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Chemical Composition, Anti-Inflammatory and Analgesic Activities of Extracts and Fractions of *Vitex negundo*

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Authors' contributions

This work was carried out in collaboration among all authors. Author ZMPR created the study design, assisted and supervised the laboratory work, performed data analysis and interpretation, wrote and edited the manuscript, and submitted a request for funding approval. Authors CGT, JDC and FA conducted experiments, troubleshot laboratory issues, and contributed to the discussion and insights. Authors CGT and JDC assisted in the paper review. All authors read and approved the final manuscript.

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ABSTRACT

This study aims to determine chemical components and demonstrate the anti-inflammatory and analgesic activities of *Vitex negundo* species native to the Philippines. Using high-performance liquid chromatography (HPLC) and liquid chromatography-tandem mass spectrometry (LC-MS/MS), eleven compounds were tentatively identified from the iridoids, flavonoids, and phenolics group. The anti-inflammatory activity was determined by measuring the inhibition of proinflammatory enzymes, lipoxygenase (LOX), and cyclooxygenase (COX-2). For LOX inhibitory

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effect, crude methanol extract has an IC_{50} value of 440.9 µg/mL. Solid-phase extraction (SPE) fractionation results in IC_{50} values of 196.6 µg/mL and 150.0 µg/mL for the 50% methanol-water and 100% methanol fractions, respectively. The IC_{50} value of crude hexane extract is 519.7 µg/mL. SPE produced lower IC_{50} values; 397.5 µg/mL for 2% IPA-hexane fraction and 142.9 µg/mL for 50% IPA-hexane fraction. The inhibition against COX-2 was effective in the 100% methanol fraction with an IC_{50} of 34.27 µg/mL and 2% IPA-hexane with an IC_{50} of 6.57 µg/mL. SPE fractions demonstrated more potent anti-inflammatory effects via in-vitro inhibition of LOX for the 100% methanol-water and 50% IPA-hexane or COX-2 for the 100% methanol and 2% IPA-hexane. The analgesic action was evaluated using acetic acid-induced writhing in mice. The percentage inhibition of writhing at all dose levels of methanol and hexane extracts ranged from 90-96% ($p<0.05$). The SPE fractions from methanol extracts showed inhibition of 73-97% ($p<0.05$). Both extracts and fractions showed strong analgesic effects in an *in-vivo* animal model.

Keywords: Vitex negundo; extracts; fractions; SPE; HPLC; LC-MS/MS; anti-inflammatory; analgesic.

1. INTRODUCTION

Vitex negundo (VN) is a member of the Vitex genus in the Verbenaceae family. It is a 2 to 5-meter tall, erect, branching tree or aromatic shrub. The leaves have a classic five-foliolate arrangement. VN is native to South Asia, China, Indonesia, and the Philippines and can be found in tropical to temperate climates [1].

VN has been studied for its antitussive [2], anti-asthmatic [3], and the mechanism underlying its efficacy in hyperactive respiratory disease properties [4]. The plant has been utilized as herbal medicine in the Philippines, supported by the Department of Health. In addition, medical researchers examined its clinical efficacy as a treatment for cough and asthma [5].

Review papers of various studies claim that VN also possesses anti-inflammatory and analgesic effects [6,7]. In a mouse model study of OVA-LPS-induced allergic asthma, the hydroalcoholic extract of VN leaves has complex preventative and inhibitory effects on the formation and progression of inflammation associated with the allergic airway. It has been shown to reduce inflammatory aggravation and lung injury by modulating the AMPK/PI3K/Akt/p38-NF-B and TGF/Smad/Bcl2/ caspase/LC3 cascades, as well as the activation of alveolar macrophages [8,9]. In another study, the freeze-dried aqueous leaf extract of VN suppressed inflammatory responses by human leukocytes via multiple *in vitro* mechanisms, including inhibition of cytokines interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) secretion, phagocytosis of human neutrophils, reactive oxygen species (ROS) and nitric oxide (NO) production, and induction of membrane stabilization [10]. The antinociceptive efficacy of ethanolic leaf extract of VN has been studied using the tail-flick test in rats and acetic acid-induced writhing in mice. Observations reveal that the extract has central and peripheral analgesic action [11]. A bioassay-guided separation study from the acetoacetate fraction of VN seeds yielded a lignan molecule that exhibited strong analgesic efficacy in mice models of nociception generated by chemical stimuli [12]. The phytochemicals in a medicinal plant are responsible for its therapeutic effects, providing biological benefits for humans. Analyses of the phytochemistry of VN leaves have led to the detection of phytochemicals such as triterpenoids, alkaloids, flavonoids, tannins, and iridoid glycosides [13]. The anti-inflammatory and analgesic action of VN seeds has been attributed to several lignan compounds [12].

In the Philippines, VN (known as Lagundi) is widely known as a cough medicine. However, little is known, and no established scientific report exists on the chemical components and other activities of native VN leaves. The present study aims to determine the chemical composition of VN using chromatographic and mass spectrometric analytical methods and to investigate the potential anti-inflammatory and analgesic action of the extracts and fractions by *in-vitro* and *in-vivo* experiments.

2. MATERIALS AND METHODS

2.1 Plant Materials

VN was acquired from a Palawan Center for Appropriate Rural Technology, Inc. farm in Bacungan, Puerto Princesa, Philippines. Plants were authenticated and validated by the Department of Pharmacology of the University of the Philippines, Manila. The leaves of VN were

harvested just before the onset of flowering. Processing of raw materials entails the following steps: (1) harvesting was carried out by the farmers in the morning, and the harvested leaves were placed in new plastic bags, (2) harvests were then transported to the facility's garbling and washing sections, (3) air-drying was completed within two hours from harvesting or up to a moisture level between 7-8%, (4) leaves are placed in food-grade polyethylene bags, sealed, and delivered to the processing facility after they have dried and cooled, (3) dried plant samples were subjected to ozonation, pulverized to the necessary mesh size, and dried in an industrial oven to a maximum of 5% moisture content.

