Original Research Article

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

ABSTRACT

Aims: *Vitex negundo* is a plant native to the Philippines traditionally used as an herbal cough remedy. This research aims to demonstrate other medicinal uses of locally cultivated *Vitex negundo*, including analgesic and anti-inflammatory properties, and to determine the chemical components present.

Study design: The powdered leaves were extracted using ultrasonic-assisted extraction with methanol and hexane, and the resulting extracts were fractionated using Solid-phase extraction (SPE). The extracts and their fractions were subjected to chemical and biological analysis.

Place and Duration of Study: The study was carried out between October 2019 and March 2022 at the Synnovate, Pharma; Biological Sciences Department, Medical Affairs Division, Unilab; and Pascual Pharma R&D Laboratory.

Methodology: The analysis of the chemical profile involved High-performance liquid chromatography (HPLC) and Liquid chromatography-tandem mass spectrometry (LC-MS/MS). Inhibition of lipoxygenase (LOX) and cyclooxygenase-2 (COX-2) enzymes assessed the inflammatory action. Inhibition of Acetic acid-induced writhing in mice evaluated the analgesic activity.

Results: Our results showed multi-component peaks in the extracts, out of which the identified compounds were 3,4-Dihydroxybenzoic acid, Negundoside, 4-Hydroxybenzoic acid, Agnuside, (p(1r,3R,4s,5S)-4-{[(2E)-3-(3,4-Dihydroxyphenyl)-2-propenoyl] oxy}-1,3,5trihydroxycyclohexanecarboxylic acid, luteolin 6-C-glucoside, homovitexin, luteolin 4'-O-glucoside, luteolin 8-C-glucoside, kaempferol 3-glucuronide, and chrysosplenol D.

In the LOX inhibition, the IC_{50} value for the methanol extract is 440.9 µg/mL after SPE fractionation; the IC_{50} values for Methanol fraction 1 (50% methanol-water) and Methanol fraction 2 (100% methanol) are 196.6 µg/mL and 150.0 µg/mL, respectively. The hexane extract's IC_{50} value was 519.7 g/mL. SPE produced fractions with lower IC_{50} values; hexane fraction 1 (2% IPA-hexane) is 397.5 µg/mL, and fraction 2 (50% IPA-hexane) is 142.9 µg/mL.
Methanol and hexane crude extracts showed interference in the COX-2 inhibition assay. In the SPE fractions, Methanol fraction 1(50% methanol-water) did not exhibit any activity at 150 µg/mL and 450 µg/mL. Methanol fraction 2 (100% methanol) has an IC_{50}=34.27 µg/mL, which showed better activity than its counterpart. Hexane Fraction 1 (2% IPA-hexane) effectively inhibited COX-2 with an IC_{50} value of 6.57 µg/mL. While with Hexane Fraction 2 (50% IPA-hexane), no dose-response was observed from 5 to 150 µg/mL.

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.

Conclusion: An analytical method was developed for HPLC and LC-MS/MS analysis allowing the separation of compounds in the methanol and hexane extracts of *Vitex negundo*. Several compounds were identified in the methanol extract. Some fractions produced from SPE demonstrated better anti-inflammatory effects via *in-vitro* inhibition of LOX or COX-2 enzymes. Both extracts and fractions showed potent analgesic effects in an *in-vivo* animal model. Further research is recommended to isolate and standardize the bioactive components.

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 light of the predominant use of VN in the Philippines (known as Lagundi) as a cough medicine, the present study aims to determine the phytochemical composition of the extract and fraction of VN using chromatographic and mass spectrometric analytical methods, followed by an investigation of their potential anti-inflammatory and analgesic action by *in-vivo* and *in-vitro* experiments. Little is known, and no established scientific report exists on the chemical components and activities of the Philippine variety of VN leaves.

