Phytochemical investigation on *Vitex negundo* leaves and their anti-inflammatory and analgesic activities

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The phytochemical investigation on *Vitex negundo* leaves has led to the isolation of one new iridoid glucoside (8α-hydroxy-4-carboxyl-5βH-9βH-iridoid-1α-O-(6′-O-(6,7-dihydrofoliamenthonyl)-β-D-glucopyranoside, 3), together with three known compounds, namely agnuside (1), 6′-O-E-caffeoylmuusaeonidic acid (2), and 3,5-dicafeoylquinic acid (4). The HPLC analytical study was also performed to quantify the content of agnuside (1) in dried leaves. The results indicated the very high content of 1 (3.04 ± 0.02%). The method was also validated by various parameters, including linearity (R\(^2\)= 0.9999), precision (intra-day RSD ≤ 2.50%, inter-day RSD= 0.76%), and accuracy (recovery rates 96.58-101.86%). The animal testing data showed that the extract did not reduce pain at the doses of 9.6 and 28.8 g/kg (leaf weight/body weight) in the hot plates and pain measuring models but showed the pain reduction in the acetic acid-induced pain model. The extract at the dose of 5.6 g/kg (leaf weight/body weight) also had effects on the acute inflammation in the carrageenin-induced edema model. The extract at the dose 9.6 and 28.8 g/kg (leaf weight/body weight) also showed significant chronic anti-inflammation, comparable to methylprednisolon on the mouse peritoneal.

**Keywords:** *Vitex negundo*, Iridoid, HPLC quantification, Anti-inflammatory, Analgesic.

**INTRODUCTION**

*Vitex negundo* L. was a shrub or small tree which was available in the plains, mountainous area and in the midland of Vietnam. It has been widely used in treatment of many diseases such as arthritis, flu, fever, cough, asthma, sprains, and colitis (National Institute of Medicinal Materials, 2006; Vu, 2007). The published chemical studies on *V. negundo* showed the wide spectrum of its compounds, such as iridoid glycosides, quinic acid derivatives, flavonoids, and lignans (Hu et al., 2016; Hu et al., 2017; Zheng, C.J. et al., 2015). There were also pharmacological studies on analgesic and anti-inflammatory properties of this plant (Chattopadhyay et al., 2012; Dharmasiri et al., 2003; Gill et al., 2018).

In Vietnam, so far, no study has been conducted on the compounds of this plant as well as its pharmacological activities. With the potential clinical application and consideration of available studies on this plant, the present study was conducted to give more insights into compounds of *V. negundo* collected in Vietnam, analytical method for quantification of main compounds, as well as its analgesic and anti-inflammatory properties in the different animal models, in order to provide firm scientific support for traditional usage, clinical application, and quality control procedure in Vietnam.

**MATERIAL AND METHODS**

**General experimental procedures**

NMR experiments were conducted on a Bruker Avance III HD (400 MHz) spectrometer (Bruker)
operating at 400 MHz (¹H) and 100 MHz (¹³C), the samples were dissolved in methanol-d₄. HRESIMS spectra were recorded on a micrOTOF-Q II (Bruker) operated by Hystar software. Optical rotation was measured on a Jasco P-2000 polarimeter with 10 cm path length. Chromatography was performed on open column, with normal-phase and reverse-phase columns (RP-C₁₈), as well as with Sephadex LH-20 (Sigma-Aldrich). The HPLC analysis was performed on an Agilent HPLC system 1200 series (Agilent, USA). All of solvents used for extraction and isolation were redistilled for suitable quality. Solvents for analytical experiments were obtained from Merck.

Plant material

The *Vitex negundo* leaves were collected in the outskirts of Hanoi, Vietnam. The sample was taxonomically authenticated by Prof. Phuong Xuan Vu from the Institute of Ecology and Biological Resources, Hanoi, Vietnam. The voucher specimens (HMU-VN2018-01) was deposited in the Institute of Ecology and Biological Resources, Hanoi, Vietnam.