2.2 Chemicals

HPLC-grade acetonitrile, methanol, hexane, and 2-propanol were obtained from Theo-Pam Trading, Philippines. The analytical-grade chemicals were purchased from Belman Laboratories, Philippines. VN standards Negundoside and Kaempferol (Sigma-Aldrich) were purchased from Merck, Philippines. Agnuside, 3,4-Dihydroxybenzoic acid, and 4-Hydroxybenzoic acid were purchased from ChemFaces Biochemical Co., Ltd., China. For the LOX assay, Glycine max (soybean), Lipoxygenase (Cat No: L7395), Linoleic acid (Cat No: L1376), and Indomethacin (Cat No: I8280) were from Sigma-Aldrich. For COX assay, the COX-2 (human) Inhibitor Screening Assay Kit (Cat No: 701080, Cayman Chemicals) was supplied by Infinnomed Enterprise, Philippines. λ-Carrageenan (Cat No: 22049, Sigma-Aldrich) was provided by Chemline Scientific Corporation, Philippines.

2.3 Preparation of Standards

Stock solutions of standard compound Agnuside, 3,4-Dihydroxybenzoic acid, 4-Hydroxybenzoic acid (1 mg/mL), Kaempferol (0.5 mg/mL), Negundoside (0.1 mg/mL) were prepared separately in methanol. All the standard solutions were stored at 4°C until use. An appropriate volume of the individual stock standard was taken to prepare a mixed standard solution with the desired concentration suitable for chromatographic identification. Before analysis, the prepared mixed standard solution was sonicated and filtered through a 0.20 µm polytetrafluoroethylene polymer (PTFE) membrane filter.

2.4 Preparation of Plant Extracts

The powdered leaves of VN were extracted using pure methanol or hexane (25 g in 200 mL solvent) and an ultrasonic bath (MRC Scientific Instruments Professional Ultrasonic, UK) operating at 40 kHz, 30°C for 60 minutes. After ultrasonic-assisted extraction (UAE), the mixture was filtered twice using cheesecloth followed by filter paper, concentrated, and dried using a rotary evaporator (IKA, Germany). The resulting concentrate was kept in an amber bottle at 4°C for up to one month. In each subsequent assay, an extract was freshly prepared.

2.5 Solid Phase Extraction (SPE) Fractionation of the UAE Extracts

A 25 mL of 10mg/mL sample solution of methanol extract was fractionated using SPE. The Strata-X Polymeric Reversed-Phase (500mg/6mL) cartridge was attached to an SPE vacuum manifold (Phenomenex), conditioned with 100% methanol, and equilibrated with 100% ultrapure water. Next, the extract solution was slowly passed through the preconditioned cartridge. SPE column was eluted with two solvent mixtures of 50% methanol-water (SPE Methanol fraction 1) and 100% methanol (SPE Methanol fraction 2) with decreasing polarity. A 50mL 20mg/mL sample solution was fractionated for the hexane extract. The Strata Si-1 Normal Phase (1g/6mL) cartridge was attached to an SPE vacuum manifold (Phenomenex), conditioned with 100% dichloromethane, and equilibrated with 100% hexane. Next, the extract solution was slowly passed through the preconditioned cartridge. SPE column was eluted with two solvent mixtures of 2% IPA-hexane (SPE Hexane fraction 1) and 50% IPA-hexane (SPE Hexane fraction 2). Each collected fraction was evaporated to dry using a rotary evaporator at 80 rpm, a 40°C water bath, and a 10°C chiller. The fractions were subjected to HPLC analysis and biological assays.

2.6 High-performance liquid Chromatography (HPLC)

HPLC analysis was performed to obtain chromatographic fingerprints of VN extracts. Methanol (2.5 mg/mL) or hexane (10 mg/mL) VN extracts dissolved in methanol were passed through a 0.2µm PTFE membrane filter for HPLC injection. Each extract and the standard mixture were subjected to HPLC analysis separately. The

HPLC system consisted of a separation module (Shimadzu Prominence) equipped with LabSolutions software (Shimadzu) with a binary pump, needle-in-flow path autosampler, and a photodiode array (SPD-M20A) detector. The analysis was performed on a Waters Xbridge C18 (4.6×250 mm, 5.0 µm) column using 0.5% phosphoric acid (solvent A) and acetonitrile (solvent B) as mobile phase in gradient elution. All the mobile phase solvents were passed through a 0.45 µm membrane filter. Mixed standard (10 µL) and samples (5 or 10 µL) were injected at a flow rate of 0.5 mL/min into the HPLC. The column oven was at 30°C, and the HPLC peaks were observed at 254 nm. The mobile phase gradient elution used was: 0-10 min: 85-60% A; 10-30 min: 60-45% A; 30-40 min: 45-25% A; 40-60 min: 25-20% A; 60-70 min: 20-5% A; 70-75 min: 5-85% A.

2.7 Semi-Preparative HPLC

The SPE fractions of methanol or hexane VN extracts were dissolved in methanol. The same HPLC system was used, but the analysis was done on a Waters XBridge C18 OBD Semi-Preparative Column (10mx250mm, 5.0µm) using 0.1% formic acid (solvent A) and acetonitrile (solvent B) as mobile phase in gradient elution. All the mobile phase solvents were passed through a 0.45 µm membrane filter. Samples (50 µL) were injected into the HPLC using a flow rate of 2.3629 mL/min with a delay volume of 572 µL and a sampling time/time constant of 0.160 sec. The column oven was at 30°C, and the HPLC peaks were observed at 254 nm. The solvent gradient elution used for methanol SPE fractions was: 0-10 min: 85% A; 10-30 min: 85-60% A; 30-50 min: 60-45% A. The solvent gradient elution used for hexane SPE fractions was: 0-10 min: 85-60% A; 10-30 min: 60-45% A; 30-40 min: 45-25% A; 40-65 min: 25-20% A; 65-75 min: 20-5% A. All changes were based on scale-up methods from analytical to preparative HPLC.