2. MATERIAL 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. Prostaglandin was determined by competitive Enzyme-linked Immunosorbent Assay (ELISA) after reaction quenching. The Biotek Synergy HT plate reader was used to measure 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

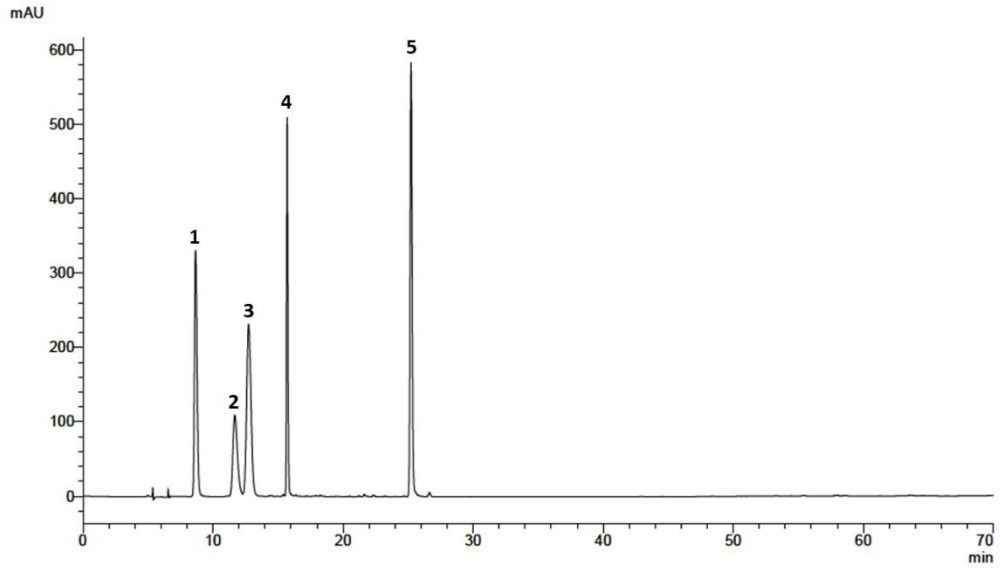
3.1 Chemical Composition of VN Extracts

The chemical compounds found in VN extracts were analyzed through HPLC. The extraction yield of methanol is higher than hexane. Figure 1A displays the chromatograms of available standard reference compounds for the VN sample. The chromatograms of methanol and hexane extracts of VN (Figures 1B and 1C) showed relatively good peak resolutions. The eluted compounds in methanol extract (Figure 1B) were detected in the 8-22 min range indicating polar compounds. The eluted compounds in the hexane extract (Figure 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 (Figure 1B and 1C). There were more peaks observed in methanol extracts than in hexane extracts. The total phenolic and flavonoid content of VN extracts was higher in methanol than in hexane extracts (data not shown). Previous studies on using different solvents to extract *Severinia buxifolia* revealed that methanol, distilled water, and ethanol had higher extraction yields than chloroform, dichloromethane, and acetone, showing that the extraction efficiency for the specific plant sample favored the high polar solvents. Furthermore, in their study, methanol was the most effective solvent for extracting bioactive chemicals. It produced the maximum extraction yield and the highest concentration of phenolics, alkaloids, flavonoids, and terpenoids [16].

Compounds identified may contribute to the action of VN extracts. Agnuside, an iridoid glycoside, exhibited the highest HPLC peak intensities in the VN extracts studied. It has been suggested that Agnuside can be potentially used to treat arthritis. 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]. In a different study, the compounds 4-Hydroxybenzoic acid, 3,4-Dihydrobenzoic acid, Negundoside, and Luteolin were isolated from the active fractions of VN and 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, including 4-Hydroxybenzoic acid, Negundoside, and Luteolin, were low, ranging between 24 and 27% [18].

A.