Extraction and isolation

The dried *V. negundo* leaves (500 g) were extracted by MeOH in ultrasonic bath (3 times × 2 L × 2h). The extracts were combined and evaporated under the low pressure to obtain 70.8 g of green slurry. The crude extract was suspended in distilled water and partitioned with EtOAc. The organic solvent layer was then evaporated to yield EtOAc-soluble portion (22.5 g). The EtOAc portion was fractionated by normal-phase silica gel column (5×40 cm, 40-63 μm), eluting with gradient solvent system of EtOAc/MeOH (from 30:1 to 0:1) to obtain 10 subfractions (F1-10). Fraction F5 (1.2 g) was firstly subjected into RP-C₁₈ column (1.5×60 cm, 40-63 μm) with gradient MeOH/H₂O (from 30-80%), then purified by Sephadex LH-20 column (1.5×60 cm, MeOH) to obtain compounds 2 (24.4 mg) and 4 (48.7 mg). Fraction 6 (2.5 g) was also purified using the same procedure to obtain compounds 1 (10.7 mg) and 3 (7.7 mg).

8α-hydroxy-4-carboxyl-5βH-9βH-iridoid-1α-O-(6′-O-(6,7-dihydrofoliamenthonyl)-β-d-glucopyranoside (3)

Brownish gum; -8.8 (c 0.05, MeOH); UV λmax 236, 262 nm; CD (40 μM, MeOH, mdeg) 203 (+0.44), 229 (-6.02), 255 (+0.64); ¹H and ¹³C NMR see Table I; HR-ESIMS m/z 543.2418 [M-H] (calcd. for C₂₆H₃₉O₁₂ 543.2447).

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**TABLE I -** NMR data for 3 (400/100 MHz, MeOH-d₄)
HPLC quantification method

Standard solution

Reference compound 1 was isolated in-house by various means of chromatography. The standard compound had a purity ≥ 95% as determined by HPLC and NMR. The stock standard solution was freshly prepared in methanol with concentration approximately 1.0 mg/mL. These steps of standard solution were prepared from the stock by diluting with methanol.

Sample preparation

Powdered plant material (50 mg) was extracted sonically with methanol (3.0 mL × 20 min × 3 times). After centrifugation (2800 rpm, 2 min), the supernatants were combined in a 10 mL volumetric flask. Before analysis, each sample solution was filtered through 0.4 µm membrane.

Analytical conditions

The optimal separation was determined as follows: on column Phenomenex Synergi 4u MAX-RP 80A (150×4.60 mm, 4µm) protected by a 0.2 µm guard filter (Waters) at 40°C. The mobile phase was consisted of water containing 0.02% trifluoroformic acid (A) and acetonitrile (B). The gradient was from 12B/88A to 16B/84A in 35 min. The column was then washed with acetonitrile and re-equilibrated with initial solvent system for 15 min before the next analysis. The injected volume of sample was 10 µL, the flow rate was set at 0.6 mL/min. The UV detector was set at 258 nm

Method validation

The method validation was conducted for various criteria, including linearity, the limits of detection (LOD) and quantification (LOQ), accuracy, and precision. The calibration curve was established with a stock solution of compound 1 (approximately 1.0 mg/mL) prepared in methanol and five further levels obtained by serially diluting with methanol (1:1). Linearity was calculated based on six concentrations and their respective HPLC peak area. The LODs and LOQs were visually determined as 3-times and 10-times signal-to-noise ratio, respectively. Peak purity was determined by ‘Peak Purity’ function in Agilent software with threshold set at 950. Intra- and inter-day precisions were evaluated on three consecutive days, each sample was examined in triplicate. The accuracy of method was evaluated by spiking three different volumes of 1 (1 mg/mL) into plant materials sample (high spike: 1000 µL; medium spike: 500 µL; low spike: 250 µL).

Statistical analysis

All samples were analyzed in triplicate. The analyzed data was expressed in the form “mean ± standard deviation (SD)”. The data was calculated and processed by Microsoft Excel 2016 (Microsoft, USA).

