOHEC > EtOHAM.

The injection of carrageenan in the control group caused edema, which increased gradually until it reaches a maximum size of 0.234 mm after 5 h. Earlier administration, the effect of the reference drug (indometacin) resulted in significant inhibition of the development of edema in a progressive manner (Table 5). The inhibitory effect of indometacin (10 mg/kg) occurred in the first hour after carrageenan injection and was maintained at almost the same level for 5 h, with inhibition percentages ranging from 63.95% to 83.89%. Pre-treatment of the rats with the ethanol extracts resulted in a dose-dependent reduction of edema with inhibition percentages ranging between 10.25% and 32.55% (EtOHVT), 6.86% and 43.89% (EtOHEC), and 13.67% and 34.74% (EtO-

HAM) at 200 mg/kg bw; 21.31% and 41.86% (EtOHVT), 16.66% and 46.61% (EtOHEC), and 10.65% and 67.44% (EtOHAM) at 400 mg/kg bw. The inhibitory activity of EtOHAM at 400 mg/kg bw (67.44%) was greater than indometacin (63.95%) after 1 h.

Table 5: Effect of the studied plant ethanol extracts on carrageenan-induced rat paw edema.

Treatment	Edema or change in paw size after time t (mm) and % inhibition					
	Dose (mg/kg)	1 h	2 h	3 h	4 h	5 h
Control	–	0.17 ± 0.05	0.21 ± 0.05	0.23 ± 0.04	0.20 ± 0.06	0.23 ± 0.04
Indometacin	10	0.06 ± 0.03 ^a (63.95)	0.06 ± 0.03 ^a (71.69)	0.05 ± 0.03 ^b (76.27)	0.05 ± 0.03 ^a (83.89)	0.05 ± 0.03 ^b (76.22)
<i>V. thyriflora</i>	200	0.11 ± 0.07 (32.55)	0.15 ± 0.09 (29.24)	0.18 ± 0.10 (22.03)	0.16 ± 0.09 (19.60)	0.21 ± 0.12 (10.25)
	400	0.10 ± 0.02 (41.86)	0.14 ± 0.03 (32.07)	0.15 ± 0.04 (35.59)	0.15 ± 0.06 (23.52)	0.19 ± 0.02 (21.31)
<i>E. cylindricum</i>	200	0.11 ± 0.05 (34.88)	0.12 ± 0.08 (43.39)	0.21 ± 0.07 (11.01)	0.19 ± 0.09 (6.86)	0.17 ± 0.07 (25.64)
	400	0.09 ± 0.01 (46.51)	0.11 ± 0.04 (46.22)	0.12 ± 0.04 (46.61)	0.17 ± 0.05 (16.66)	0.18 ± 0.06 (26.22)
<i>A. mannii</i>	200	0.12 ± 0.04 (30.23)	0.15 ± 0.07 (25.47)	0.15 ± 0.03 (34.74)	0.15 ± 0.07 (26.47)	0.20 ± 0.06 (13.67)
	400	0.05 ± 0.03 ^b (67.44)	0.10 ± 0.04 (49.05)	0.15 ± 0.04 (36.44)	0.17 ± 0.05 (13.72)	0.21 ± 0.04 (10.65)

^ap < 0.05, ^bp < 0.001, ^cp < 0.01, compared to control group (water), n = 6 (Student-Newman-Keuls test), mean ± SD.

Analgesic activity

Acetic acid injection intraperitoneally caused 41 abdominal writhings within 15 min in the control group (Table 6). The extracts significantly inhibited the writhing in a dose-dependent manner with inhibition percentages ranging from 28.98% to 65.21% (200 mg/kg) and 51.20% to 76.81% (400 mg/kg). EtOHAM (65.21%) showed a similar activity to diclofenac sodium (65.70%) at 200 mg/kg. The activity of EtOHAM (76.81%) was better than diclofenac sodium at 400 mg/kg. Inhibitory effects of the extracts against abdominal writhing decreased in the following order: EtOHAM > indometacin > EtOHVT > EtOHEC.

Table 6: Effect of the studied plant ethanol extracts on acetic acid-induced rat writhing reflex.

Treatment	Dose (mg/kg)	Number of writhing	% inhibition
Control	–	41.40 ± 9.81	0.00
Diclofenac sodium	100	14.20 ± 2.77 ^a	65.70
<i>V. thyriflora</i>	200	28.80 ± 5.93 ^b	30.43
	400	19.00 ± 6.00 ^a	54.10
<i>E. cylindricum</i>	200	29.40 ± 2.96 ^b	28.98
	400	20.20 ± 7.25 ^a	51.20
<i>A. mannii</i>	200	14.40 ± 5.77 ^{a,d}	65.21
	400	9.60 ± 4.66 ^a	76.81

^ap < 0.001, ^bp < 0.01, ^cp < 0.05 compared to control group, ^dp < 0.01 compared to treated group (200 mg/kg), n = 6 (Student-Newman-Keuls test), mean ± SD.