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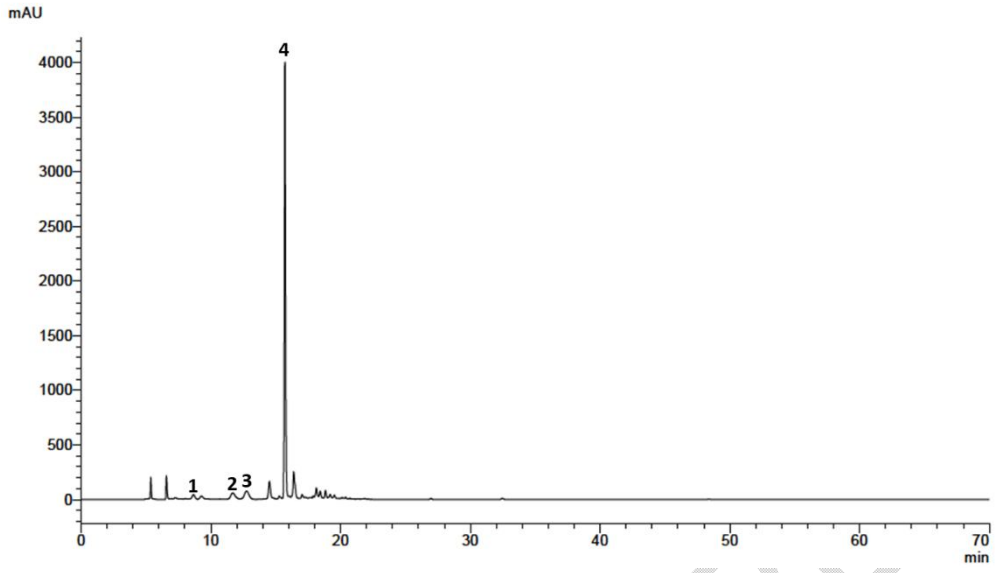

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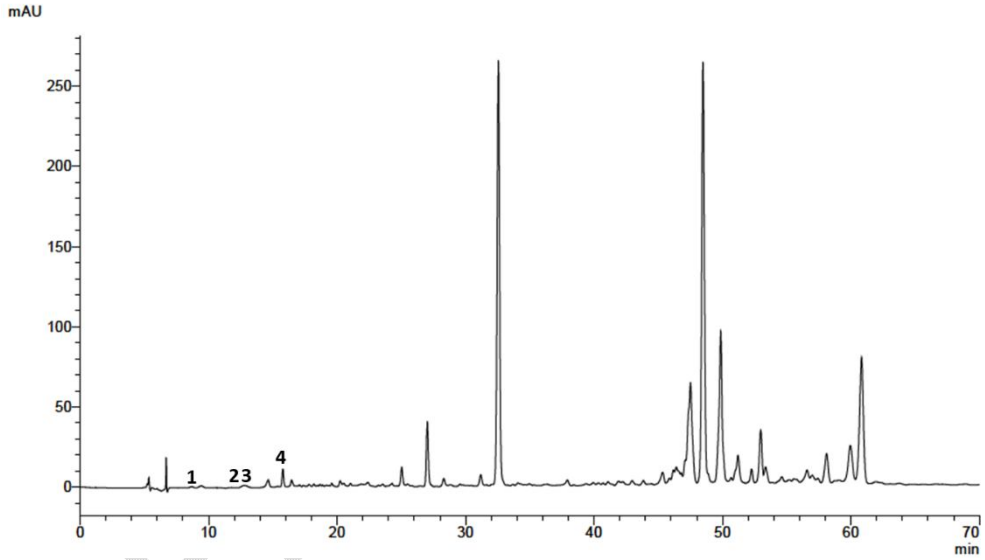

Figure 1. HPLC Chromatograms of A. Standard compounds: 3,4-Dihydroxybenzoic acid (1), Negundoside (2), 4-Hydroxybenzoic acid (3), Agnuside (4), Kaempferol (5); B. methanol; and C. hexane extract of *Vitex negundo*.

LC-MS/MS untargeted system was performed to obtain information about the chemical composition of VN extracts. Plant components were analyzed from extracts or HPLC fractions (data not shown). Figure 2 represents the chromatograms with MS and UV detector of methanol (Figure 2A) and hexane (Figure 2B) VN extracts. Table 1 shows the chromatographic and mass spectral properties of the identified peaks. As shown in Table 2, nine (9) compounds were identified by manually comparing retention times and MS spectra to available reference standards or tentatively assigned based on the MS data library using MS-DIAL software.

The chemicals contained in VN 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). The data confirmed the presence of negundoside, agnuside, 3,4-dihydroxybenzoic acid, and 4-hydroxybenzoic acid in the VN methanol extract or HPLC fraction, which were identified in the HPLC analysis. Several detected compounds have also been reported to be present in VN. In a different study, 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 [19]. Iridoids and flavonoids have reportedly been found in the leaves of VN. Negundoside and Agnuside were determined as two iridoids. The flavonoids were vitexicarpin, isoorientin, vitexin, and isovitexin [20]. Unidentified peaks not matched in the library will require further characterization.