Extract preparation for animal testing

Plant materials (5 kg) were crushed into small pieces and extracted by 70% aqueous ethanol under flux 3 times (solvent/ material ratio= 6/1). The combined extract was then evaporated under reduced pressure to two thirds of original volume and stirred for 24 hours. After that, the extract was filtered to remove settled resin and evaporated to remove completely organic solvent in order to obtain concentrated extract.

Experimental animals

Healthy mature Swiss mice (weights, 18-22 g), and mature Wistar rats (weights, 100-150 g), of both sexes were provided by the National Institute of Hygiene and Epidemiology (Hanoi, Vietnam). Animals were acclimated 5-7 days before experiments.

The experiments were carried out in the Department of Pharmacology, Hanoi Medical University, Vietnam. Animal research protocols were approved by the Ethical Council of Hanoi Medical University, Vietnam.

Hot plate test and gauge pain threshold

The mice were divided into 4 groups and administrated orally using gavage. Group I drank
distilled water as the control at the dose of 0.2 mL/10g; Group II drank codeine phosphate at a dose of 20 mg/kg; Group III, IV drank extract of *V. negundo* leaves at the dose of 9.6, 28.8 g/kg (leaf weight/body weight), respectively.

The heat sensitivity of mice was evaluated by hot plate test to measure the response time to temperature of mice one hour before and after the last treatment. The animals are plated on the hot plate (always maintained at 56°C) and the time until either licking or jumping occurs was recorded by stopwatch. The mice that responded too quickly (before 8 seconds) or too slowly (after 30 seconds) were then eliminated. The reaction time with heat stimulation before and after the administration of the *V. negundo* extracts (in seconds) was then compared among mice groups (Ankier, 1974; Vogel, 2008).

The pressure pain threshold was measured using Dynamic Plantar Aesthesiometer in which an ascending force was applied to the right paw of the mice. The distance was recorded by observing on the scale the force at which the animal felt pain (Ankier, 1974).

**Acetic acid-induced writhing**

White mice were divided into 4 groups each containing ten rats: group I drank distilled water as the control at the dose of 0.2 mL/10g; group II drank aspirin 150 mg/kg; group III, IV drank extract of *V. negundo* leaves at the dose of 9.6, 28.8 g/kg (leaf weight/body weight), respectively. The mice in the groups I, III and IV were drinking distilled water or the reagents once a day in the morning for three consecutive days. On the third day, one hour after taking the substance, 0.2 mL of acetic acid 1% was injected to mice’s abdominal cavities. The number of cramping pain episodes in each mouse was counted every 5 minutes for 30 minutes after the injection of acetic acid, then was compared to the control groups (Koster, Anderson, de Beer, 1952).

**Anti-inflammatory activity**

The acute anti-inflammatory activity of the *V. negundo* leaves were evaluated in the carrageenan-induced rat paw edema model and the peritonitis rat model (Winter et al., 1962). Each model was divided into 4 groups: Group I drank distilled water as the control at the dose of 1 mL/100g; Group II drank aspirin 200 mg/kg; Group III, IV drank the *V. negundo* leaves at the doses of 5.6 and 16.8 g/kg/day (leaf weight/body weight/day), respectively. Rats were given the *V. negundo* leaves or water or aspirin for 4 days before inducing inflammation. For the carrageenan-induced rat paw edema model, on the fourth day, one hour after taking the *V. negundo* leaves, 0.05 mL of 1% solution of carrageenan was injected into the right hind soles of the rat. The volume of rat paw was observed before inducing inflammation (V0), 2 hours after inducing inflammation (V2), 4 hours (V4), 6 hours (V6) and 24 hours (V24) by using a Plethysmometer.