2.8 Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

LC-MS analysis of methanol and hexane VN extracts was carried out at Pascual Pharma Laboratory using an ESI-QTOF-MS/MS system comprised of a Waters ACQUITY I-Class UPLC coupled with a Waters Xevo G2-S QTOF mass spectrometer. Two (2) µL samples were separated on reverse-phase Waters ACQUITY HSS C18 column (2.1×100 mm, 1.8 µm) at 30°C with gradient elution at a flow rate of 0.25 mL/min. The mobile phase was 0.1% formic acid-water (solvent A) and 0.1% formic acid-acetonitrile (solvent B). Using columns calculator ver. 2.0.53.0 (Waters Corporation), the HPLC gradient method was converted into a UPLC method to match the LC-MS system with minimal optimization. Data processing was performed using MassLynx 4.1. The acquisition parameters were: data range, 100-1500 Da; applied source temperature, 20 °C; desolvation temperature, 450 °C; cone gas (argon) flow rate, 50 L/h; desolvation gas (nitrogen) flow rate, 600 L/h; Electrospray ionization, positive mode capillary voltage, 3.0 kV and cone voltage, 80 V (source offset, 80 V). MSE mode, low and high collision energy scans; low energy scan, 6 eV, and high energy scan, 30 to 50 eV; scan time, 0.1 s. The RAW files output was converted to ABF for peak alignment, peak picking, and identification processing using MS-DIAL software. The spectral databases include the following libraries: GNPS, Sumner, ReSpect, MassBank EU, Massbank NA, Faulkner Legacy, NIH Natural Products, Prestwick Phytochemicals, and Dorrestein/FDA Natural Products. Several MS-DIAL-processed data were additionally processed in GNPS for compound matching.

2.9 Lipoxygenase (LOX) Inhibitory Activity

In a 96-well quartz plate, 25 µL of the sample (extract solution or SPE fraction) and 100 µL of lipoxygenase solution (0.0045 mg/mL) in borate buffer (pH 9.0) were incubated at room temperature for 5 min. Then, 25 µL of linoleic acid (0.84 mg/mL) was added and thoroughly mixed. The absorbance at 234 nm was measured using the Biotek Synergy HT microplate reader. Borate buffer was used as a sample blank, while 5% DMSO-borate buffer was used as a control (enzyme + substrate only). Percentage inhibition was calculated as follows:

% Inhibition= [A (control)– A (test group)]/ A (control) x 100, where A = Mean absorbance. Indomethacin was used as a positive control. A dose-response curve was plotted to determine the IC_{50} values.

2.10 Cyclooxygenase (COX) Inhibitory Activity

The cyclooxygenase reaction was done, and all reagents were prepared as described in the

Cayman COX-2 human inhibitor screening assay kit. The assay measures prostaglandin F2α (PGF2), formed by $SnCl_2$ reduction of prostaglandin H2 (PGH2) from the COX-2 reaction. The sample fractions were screened at 150 and 450 µg/mL; then, a dose-response curve was performed along with Indomethacin. After reaction quenching, prostaglandin was determined by competitive Enzyme-linked Immunosorbent Assay (ELISA). The Biotek Synergy HT plate reader measured the absorbance at 405 nm. Percentage COX-2 inhibition and IC_{50} values were calculated.

2.11 Experimental Animals

The animals were sheltered in a room kept at 22±3°C and 50-60% relative humidity, with a light/dark cycle of 12 h: 12 h, and fed a standard laboratory diet (Sarimanok, UNAHCO) and sterile water in unlimited quantities. Before subjecting them for experimentation, the animals were given seven days to get acclimatized to laboratory conditions.

2.12 Acute Oral Toxicity

The acute oral toxicity of VN extract and SPE fractions was determined using the "Up-and-Down" method outlined in OECD Guideline 425 [14,15]. The animals used for testing were Swiss Webster mice (healthy, nulliparous, non-pregnant, 8-12 weeks old) weighing between 20-25 grams. They were provided water but fasted for three to four hours before drug administration. The test samples were administered in a single dose by oral gavage using a stomach tube. The study utilized a limit test (2000 mg/kg) and the main test (175, 550, and 2000 mg/kg) for test samples with lethality after the first animal dosing in the limit test. The general behavior of mice and clinical signs of toxicity were monitored constantly for the first hour and every 30 minutes for the next 6 hours following the treatment. Each animal was checked 24 hours after the treatment for any toxic manifestations and daily for 14 days after dosing. The experimental parameters include changes in the skin, fur, eyes, mucous membranes, respiratory and circulatory systems, salivation, diarrhea, lethargy, and convulsions. The type and intensity of clinical signs and lesions were recorded individually.

2.13 Acetic Acid-Induced Writhing in Mice

The analgesic activity was determined in female Swiss Webster mice (healthy, nulliparous, 8-12 weeks old) weighing 25-30 grams. They were provided water but fasted for three to four hours before drug administration. Injections of 0.6% acetic acid (10 mL/kg body weight) intraperitoneally into mice produced writhing. A painful reaction and acute inflammation manifest in the peritoneal region, causing a writhing reflex. Only animals that writhed within 15 minutes were included in the trial after a pre-screening. The selected animals were given 48 hours of rest. The pre-screened animals were divided into six groups at random. The test drugs were orally supplied to the mice 30 minutes before the acetic acid injection. Individual mice were placed in a glass viewing jar immediately after injection, and five minutes were allowed to pass. The animals were then observed for twenty minutes, during which each animal's number and time of writhing were recorded. As an indicator of analgesia, the percentage of inhibition of writhing was determined as follows:

% Inhibition= [W (Control) – W (test group)] / W (Control) Where W = Mean number of writhes

2.14 Statistical Analysis

Results were expressed as mean, standard deviation (SD), and standard error of the mean (SEM). Statistical analysis was performed using an Analysis of Variance (ANOVA) or Kruskal-Wallis in the SPSS statistics software (version 27). $P < 0.05$ was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1 Chemical Composition of VN Extracts

The chemical compounds found in VN extracts were analyzed through HPLC. The extraction yield of methanol is higher than hexane. Fig. 1A displays the chromatograms of available standard reference compounds for the VN sample. The chromatograms of methanol and hexane extracts of VN (Fig. 1B and 1C) showed relatively good peak resolutions. The eluted compounds in methanol extract (Fig. 1B) were detected in the 8-22 min range indicating polar compounds. The eluted compounds in the hexane extract (Fig. 1C) were detected in the 8-62 min range. Comparison of the peak retention times of these reference compounds under the same HPLC system conditions indicated that four peaks at 8.7, 11.7, 12.8, and 15.7 min could be 3,4-Dihydroxybenzoic acid (1), Negundoside (2),

4-Hydroxybenzoic acid (3), and Agnuside (4), respectively in both methanol and hexane extracts of VN (Fig. 1B and 1C). The intensity of the peaks seen in methanol extracts was higher than in hexane extracts, demonstrating that the high polar solvents were more effective at extracting the particular plant material. Methanol, distilled water, and ethanol had higher extraction yields than chloroform, dichloromethane, and acetone, according to prior research on the extraction of Severinia buxifolia. Methanol was also shown to be the most efficient solvent in their investigation for extracting bioactive compounds. It generated the largest concentration of phenolics, alkaloids, flavonoids, and terpenoids and the highest extraction yield. The same finding in our investigations showed that methanol extracts of VN had higher total phenolic and flavonoid contents than hexane extracts (data not shown) [16].