Antipyretic activity

Experimental rats showed an average increase in rectal temperature of 0.93 °C after 18 h of yeast injection (Figure 2). The temperature gradually increased and reached a maximum (2.15 °C) after 4 h in the control group. In the group treated with the standard drug (aspirin), the rectal temperature of the animals returned to normal after 5 h. Except for EtOHVT at the dose of 200 mg/kg, the other extracts significantly reduced the rectal temperature

of the rats compared to the control group. EtOHAM showed a similar antipyretic effect ($p < 0.001$) to aspirin at 400 mg/kg. There was a sharp decrease in rectal temperature 1 h after administration of EtOHAM at 400 mg/kg, which was further followed by a gradual decrease in the tendency up to 5 h. Inhibitory effects of the extracts against fever decreased in the following order: aspirin > EtOHAM > EtOHEC > EtOHVT.

Figure 1: Effect of the studied plant ethanol extracts on time-course curves (A) and rectal temperature reduction (B) in the protocol of the brewer's yeast-induced rat pyrexia. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control; # $p < 0.05$ compared to treated group (400 mg/kg), $n = 6$ (Student-Newman-Keuls), Mean $\pm$ SD; VT 200: *V. thyriflora* (200 mg/kg), VT 400: *V. thyriflora* (400 mg/kg), EC 200: *E. cylindricum* (200 mg/kg), EC 400: *E. cylindricum* (400 mg/kg), AM 200: *A. mannii* (200 mg/kg), AM 400: *A. mannii* (400 mg/kg). All extracts reduced the rectal temperature of the rats compared to the control group.

Discussion

The identification of bioactive principles of medicinal plants is crucial for the standardization of herbal medicines. The presence of numerous biologically active secondary metabolites in the various plant extracts is responsible for their pharmacological properties. Several investigators reported the presence of these compounds in the Verbenaceae, Meliaceae, and Annonaceae families to which belong the studied plants [38]. Kuete et al. [23] working on the methanol extract of *A. mannii* leaves revealed the presence of alkaloids, steroids, and tannins and the absence of flavonoids. Ngnokam et al. [39] showed the presence of steroids in methylene chloride extract of the stem barks of *E. cylindricum*. Kopa et al. [14] revealed the presence of alkaloids, flavonoids, steroids, and glycosides in the ethanol extract of *V. thyriflora* and also steroids in the methanol-methylene chloride extract of the stem barks of *V. thyriflora* [40].

The TFC of the extracts was significantly higher than that of TPC, which was proportional to their reducing power. The TFC method involves hydrolysis, which liberates even bound flavonoids, unlike the TPC method that analyzes only free phenol compounds. Phenolic compounds act as antioxidants, radical scavengers, metal chelators, mediators, and enzyme inhibitors. They end free radical chain reactions by hydrogen transfer and by transforming these reactive species into more stable non-radical products. Some studies showed a correlation between phenolic content and antioxidant capacity [41], [42], [43]. Antioxidant activity was reported to increase proportionally with increased phenolic content [44]. Radical scavenging activity of the extracts may be due to their electron donor capacity by the DPPH as well as the H_2O_2 method. This could be explained by the difference in the stoichiometry of the reactions between antioxidant compounds in the extract and various radicals. Other factors such as radicals' stereoselectivity or differential solubility of the compounds (methanol for the DPPH test and distilled water for the H_2O_2 test) could also influence the ability of the extract to reduce different radicals when the latter contains a variety of antioxidants [45]. Although phenolic compounds possess an antioxidant potential, it is possible that antioxidant properties of the studied extracts are at least, in part, due to the presence of their non-phenolic compounds such as carbohydrates, alkaloids, and steroids. These compounds possess antioxidant activity by suppressing the initiation or propagation of chain reactions [46]. Catechin and its derivatives (epicatechin, androlide G) isolated from the stem bark of *E. cylindricum* were reported to be effective scavengers of ROS and may also function indirectly as antioxidants through their effects on transcription factors and enzyme activities [47].

Several anti-inflammatory drugs showed dose-dependent ability to inhibit thermally-induced protein denaturation. The mechanism of denaturation probably involved alteration of the electrostatic hydrogen, hydrophobic and disulfide bonding due to the inflammation response. The extracts could inhibit protein denaturation by interacting with amino acids that are exposed and denatured on heating such as lysine and