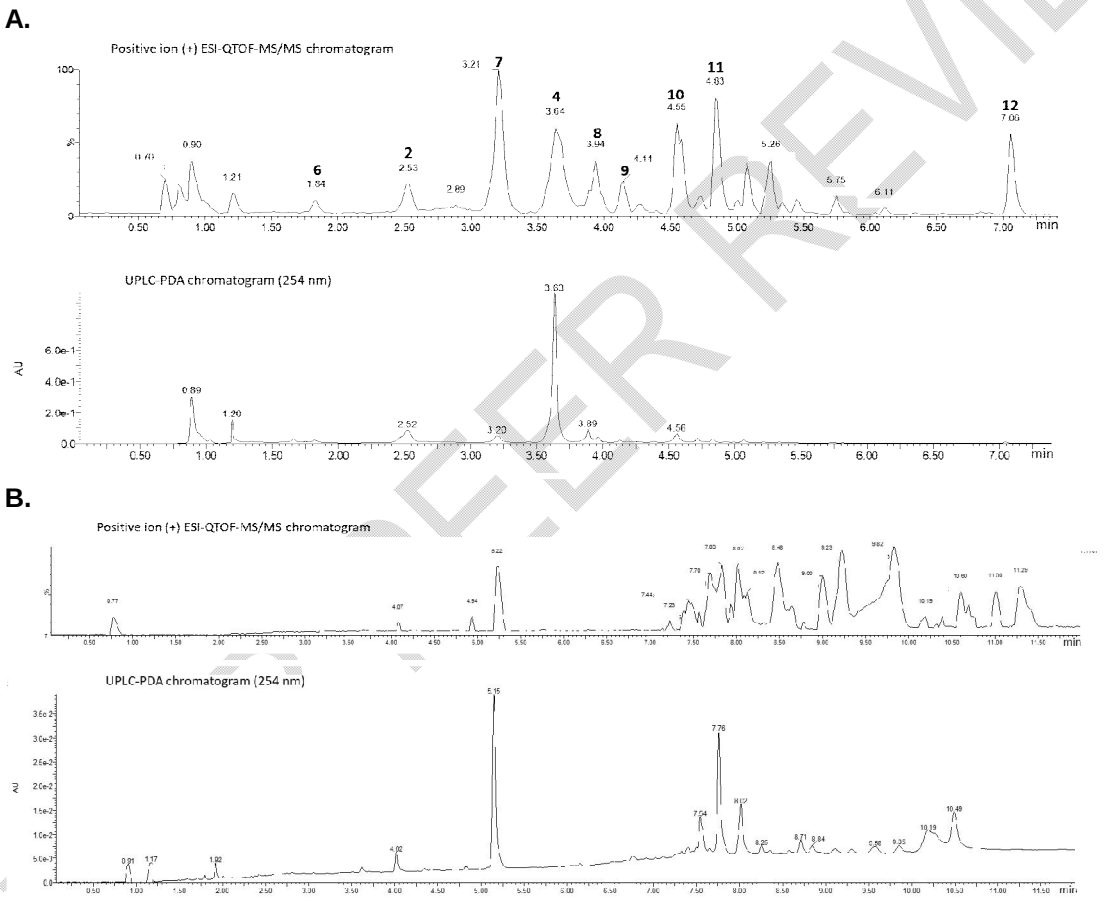

Figure 2. LC-MS/MS analysis of A. methanol and B. hexane extracts of *Vitex negundo* showing the positive ion (+) ESI-QTOF-MS/MS and UPLC-PDA chromatograms

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	Fragments	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, 299.05, 182.99, 141.96	luteolin 6-C-glucoside
4	3.64	$[M+Na]^+$	489.1375	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

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.