The increase of rat paw volume was calculated by following formula

\[ \Delta V\% = \frac{V_i - V_0}{V_0} \times 100 \]

V<sub>0</sub>: rat paw volume before inducing rat paw edema  
V<sub>i</sub>: rat paw volume after inducing rat paw edema

The anti-inflammatory activity of the drugs was expressed as percentage inhibition (I%), which was calculated as follows:

\[ I\% = \frac{\Delta V_c\% - \Delta V_t\%}{\Delta V_c\%} \times 100 \]

\( \Delta V_c\% \): Average increase in rat paw volume in control group  
\( \Delta V_t\% \): Average increase in rat paw volume in treated group

In the peritonitis rat model, on the fourth day, one hour after taking the *V. negundo* leaves, 2 mL of solution of carrageenan (50 mg carrageenan and 1.4 ml formaldehyde mixed enough in 100ml saline solution) was injected into the peritonitis of the rat. One day after causing inflammation, inflammatory exudate was taken from the abdomen of the mouse. The number of leukocytes in 1 mL of inflammatory exudate was counted (Vogel, 2008).

The chronic anti-inflammatory effects were studied on experimental granulomas model (Vogel, 2008). In
In detail, chronic inflammation was induced by implanting sterile asbestos fibers weighed 6 mg which were soaked in 1% carrageenan into the skin of each mouse nape. The treatment groups included Group I drank distilled water 0.2 mL/10g; Group II drank methylprednisolone 10 mg/kg; Group III, IV drank the \textit{V. negundo} leaves at the doses of 9.6, 28.8 g/kg/day (leaf weight/body weight/day), respectively. The mice then drank distilled water or the reagents continuously for 10 days. On the eleventh day, mice were sacrificed in order to collect granuloma. Three granulomas in each group were randomly selected for microscopic pathological observation. The remaining granulomas were dried at 56°C for 18 hours then weigh after being dried. Results were expressed as percentage inhibition of granuloma in drug treated groups compared to the control group, which was calculated as follows:

\[
\text{inhibition of granuloma} = 1 - \frac{\text{GT}}{\text{GC}} \times 100
\]

GT: granuloma tissue weight in treated group, GC: granuloma tissue weight in control group.

**Statistical analysis**

Data was shown as mean ± standard error (mean ± SE). The data was evaluated Independent Samples T-Test using SPSS program (version 18. SPSS Inc., USA). The differences were statistically significant with \( p < 0.05 \).

**RESULTS AND DISCUSSION**

**Structural elucidation of new compound**

Phytochemical investigation on \textit{V. negundo} leaves have resulted in the isolation of 4 compounds (1-4) (Figure 1). The structures of isolated compounds were determined by NMR spectroscopic analysis and comparison with published data, specifically, agnuside (1), 6’-O-E-caffeoylmussaenosidic acid (2), 8α-hydroxy-4-carboxyl-5βH-9βH-iridoid-1α-O-(6’-O-(6,7-dihydrofolicamthonyl)-β-ᴅ-glucopyranoside (3), and 3,5-dicafeoylquinic acid (4). Compound 3 was identified as a new natural product.