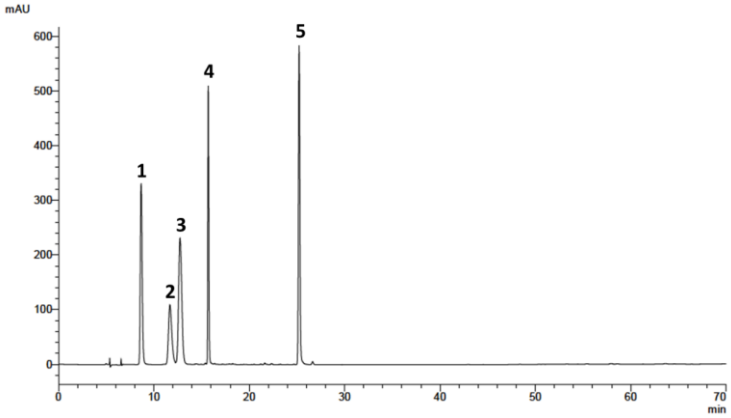
It is likely that these compounds identified may contribute to the action of VN extracts. This includes Agnuside, which exhibited the highest HPLC peak intensities. In a test for polyarthritis in rats, Agnuside isolated from VN substantially inhibited the expression of leukotriene B_4 and other proinflammatory mediators, exerting anti-inflammatory effects via its ability to regulate the balance of T-cell-mediated cytokines (Th1/Th2). Furthermore, Agnuside's non-significant suppression of PGE_2 at various levels was said to have no ulcerogenic potential since PGE_2 protects the gastric mucosa [17]. Thus, Agnuside can be potential use to treat arthritis. More compounds like 4-Hydroxybenzoic acid, 3,4-Dihydrobenzoic acid, Negundoside, and Luteolin were also isolated from the active fractions of VN. They were shown to be involved in the anti-inflammatory action of the leaf extract of VN on TPA (tetradecanoylphorbol acetate)-induced mouse ear inflammation via topical administration. With a 41% inhibition, 3,4-dihydrobenzoic acid showed moderate effectiveness in TPA-induced edema. The activities of the other three components were low, ranging between 24 and 27% [18].

LC-MS/MS untargeted system was performed to uncover more information about the chemical composition of VN extracts, especially those unidentified peaks. Plant components were analyzed from extracts or HPLC fractions. Fig. 2 represents the chromatograms with MS and UV detector of methanol (Fig. 2A) and hexane (Fig. 2B) VN extracts. Various peaks were observed in the chromatograms of both extracts. Table 1 shows the chromatographic and mass spectral properties of the identified peaks. As shown in Table 1, nine (9) compounds were characterized from the methanol extracts by manually comparing retention times and the observed molecular (precursor) ions, mass, and fragmentation patterns (i.e., product ions) to available reference standards or tentatively assigned based on the MS data library using MS-DIAL software. The compounds were determined as (1r,3R,4s,5S)-4-{[(2E)-3-(3,4-Dihydroxyphenyl)-2-propenoyl] oxy}-1,3,5trihydroxycyclohexanecarboxylic acid (6), negundoside (2), luteolin 6-C-glucoside (7), agnuside (4), homovitexin (8), luteolin 4'-O-glucoside (9), luteolin 8-C-glucoside (10), kaempferol 3-glucuronide (11), and chrysosplenol D (12). Peak number 4 gave a molecular ion $[M+Na]^+$ at m/z 489 and fragment ions at m/z 287 and m/z 131. This result is compatible with agnuside, an iridoid glucoside, in other *Vitex* species [19]. Homovitexin (8), often referred to as isovitexin, displayed a molecular ion $[M+H]^+$ at m/z 433 and luteolin 4'-O-glucoside (9) at m/z 449. These compounds were previously isolated from *Vitex* with the same molecular weight, but the latter is determined as luteolin-7-O-glucoside [20]. The LC-MS/MS confirmed the presence of negundoside, agnuside, 3,4-dihydroxybenzoic acid, and 4-hydroxybenzoic acid from the isolated HPLC peak fractions by comparing them with the standards (data not shown). Several detected compounds have also been reported to be present in VN. In other studies, the bioactivity-guided analysis of VN methanol extracts identified chrysoplenetin and chrysosplenol D as active compounds with significant selective cytotoxic activity against PANC-1 human pancreatic cancer cells [21]. The leaves of VN have reportedly been found to contain iridoids and flavonoids. It was determined that Negundoside and Agnuside are two iridoids. The flavonoids included vitexicarpin, isoorientin, vitex, and isovitexin [22]. In an attempt to elucidate the chemical biomarkers, unidentified peaks in this study not matched in the library will require further characterization. The use of more standard compounds, mass spectral libraries, and other spectroscopy techniques will be helpful.

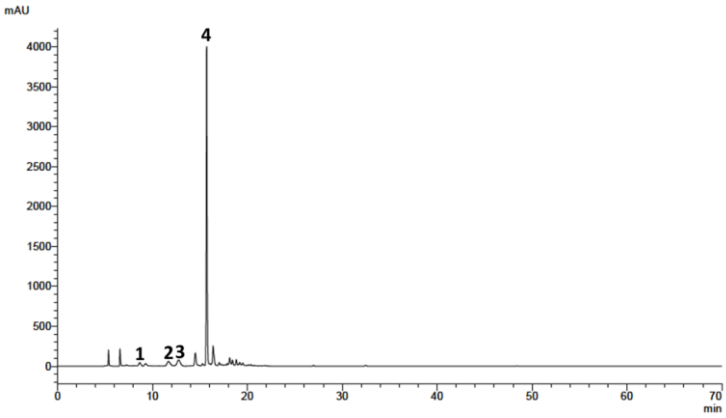
3.2 Chemical Profile of VN Fractions

Solid-Phase extraction (SPE) is becoming known as a method for separating and concentrating components from liquid samples. It avoids many

issues with liquid/liquid extraction, such as incomplete phase separations, less-than-quantitative recoveries, emulsion formation, and the disposal of large amounts of organic solvents. Methanol and hexane extracts of VN obtained under the Ultrasonic-assisted extraction were subjected to SPE to remove matrix compounds and further fractionate and separate the compounds in each extract based on their polarity.