threonine [48]. The erythrocyte membrane is a lysosomal membrane analog [43]. The lysosomal enzymes released during inflammation produce a variety of damage. The activity of these extracellular enzymes would be related to acute or chronic inflammation. There is evidence that these enzymes play an important role in the development of acute and chronic inflammation. Most anti-inflammatory drugs inhibit the release of these enzymes or stabilize the lysosomal membrane, which is one of the major events responsible for the inflammatory process [49]. Compounds with membrane-stabilizing properties are well known for their ability to interfere with the release of phospholipases that trigger the formation of inflammatory mediators [49]. All the extracts showed the membrane-stabilizing properties, which suggest that their anti-inflammatory activity observed in this study, may be related to the inhibition of the release of phospholipases that trigger the formation of inflammatory mediators. The induced inflammatory response is triphasic, characterized by the formation of marked edema resulting from the rapid production of several inflammation mediators. The first phase (90 min) involves the release of histamine and serotonin; the second phase (90–150 min) is mediated by kinins (bradykinin), and the third phase (after 180 min) by prostaglandins and nitric oxide produced by isoforms of cyclooxygenase and inducible NO synthase, respectively [50]. Although the actual mechanism of action of EtOHAM, EtOHEC, and EtOHVT in inflammation is unknown, these extracts inhibited the early phase of edema. The maximum anti-inflammatory activity occurred between 1 h and 2 h at the different doses tested. This suggests that these ethanol extracts would probably act by inhibiting the release and/or action of histamine, serotonin, and kinin. However, the exact mechanism needs to be established through further investigations. Secondary metabolites such as alkaloids, flavonoids, tannins, or steroids present in the three extracts, could be responsible for the observed anti-inflammatory activity [51]. Flavonoids may inhibit enzymes such as aldose reductase, xanthine oxidase, phosphodiesterase, Ca^{2+} -ATPase, lipoxygenase, and cyclooxygenase; as well as other mediators of the inflammatory process, such as protein C or adhesion of reactive molecules [52]. Steroids are known to attenuate inflammation by inhibiting phospholipase A2, which hydrolyzes arachidonic acid from membrane phospholipids, and the subsequent formation of prostanoids and leukotrienes through the pathways of cyclooxygenase and lipoxygenase and immune dysfunction in experimental models [53]. Kouam et al. [20] showed that the sapelenins G-J (acyclic triterpenoids) isolated from the stem barks of *E. cylindricum*, exhibit anti-inflammatory activities by suppressing the secretion of interleukin 17 by phytohemagglutinin-stimulated human peripheral blood mononuclear cells. Sapelenin G showed high activity comparable to reference cyclosporin A without any cytotoxic effects. Nakanishi et al. [54] reported that catechin reduces significantly the expression of pro-inflammatory cytokines (interleukin 6 and interleukin 8) and adhesion molecules (intercellular adhesion molecule-1 and vascular cell adhesion molecule-1) in human dental pulp cells stimulated with lipopolysaccharide or peptidoglycan. It also exhibits anti-inflammatory effects in BV-2 cells and 3T3-L1 adipocytes by suppressing the production of pro-inflammatory mediators (nitric oxide, tumor necrosis factor- α , and ROS) and mitigation of nuclear factor-kB through protein kinase B, extracellular signal-regulated kinase, p-38 mitogen-activated protein kinase, and adenosine monophosphate-activated protein kinase pathways [55], [56]. Catechins can stabilize the structure of the gastrointestinal micro-ecological environment via promoting the proliferation of beneficial intestinal bacteria and regulating the balance of intestinal flora, so as to relieve the inflammatory bowel disease [57]. Catechins could be also a key mediator in cardiovascular health via mechanisms of blood pressure reduction, flow-mediated vasodilation, and atherosclerosis attenuation [58].

It was suggested that acetic acid would act by releasing endogenous mediators that stimulate nociceptive neurons. Writhing is induced following activation of the local peritoneal receptor and involves prostanoid mediators. In rats, there is an increase in the peritoneal fluid of prostaglandins E2 and F2, as well as products of lipoxygenase, the release of sympathetic mediators of the nervous system. The nociceptive properties of acetic acid may also be due to the release of cytokines, such as nuclear factor-kB, tumor necrosis factor α , interleukin 1 β , and interleukin 8, by peritoneal macrophages and resident mast cells [59]. The inhibitory effects on inflammatory pain and abdominal writhings produced after acetic acid administration may, therefore, be due to their ability to interfere with the activation of nociceptors by one of these endogenous mediators or to remove the sensitization of nociceptors to prostaglandins.

It was documented that yeast-induced fever increases the production of prostaglandins, which, in turn, stimulate the thermoregulatory center to increase body temperature [60]. The hypothermic activity of the extracts may be due to their action on cyclooxygenase 2, which would reduce the concentration of prostaglandin E2 in the brain or increase the inherent production of the body's own antipyretic substances such as arginine and vasopressin [61].

Conclusions

The present study demonstrated that EtOHVT, EtOHEC, and EtOHAM possess good antioxidant, anti-inflammatory, analgesic, and antipyretic effects. These effects might be attributed to the presence of biologically active compounds. However, among the three plants studied, ETOHAM could be recommended in the effective treatment of inflammation in traditional medicine.

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