![FIGURE 1 - Structures of compounds isolated from \textit{V. negundo} leaves.](image-url)
Compound 3 was isolated as a brownish gum. Its chemical formula was determined as C_{26}H_{40}O_{12} by a negative charged peak [M-H] at m/z 543.2418 (calcd. for C_{26}H_{39}O_{12} 543.2447) in the HR-ESI mass spectrum. The "^1H NMR spectrum showed two olefinic protons [δ_H 7.22 (1H, d, J= 0.8 Hz) and δ_H 6.77 (1H, dt, J= 1.6, 7.6 Hz)], one characteristic proton [δ_H 5.49 (1H, d, J= 2.0 Hz)], one anomeric sugar proton [δ_H 4.84 (1H, d, J= 7.6 Hz)], and three methyl groups [δ_H 1.79 (3H, s), 1.25 (3H, s), and 0.95 (3H, d, J= 6.4 Hz)]. The "^{13}C NMR spectrum showed 26 signals, including two carbonyl (δ_c 168.7 and 167.2), four olefinic (δ_c 149.8, 143.2, 126.9, and 112.6), and 9 oxygenated (δ_c 96.3, 93.4, 78.3, 77.1, 74.5, 73.4, 70.4, 61.3, and 59.6). The COSY indicated three chain coupling systems, including H-1/ H-5/ H-6/ H-7; H-1'/ H-2' / H-3'/ H-4' / H-5'/ H-6'; and H-3'' / H-4'' / H-5'' / H-6'' (H-10'') / H-7'' / H-8'' (Figure 2). The key HMBC correlations of H-1 (δ_H 5.49) / C-3 (δ_c 149.8), H-3 (δ_H 7.22) / C-11 (δ_c 168.7) and C-5 (δ_c 29.6), and H-10 (δ_H 1.25) / C-7 (δ_c 40.2), C-8 (δ_c 78.3), and C-9 (δ_c 51.1) strongly suggested an iridoid skeleton (Figure 2). The sugar moiety was connected to the aglycon at C-1 position, which was determined by an HMBC correlation from H-1 (δ_H 5.49) to C-1' (δ_c 96.3). Additionally, the down fielded chemical shift of H-2' (δ_H 4.77) and an HMBC correlation of H-2' (δ_H 4.77) / C-1'' (δ_c 167.2) indicated the esterification at C-2 position sugar. The substitution was identified as 6,7-dihydrofoliamenthonyl by a COSY chain coupling network H-3'' / H-4'' / H-5'' / H-6'' (H-10'') / H-7'' / H-8'' and series of HMBC correlations of H-9'' (δ_H 1.79) / C-1'' (δ_c 167.2), C-2'' (δ_c 126.9), and C-3'' (δ_c 143.2) (Wang et al., 2017).

The relative configuration of 3 was established by NOESY and coupling constant analysis. The NOESY cross peaks of H-9/ H-5, H-10, and H-1 indicated that these four protons were in the same orientation (Figure 3). The small coupling constant of H-1 [δ_H 5.49 (1H, d, J= 2.0 Hz)] indicated the cis-orientation of H-1 and H-9, which also supported the relative configuration (Yang et al., 2006). The sugar was determined as β-ᴅ-glucopyranose by NOESY correlations of H-1'/ H-3'' and H-5'', H-2''/ H-4'', and by large coupling constant of anomeric proton (J= 7.6 Hz), as well as by comparison with published data (Xiong et al., 2015). Collectively, the structure of 3 was established as 8α-hydroxy-4-carboxyl-5βH-9βH-iridoid-1α-O-(6'-O-(6,7-dihydrofoliamenthonyl)-β-ᴅ-glucopyranoside, a new iridoid glucoside.
**HPLC quantification and method validation**

The optimal separation was determined as follows: on column Phenomenex Synergi 4u MAX-RP 80A (150×4.60 mm, 4µm) protected by a 0.2 µm guard filter (Waters) at 40°C. The mobile phase was consisted of water containing 0.02% trifluoroformic acid (A) and acetonitrile (B). The gradient was from 12B/88A to 16B/84A in 35 min. The column was then washed with acetonitrile and re-equilibrated with initial solvent system for 15 min before the next analysis. The injected volume of sample was 10 µL, the flow rate was set at 0.6 mL/ min (Figure 4). The UV detector was set at 258 nm. The content of I in experimental sample was determined 3.04 ± 0.02% (dried weight).

The proposed method was also validated by various parameters, including linearity (R²= 0.9999), limits of detection (LOD= 0.045 µg/mL), limits of quantification (LOQ= 0.135 µg/mL), precision (intra-day RSD ≤ 2.50%, inter-day RSD= 0.76%), and accuracy (recovery rates of high spike 96.58%, medium spike 101.86%, and low spike 98.94%) (Table II). All parameters were in good range for validation.

**FIGURE 4** - HPLC chromatogram of standard compound I and methanol plant extract. HPLC conditions: Agilent HPLC system 1200 series system (USA); Column: Phenomenex Synergi 4u MAX-RP 80A (C12, 150×4.60 mm, 4µm); column temperature: 40°C; mobile phase 12B/88A to 16B/84A in 35 min [0.02% trifluoroformic acid in water (A) and acetonitrile (B)]; injection volume: 10 µL, flow rate: 0.6 mL/ min; UV wavelength: 258 nm; Sample: 50 mg leaves/ 10 mL MeOH.
TABLE II - Validation parameters of HPLC quantification of *V. negundo* leaves

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<sup>1</sup> expressed as recovery rates in percent.