A.



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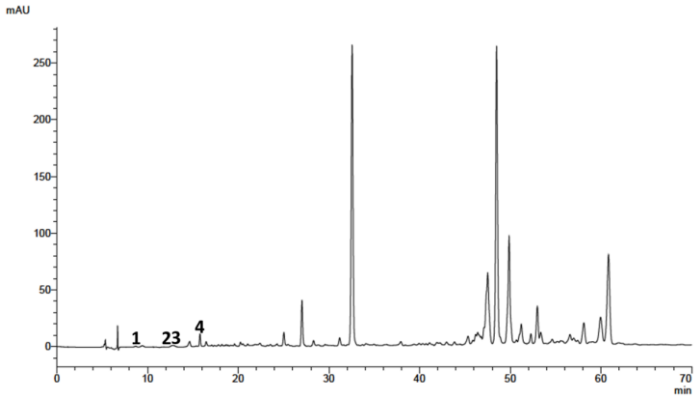

C.

Fig. 1. HPLC Chromatograms of A. Standard compounds: 3,4-Dihydroxybenzoic acid (1), Negundoside (2), 4-Hydroxybenzoic acid (3), Agnuside (4), Kaempferol (5); B. methanol; and C. hexane extract of *Vitex negundo*

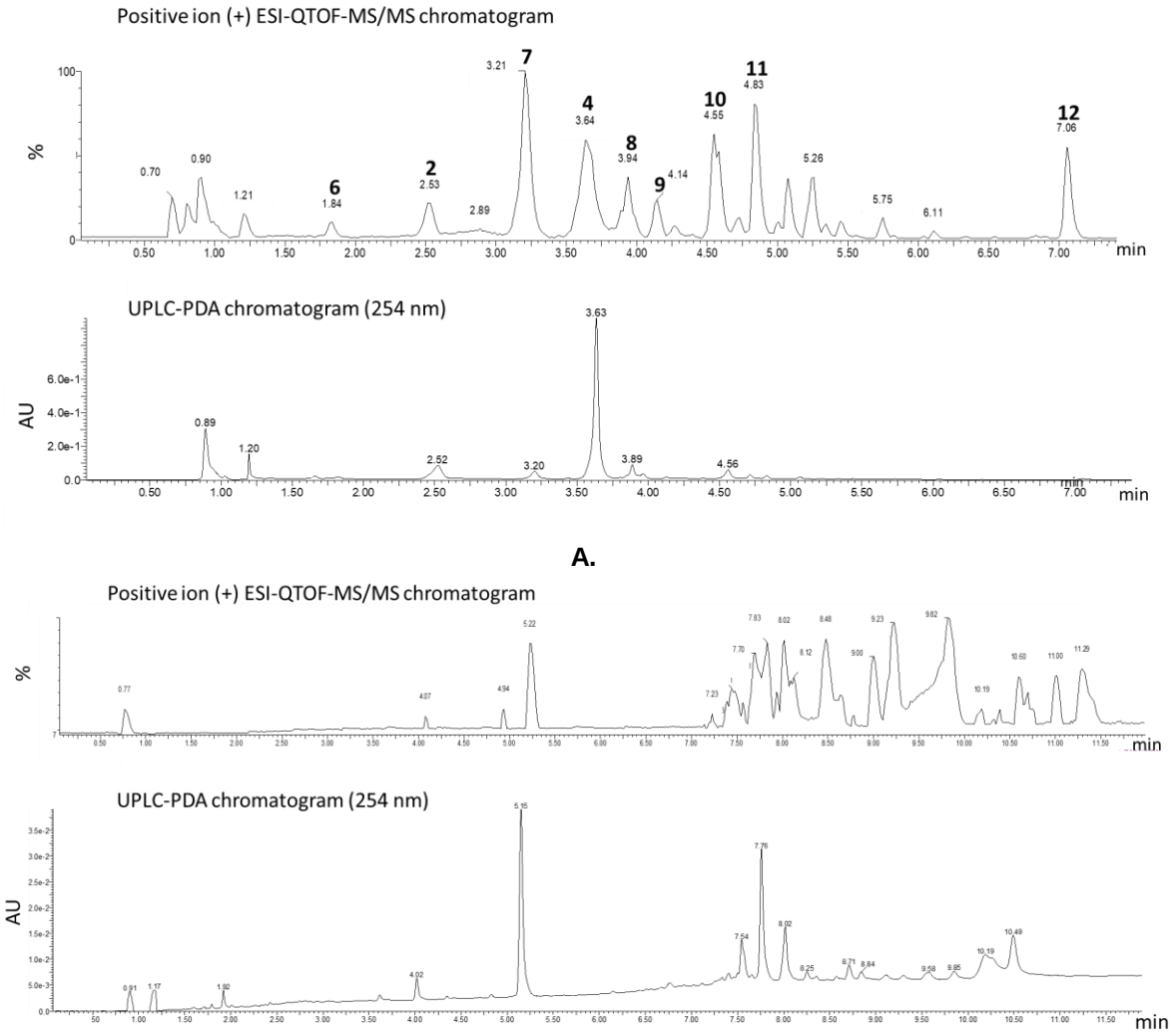

Fig. 2. LC-MS/MS analysis of A. methanol and B. hexane extracts of *Vitex negundo* showing the positive ion (+) ESI-QTOF-MS/MS and UPLC-PDA chromatograms

Table 1. Chromatographic and mass spectral properties of peaks identified from *Vitex Negundo* using LC-MS/MS analysis