Figures 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 (Figure 3B, left). In contrast, fraction 2 used a 50% IPA-hexane solvent to isolate the less non-polar compounds (Figure 3B, right). SPE fractions were used in *in-vitro* and *in-vivo* bioassays to deduce further the complex constituents associated with plant activity. In addition, it is important to determine which fractional components are more responsible for the activity.

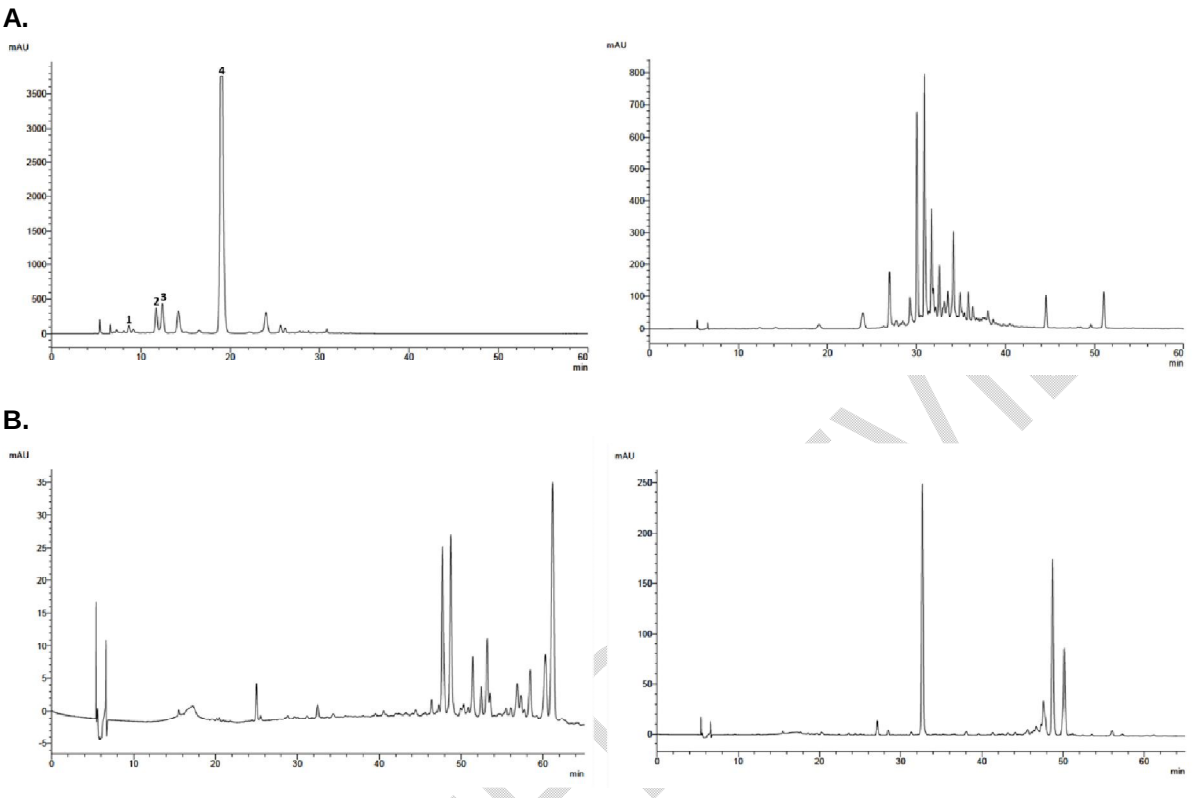

Figure 3. HPLC Chromatograms of A. SPE fraction from methanol extract of *Vitex Negundo*: VN methanol SPE fraction 1(left), VN methanol SPE fraction 2 (right). B. SPE fraction from hexane extract: VN hexane SPE fraction 1 (left); VN hexane SPE fraction 2 (right)

VN: Vitex negundo, SPE: Solid Phase Extraction

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, and Methanol fraction 2 (100% methanol) with 150.0 µg/mL, indicating that less polar components of VN methanol extract may have higher inhibition activity than its more polar components. The IC_{50} value of the hexane extract of VN was 519.7 µg/mL, and SPE yielded fractions with higher activity than the crude extract. 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 imply that the polar component of VN Hexane fractions inhibits LOX activity more effectively.