<sup>2</sup> maximum relative standard deviation (peak area) within one and three consecutive days (n = 3).

Abbreviations: y = peak area, x = concentration (mg/mL), σ<sub>rel</sub> = relative standard deviation, R<sup>2</sup> = determination coefficient, LOD = limit of detection, LOQ = limit of quantification.

**Analgesic activity**

The results reported in the Figure 5 showed that the extract of *V. negundo* leaves in both 2 doses of 9.8 g/kg/day (leaf weight/body weight/day) and 28.8 g/kg/day (leaf weight/body weight/day) taken orally for 3 consecutive days were significantly effective in reducing a number of writhing induced by 0.2 mL acetic acid 1% at all times of study, compared to control group (p < 0.05, p < 0.01, and p < 0.001), indicating the peripheral analgesic activity of the extract. However, the Figure 6 and Figure 7 showed that the extract of *V. negundo* leaves showed no central analgesic activity in hot plate method and pressure pain threshold on mice with the doses of 9.6 and 28.8 g/kg/day (leaf weight/body weight/day) orally for three consecutive days.
FIGURE 5 - Effects of the *V. negundo* leaves on acetic acid-induced writhing in mice
(Presented as Mean ± SE (n=10). Statistical analysis: *: p < 0.05, **: p < 0.01, ***: p < 0.001, T-test as compared to control)

FIGURE 6 - Analgesic effect of the *V. negundo* leaves on mice by the hot plate method.
(Presented as Mean ± SE (n=10). Statistical analysis: *: p < 0.05, T-test as compared to control)
Acute anti-inflammatory activity

Acute anti-inflammatory effects were measured on the white rat with carrageenan-induced edema paw model. The reduced percentages of edema paw volume were presented in the Figure 8. In the control group (group 1), carrageenan injection was used to induce a local edema, which progressively increased after 2, 4, and 6 hours to 42.1, 60.8, and 69.0%, respectively. Afterwards, the edema decreased, but still remained after 24 hours. The pretreatment by administering the *V. negundo* leaves orally with the dose of 5.6 g/kg (leaf weight/body weight) on rat significantly decreased the paw edema levels by 10.5, 18.3, and 41.2% after 2, 4, and 6 hours, respectively.

**FIGURE 7** - Analgesic effect of the *V. negundo* leaves on mice by pressure pain threshold.

(Presented as Mean ± SE (n=10). Statistical analysis: **: p < 0.01, T-test as compared to control)
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**FIGURE 8** - Effects of the *V. negundo* leaves on edema volume in carrageenan-induced inflammation.

(Presented as Mean ± SE (n=10). Statistical analysis: *: p < 0.05, **: p < 0.01, T-test as compared to control)

**Influence of *V. negundo* leaves on the number of leukocytes in inflammatory exudate**

The results in the Figure 9 showed that the extract of *V. negundo* leaves with the dose of 16.8 g/kg (leaf weight/body weight) reduced significantly the number of leukocytes in inflammatory exudate. When compared to control group (p < 0.05), the reduction was 32.23%.

**FIGURE 9** - Effects of the *V. negundo* on the number of leukocytes in inflammatory exudate.

(Presented as Mean ± SEM (n=10). Statistical analysis: *: p < 0.05, T-test as compared to control)
**DISCUSSION**

The purpose of this study was to establish a firm scientific basis for the usage of *V. negundo* for treatment of anti-inflammatory conditions in Vietnam. This was a necessary task because *V. negundo* has been widely used in Vietnamese community, however, little was known about its compounds, as well as pharmacological activity of this species cultivated in Vietnam. The phytochemical investigation on *V. negundo* leaves collected in Vietnam revealed that the major components are iridoid glucosides (1-3) and quinic acid (4) derivatives. Agnuside (1) was determined to be the major constituents in *V. negundo* leaves sample collected in Vietnam with very high content (approximately 3%). The HPLC quantification method was also validated according to ICH guideline (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, 2005)2005. Besides that, a new natural iridoid (3) was also isolated and structurally elucidated by combination spectroscopic and spectrometric methods (1D, 2D-NMR, HRMS).