No.	RT (min)	Precursor ion	Experimental Mass (m/z)	Fragments (m/z)	Identification
6	1.84	$[M+H]^+$	355.1025	163.04, 141.96	(1r,3R,4s,5S)-4-{[(2E)-3-(3,4-Dihydroxyphenyl)-2-propenoyl]oxy}-1,3,5-trihydroxycyclohexanecarboxylic acid
2	2.53	$[M+Na]^+$	519.1471	283.08, 179.08, 139.04	negundoside
7	3.21	[M+H]	449.1085	329.06,	luteolin 6-C-glucoside

No.	RT (min)	Precursor ion	Experimental Mass (m/z)	Fragments (m/z)	Identification
4	3.64	$[M+Na]^+$	489.1375	299.05, 182.99, 141.96 449.15, 287.09, 269.08, 131.05	agnuside
8	3.94	$[M+H]^+$	433.1136	283.08, 163.04	homovitexin
9	4.14	[M+H]	449.1085	287.06, 182.99	luteolin 4'-O-glucoside
10	4.55	[M+H]	499.1074	331.17, 163.04	luteolin 8-C-glucoside
11	4.83	[M+H]	463.0838	287.05, 163.04	kaempferol 3-glucuronide
12	7.06	[M+H]	361.0931	182.99, 124.09	chrysosplenol D

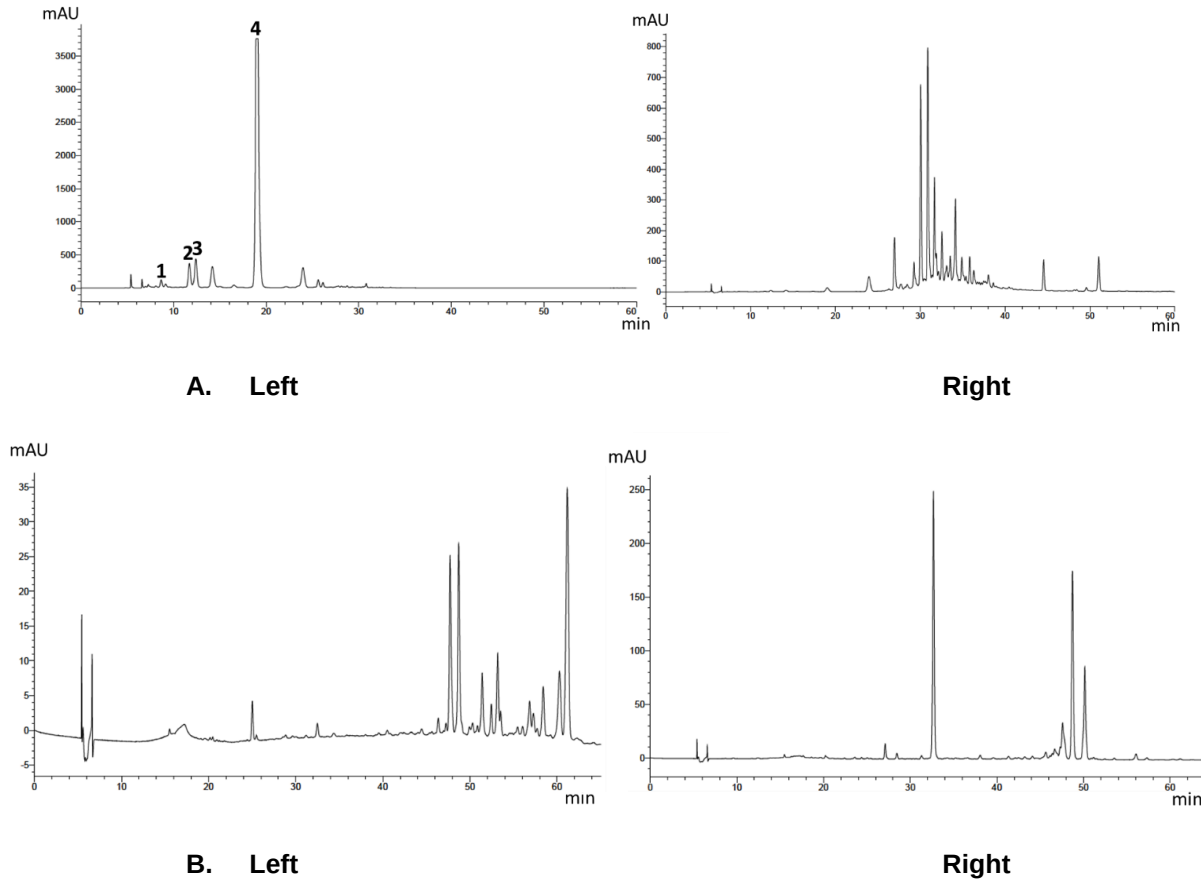

Fig. 3. HPLC Chromatograms of A. SPE fraction from methanol extract of *Vitex Negundo*: VN methanol SPE fraction 1(Left), VN methanol SPE fraction 2 (Right). B. SPE fraction from hexane extract: VN hexane SPE fraction 1 (Left); VN hexane SPE fraction 2 (Right)

VN: Vitex negundo, SPE: Solid Phase Extraction

Fig. 3 shows the HPLC chemical profiles of the VN methanol (Fig. 3A) and hexane (Fig. 3B) SPE fractions from its crude extracts. Fraction 1 of methanol extract (Fig. 3A, Left) contains more polar compounds in 50% methanol-water, including 3,4-Dihydroxybenzoic acid (1), Negundoside (2), 4-Hydroxybenzoic acid (3), and Agnuside (4) whereas fraction 2 (Fig. 3A, Right) contains the less polar part (100% methanol). After SPE, some peaks in the fraction, particularly Agnuside, were highly concentrated. Most of the more non-polar components in the hexane extract were recovered in fraction 1 using a 2% IPA-hexane solvent (Fig. 3B, Left). In contrast, fraction 2 used a 50% IPA-hexane solvent to isolate the less non-polar compounds (Fig. 3B, Right). SPE fractions were used in *in-vitro* and *in-vivo* bioassays to further deduce the complex constituents associated with plant activity. In addition, it is essential to determine which fractional components are more responsible for the activity.