Based on the findings, SPE fractionation for VN methanol and hexane extracts produced a more potent fraction than their crude counterparts. Although all four fractions derived from methanol and hexane extracts inhibited LOX, higher activity is observed in the less polar fraction of VN methanol (Methanol Fraction 2) and the more polar fraction of VN hexane extract (Hexane Fraction 2). 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 [21].

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 do not appear to be the principal 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 (Fraction 2 from methanol and Fraction 1 from hexane extracts). 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 [22].

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 I 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

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 extraction (SPE). As shown in Table 3, animals treated with methanol and hexane extracts of VN (2000 mg/kg b.w) and PEG 400 survived. Behavioral patterns did not show any abnormalities for 24 h. Animals administered with VN Methanol SPE fractions 1 or 2 at 2000 mg/kg b.w did not show lethality. However, fraction 1 exhibited toxic symptoms within six hours of injection, including hypoactivity in five mice, itching, or respiratory difficulties in one of the animals. The normal activity returned within twenty-four hours. Animals were initially given VN Hexane SPE fraction 1 at 2000 mg/kg b.w showed hypoactivity with respiratory changes before observing death within 24 hours. Therefore, the sample proceeded with the main test. A total of five animals were sequentially dosed at 175, 550, and 2000 mg/kg b.w. All animals survived; however, animals given a dose of 2000 mg/kg exhibited itching (1 of 3 animals), isolation (1 of 3 animals), and hypoactivity (3 of 3 animals) within 6 hours post-administration. On the other hand, VN Hexane SPE fraction 2 treatment in animals at 2000 mg/kg b.w survived without toxic manifestations. The results indicated that the median lethal dose (LD_{50}) of methanol and hexane extracts of VN and its SPE fractions was greater than 2000 mg/kg b.w. However, VN Methanol SPE fraction 1 and Hexane SPE fraction 1 have altered the behavior of animals, thus classified as Hazard Category 4 in the Globally Harmonized System (GHS).

Table 3. Acute oral toxicity of *Vitex negundo* methanol and hexane extracts and its fractions in Swiss Webster mice

Treatment group	Dose (mg/kg)	No. of mice survived	Behavioral Patterns			
			30 min	1 hr	6 hr	24 hr
VN Methanol	2000	5/5	None	-	None	None
VN Hexane	2000	5/5	None	-	None	None
PEG 400 (vehicle control)	-	5/5	None	-	None	None
VN Methanol SPE fraction 1	2000	5/5	-	Hypoactivity, Isolation	Respiratory changes	None
VN Methanol SPE fraction 2	2000	5/5	-	None	None	None
PG (vehicle control)	-	5/5	-	None	None	None
VN Hexane SPE fraction 1	175	1/1	-	None	None	None
	550	1/1	-	None	None	None
	2000	3/4	-	Hypoactivity, Respiratory changes, Itching, Isolation	Hypoactivity, Respiratory changes	Death
VN Hexane SPE fraction 2	2000	5/5	-	None	None	None
PVPPG (vehicle control)	-	5/5	-	None	None	None

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

3.4 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 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].

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

Group	Dose (mg/kg b.w.)	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

4. CONCLUSION

In the study, an analytical method was developed for HPLC and LC-MS/MS analysis allowing the separation of compounds in the methanol and hexane extracts of *Vitex negundo*. 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. The analgesic activity has no remarkable difference between the extracts and fractions. On the other hand, the enzyme inhibition test shows that fractionation by solid phase extraction (SPE) for methanol and hexane extracts yielded more potent fractions. Increased LOX inhibition was seen in SPE methanol fraction 2 (100 % methanol) and SPE hexane fraction 2 (50 % IPA-hexane). For COX-2 analysis, SPE methanol fraction 2 (100 % methanol) and VN hexane fraction 1 (2% IPA-hexane) showed better inhibitory activity. The study provided scientific evidence that the leaves of Philippine VN have anti-inflammatory and analgesic effects. Therefore, its crude

extracts or semi-pure fractions may be used to develop medicinal herbal preparations. Future research aims to elucidate further and isolate active compounds responsible for both activities.

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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DEFINITIONS, ACRONYMS, ABBREVIATIONS

APPENDIX