Iridoid was a group of natural compounds that was well known for analgesic and anti-inflammatory activities in in vivo models (Baghdikian et al., 1997; Lanhers et al., 1992; Wang et al., 2014; Zheng, Y. et al., 2015). The mechanism was believed via inhibition of cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and 5-lipoxygenase (5-LO) (Ryu et al., 2010). Besides that, the major composition, agnuside (1), was an iridoid glucoside linking with a 4-hydroxybenzoic acid moiety, which was well-studied for the analgesic activity in acetic acid-induced writhing model in mice (56% inhibition, p<

**Chronic anti-inflammatory effects of the *V. negundo* leaves**

The results in the Figure 10 showed that the extract of *V. negundo* leaves with the doses of 9.6 g/kg (leaf weight/body weight), and 28.8 g/kg (leaf weight/body weight), reduced 40.20% and 41.24% granulomas weight when compared to control group, which indicated the activity against chronic inflammation (p < 0.01).

**FIGURE 10 - Effects of the *V. negundo* leaves on granulomas weight.**

(Presented as Mean ± SE (n=10). Statistical analysis: **: p < 0.01, ***: p < 0.001, T- test as compared to control)
0.001 at the dose of 50 mg/kg (Okuyama et al., 1998). Therefore, the quantification of agnuside content showed significant values in quality control of this plant and its related herbal products. An HPLC quantification method was also developed and validated, which gave the result of content of agnuside (I) in dried leaves of *V. negundo* (3.04 ± 0.02%). The validation parameters were in good range for quantification (See Table II).

The extract was further examined in animal models for analgesic and anti-inflammatory activities. The *V. Negundo* leaves showed no statistically significant difference in response time in hot plate method at the both doses of 9.6 and 28.8 g/kg (leaf weight/body weight), indicating that the extract did not possess analgesic effect on the central nervous system in the experimental models. On the contrary, the extract did reduce the number of cramping pain attacks in the writhing mouse tests. The pain inducing experiment with acetic acid was representative in evaluating the peripheral analgesic activity, proving that the extract displayed effect on the peripheral mechanism at the experimental doses (9.6 and 28.8 g/kg). These results were in good agreement with previous pharmacological study results. Agnuside isolated from *V. rotundifolia* fructus showed significant writhing inhibition with oral administration at dose of 50 mg/kg (Okuyama et al., 1998). Gupta and Tandon (2005) also reported that at the dose of 500 mg/kg (extract weight/body weight), *V. negundo* leaves extract was shown to delay writhing onset and significantly reduce the number of acetic acid-induced writhings (Gupta, Tandon, 2005).

As for anti-inflammatory activities, *V. negundo* leaves extract showed potent effects in carrageenan-induced edema rat model. The volume of rat paw edema significantly decreased by 41.16% with the dose of 5.6 g/kg (leaf weight/body weight), compared to the control group. The extract also showed the reduction in the number of leukocytes in inflammatory exudate (32.23% compared to the control group) at the dose of 16.8 g/kg (leaf weight/body weight). The results strongly suggested the extract of *V. negundo* leaves had activity against acute inflammatory conditions. The extract was also shown to reduce the granulomas weight (40.20 and 41.24 %) in the chronic granulomas inflammatory mouse model at the doses of 9.6 and 28.8 g/kg (leaf weight/body weight), respectively, which was comparable to methylprednisolone at the dose of 10 mg/kg. The experiment used a carrageenan-impregnated asbestos implanted under the skin of experimental mice. The immune system of mouse would be activated but it would not be able to eliminate the inflammatory inducer, therefore causing chronic inflammatory conditions. This indicated that the extract also showed activity against chronic inflammation, and the magnitude of activity was comparable to that of methylprednisolone. Previous studies had also indicated that *V. negundo* extracts displayed potent anti-inflammation effects on the carrageenan-induced edema rat. Chattopadhyay and colleagues (2012) showed that the *V. negundo* leaf oil decreased the maximum edema to 29% at the dose of 500 µL/kg (Chattopadhyay et al., 2012). In a different case, Kulkarni (2008) reported that the 50% methanol extract of *V. negundo* leaves at a dose of 100 mg/kg decreased the edema to 69.08%. In a different case, Vinuchakkaravarthy’s group (2011) showed that tris(2,4-di-tert-butylphenyl) phosphate was isolated from the of *V. negundo* leaves, which reduced the raw paw edema volume significantly at the tested doses of 50 mg/kg and 70 mg/kg (Vinuchakkaravarthy et al., 2011). These results strongly indicated the effects of *V. negundo* leaves on the acute inflammatory conditions.