3.3 Lipoxygenase (LOX) and Cyclooxygenase (COX) Inhibitory Activity of VN Extracts and Fractions

3.3.1 LOX inhibition

The effect of VN on the activity of the lipoxygenase enzyme was examined for its anti-inflammatory properties. This enzyme is a component in the LOX pathway of arachidonic acid metabolism, which generates proinflammatory mediators such as leukotrienes. The IC_{50} value determined for VN methanol extract is 440.9 µg/mL, which is higher than the IC_{50} value after SPE fractionation (Table 2). Methanol fraction 1 (50% methanol-water) has an IC_{50} value of 196.6 µg/mL, whereas Methanol fraction 2 (100% methanol) has an IC_{50} value of 150.0 µg/mL,

These fractions have different solvent polarity, from the results indicate that less polar components from methanol extract may have higher inhibition activity than components with stronger polarity. Moreover, the IC_{50} value of the hexane extract was 519.7 µg/mL, and SPE yielded fractions with lower values. The IC_{50} of VN hexane fraction 1 (2% IPA-hexane) is 397.5 µg/mL, whereas fraction 2 (50% IPA-hexane) is 142.9 µg/mL. The results may suggest that LOX activity is inhibited more effectively by fractions of VN Hexane with more polarity.

According to the findings, SPE fractionation of VN methanol and hexane extracts yielded more powerful fractions than their crude equivalent. Although all four fractions generated from methanol and hexane extracts inhibited LOX, the least polar fraction of VN methanol (100% methanol) while the most polar fraction of VN hexane extract (50% IPA-hexane) exhibited the strongest inhibition. Therefore, it is interesting to isolate further and identify the components of these fractions in greater depth. Previous research described the chemical structures of compounds extracted from the $CHCl_3$-soluble fraction of the methanol extract of VN roots which showed inhibitory potential against the LOX enzyme. Two lignans, named Negundins A and B, were isolated along with (+)-Diasyringaresinol, (+)-Lyoniresinol, Vitrofolal E, and Vitrofolal F. The inhibition studies showed that Negundins B has potent inhibitory potential against the LOX enzyme. On the other hand, Negundin A and (+) Diasyringaresinol showed weak activity [23].

3.3.2 COX inhibition

COX enzymes catalyze the synthesis of prostaglandins (PG) from arachidonic acid. PG are proinflammatory mediators the body produces in response to infection and injury. The initial study investigated the COX-2 inhibiting activities of VN crude extracts. Methanol and hexane extract demonstrated >70% inhibition of COX-2 at 300 µg/mL (data not shown). However, the result indicated interference with the ELISA reaction, semi-pure fractions (SPE fractions or HPLC peak fractions) were more suitable for this assay. Therefore, extracts of VN were subjected to preparative HPLC, and major peaks were collected. All HPLC peak fractions purified from methanol and hexane extracts of VN did not exhibit substantial COX-2 inhibitory activity (<50% inhibition) at 150 µg/mL or a higher concentration of 450 µg/mL (data not shown). This resulting low activities of the HPLC peak fractions may be due to the reduced activity or instability of the simpler mixture (compared to crude/semi-pure extracts) or the limited viable concentrations that can be collected during preparative HPLC.

VN Methanol SPE Fraction 1(50% methanol-water) did not exhibit any activity during the screening test, even using higher concentrations at 150 µg/mL and 450 µg/mL. The compounds identified in fraction 1 of VN Methanol (3,4-Dihydroxybenzoic acid (1), Negundoside (2), 4-Hydroxybenzoic acid (3), and Agnuside (4)) did

not seem to be the main COX-2-inhibiting compounds. In dose-response experiments, only VN Methanol SPE Fraction 2, VN Hexane Fraction 1, and VN Hexane SPE Fraction 2 were further analyzed. As shown in Table 2, VN Methanol SPE Fraction 2 (100% methanol) showed an IC_{50}=34.27 µg/mL, which offered better activity than its counterpart VN Methanol SPE fraction 1. VN Hexane SPE Fraction 1 (2% IPA-hexane) effectively inhibited COX-2, which showed maximum inhibition at 40 µg/mL with an IC_{50} value of 6.57 µg/mL. While with VN Hexane SPE Fraction 2 (50% IPA-hexane), no dose-response was observed from 5 to 150 µg/mL. The findings showed that SPE fractionation of VN extracts produces more active fractions; 100% methanol and 2% IPA-hexane. It would be of interest to characterize the components that comprise these fractions. In a reported study on COX inhibitory property of VN, analyses of the dichloromethane-soluble extract of VN seeds by various spectroscopic techniques led to the isolation of five labdane diterpenes, named Negundoins A–E, a 9,10-Seco-abietane diterpene, Negundoin F, a Sandaracopimara-7,15-diene diterpene, Negundoin G, and two known diterpene derivatives. The *in-vitro* anti-inflammatory effect of these isolated compounds, Negundoins C and E, significantly reduced the iNOS and COX-2 protein levels [24].

3.4 Acute Oral Toxicity of VN Extracts and its SPE Fractions

Acute oral toxicity (AOT) tests were performed to provide preliminary findings on the toxicity level of hexane and methanol extracts of VN and their corresponding fractions obtained by solid-phase

Table 2. Lipoxygenase and Cyclooxygenase inhibitory activity of *Vitex negundo* methanol and hexane extracts and its SPE fractions

Sample	LOX Inhibition IC_{50} (95%CI) µg/ml	COX Inhibition IC_{50} (95%CI) µg/ml
VN Methanol extract	440.9 (425.8-456.5)	ND
VN Hexane extract	519.7 (463.5-582.6)	ND
VN Methanol SPE fraction 1	196.6 (186.6-207.1)	ND
VN Methanol SPE fraction 2	150.0 (140.9-159.5)	34.27 (22.2-52.7)
VN Hexane l SPE fraction 1	397.5 (373.5-422.4)	6.57 (3.6-11.0)
VN Hexane SPE fraction 2	142.9 (130.7-156.0)	ND
Indomethacin	73.7 (71.9-75.5)	0.01 (0.01-0.02)

VN: Vitex negundo, SPE: Solid Phase Extraction, ND: Not Determined

Table 3. Acute oral toxicity of *Vitex negundo* methanol and hexane extracts and its fractions in Swiss Webster mice, A. Behavioral patterns and B. Survival of animals

A. Behavioral patterns

Treatment group	Dose (mg/kg)	Behavioral patterns			
		30 min	1 hr	6 hr	24 hr
VN Methanol	2000	None	-	None	None
VN Hexane	2000	None	-	None	None
PEG 400 (vehicle control)	-	None	-	None	None
VN Methanol SPE fraction 1	2000	-	Hypoactivity, Isolation	Respiratory changes	None
VN Methanol SPE fraction 2	2000	-	None	None	None
PG (vehicle control)	-	-	None	None	None
VN Hexane SPE fraction 1	175	-	None	None	None
	550	-	None	None	None
	2000	-	Hypoactivity, Respiratory changes, Itching, Isolation	Hypoactivity, Respiratory changes	Death
VN Hexane SPE fraction 2	2000	-	None	None	None
PVPPG (vehicle control)	-	-	None	None	None

VN: Vitex Negundo, PEG: polyethylene glycol, PG: propylene glycol, PVPPG: polyvinylpyrrolidone in propylene glycol

B. Survival of animals

Treatment group	Dose (mg/kg)	No. of mice survived
VN Methanol	2000	5/5
VN Hexane	2000	5/5
VN Methanol SPE fraction 1	2000	5/5
VN Methanol SPE fraction 2	2000	5/5
VN Hexane SPE fraction 1	175	1/1
	550	1/1
	2000	3/4
VN Hexane SPE fraction 2	2000	5/5

Table 4. Analgesic activity of *Vitex Negundo* extracts and fractions in acetic acid-induced writhing in Swiss Webster mice

Group	Dose (mg/kg)	No. of writhes in 20 min	% Inhibition
VN Crude Methanol	160	3.50 ± 1.71 *	91.46 ± 4.17 *
	320	2.00 ± 1.18 *	95.12 ± 2.89 *
	500	3.83 ± 1.66 *	90.65 ± 4.05 *
VN Crude Hexane	160	4.00 ± 2.58 *	90.24 ± 6.30 *
	320	3.17 ± 1.30 *	92.28 ± 3.17 *
	500	1.33 ± 0.99 *	96.75 ± 2.41 *
PEG 400 (vehicle control)		37.17± 3.38	9.35 ± 8.25
VN Methanol SPE fraction 1	100	3.33 ± 1.12 *	90.34 ± 3.23 *
	250	9.17 ± 1.08 *	73.43 ± 3.12 *
	500	1.00 ± 0.52 *	97.10 ± 1.50 *
VN Methanol SPE fraction 2	100	2.17 ± 0.87 *	93.72 ± 2.53 *
	250	3.83 ± 1.11 *	88.89 ± 3.21 *
	500	7.83 ± 1.99 *	77.29 ± 5.77 *
PEG 400 (vehicle control)		34.50 ± 3.55	0.00 ± 10.28
Diclofenac sodium	5	0.00 ± 0.00 *	100.00 ± 0.00

*VN: Vitex Negundo, SPE: Solid-phase extraction, PEG: polyethylene glycol. Values represent the mean ± SEM (standard error mean); n=6. * P< 0.05 as compared to the control*

extraction (SPE). Initially, the limit test at 2000 mg/kg was conducted with the first animal. Another main test received a lower dose of 175, 550, and 2000 mg/kg body weight (if the first animal dies). Behavior, indicators of toxicity, and mortality were observed for 14 days after sample administration. As shown in Table 3A, animals treated with extracts (methanol and hexane) and SPE fractions (VN Methanol SPE fraction 2 and VN Hexane SPE fraction 2) did not show any abnormalities and death. However, VN Methanol SPE fraction 1 exhibited toxic symptoms within six hours but normal activity returned within twenty-four hours. At a 2000 mg/kg body weight dosage, VN Hexane SPE fraction 1 initially demonstrated mortality. Five more animals were used in the main test, some of which showed signs of toxicity. The results indicated that the median lethal dose (LD_{50}) was greater than 2000 mg/kg b.w (Table 3B). VN Methanol SPE fraction 1 and Hexane SPE fraction 1 have altered the behavior of animals and was classified as Hazard Category 4 in the Globally Harmonized System (GHS).

3.5 Analgesic Activity of VN Extracts and Fractions

The analgesic effect of methanol and hexane extracts of VN and fractions obtained by SPE of methanol extract was investigated using a Writhing Test induced by acetic acid in mice. Table 4 illustrates the total number of writhes and percentage of inhibition after its administration to different groups of treated and untreated mice throughout a 20-minute observation period. Diclofenac control at 5 mg/kg body weight inhibited writhing in test animals by 100 %, whereas vehicle control PEG 400 had no significant effect. All treatment groups demonstrated a statistically significant ($p<0.05$) reduction in the number of writhes compared to

the vehicle control groups. The percentage inhibition of writhing at all dose levels of methanol and hexane VN extracts ranged from 90-96%. The SPE fractions from methanol extracts showed 73-97% inhibition; however, there was no remarkable difference in the inhibition compared to its methanol extract. In a report on the analgesic effect of VN seeds, bio-guided analgesic separation of the VN acetoacetate fraction produced 6-hydroxy-4-(4-hydroxy-3-methoxy-phenyl)-3-hydroxymethyl-7-methoxy-3,4-dihydro-2-naphthaldehyde, which partly accounts for the analgesic effect [12]. In an *in-vivo* study, no change in activity was observed between crude extracts and fractions. For future investigations, it may be possible to observe a direct effect using commercially available standards of the detected components.

4. CONCLUSION

In the study, several compounds were identified in the methanol extract. The extracts and fractions demonstrated anti-inflammatory effects via *in-vitro* inhibition of LOX or COX-2 enzymes and analgesic activity in an *in-vivo* animal model. SPE fractions with 100% methanol and 50% IPA-hexane inhibited LOX to a greater extent. For COX-2 analysis, the SPE fractions containing 100 % methanol and 2 % IPA-hexane displayed potent inhibitory activities. The study provided scientific evidence that the leaves of Philippine VN have anti-inflammatory and analgesic effects. Future research aims to elucidate further and isolate active compounds responsible for both activities.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All experiments complied with Philippine animal welfare laws. The study protocol was reviewed and approved by UNILAB, Inc. Institutional Animal Care and Use Committee (IACUC) and the Bureau of Animal Industry (BAI).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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