However, in our experiment, there was no dose-response effect observed and the high dose (16.8 g/kg) was not effective in the carrageenan-induced edema rat model. *V. negundo* constituents, such as lignans (Singh et al., 2005), glycoside steroid, and triterpenoid glycoside (Chen et al., 2014), might stimulate the immune system with the anti-cancer potential. Therefore, it was possible that the high dose (16.8 g/kg) could stimulate these immune systems. In such cases, we did not see the anti-inflammatory effects of the *V. negundo*. Thus, in future studies, we need to conduct more experiments with the other doses as well as study detailed molecular mechanism of action of *V. negundo* leaves.

There were several studies on mechanism of anti-inflammatory activities of agnuside. Suksamrarn et al. (2002) reported the anti-inflammatory effects of agnuside in vitro, which revealed the mechanism of action through selective COX-2 inhibition using COX deficient murine
cell lines (Suksamrarn et al., 2002) while Pandey and colleagues (2012) proved that the anti-arthritic activity of agnuside was associated with the suppression of inflammatory mediators (PGE2 and LTB4) and T-cell-mediated cytokines (Th1/Th2). Moreover, agnuside was also found to inhibit vascular permeability and leukocyte migration in rat models (Pandey et al., 2012). Other groups of compounds, such as triterpenoid, lignan, labdane and megastimane derivatives, also exhibited potent inhibitory activities on NO production in lipopolysaccharide (LPS)-induced inflammation in RAW264.7 macrophages and microglial BV-2 cells (Hu et al., 2016; Li et al., 2014; Xu et al., 2019).

In conclusion, the current study provided wide range of scientific evidences to support the traditional usage and clinical application of V. negundo leaves in particular case of Vietnam, as well as a validated method for quality control for this plant and its related herbal products in the future.

ACKNOWLEDGMENTS

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SUPPLEMENTARY INFORMATION

The NMR data (1H, 13C, COSY, HSQC, HMBC, NOESY), HR-ESIMS, and CD spectra of compound 3, as well as histopathological findings of granuloma images can be found in Supplementary information.

CONFLICTS OF INTEREST

The authors declare no conflict of interest

REFERENCES


Phytochemical investigation on Vitex negundo leaves and their anti-inflammatory and analgesic activities


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SUPPLEMENTARY DATA

Figure S1.1 - $^1$H NMR spectrum of compound 3 (400 MHz, methanol-$d_4$).

Figure S1.2 - $^{13}$C NMR spectrum of compound 3 (100 MHz, methanol-$d_4$)
Figure S1.1 - 1H NMR spectrum of compound 3 (400 MHz, methanol-d4)

Figure S1.2 - 13C NMR spectrum of compound 3 (100 MHz, methanol-d4)

Figure S1.3 - COSY spectrum of compound 3 (400 MHz, methanol-d4)

Figure S1.4 - HSQC spectrum of compound 3 (400 MHz, methanol-d4)
Figure S1.5 - HMBC spectrum of compound 3 (400 MHz, methanol-d4)

Figure S1.6.1 - NOESY spectrum of compound 3 (aglycon, 400 MHz, methanol-d4)
Figure S1.6.2 - NOESY spectrum of compound 3 (sugar, 400 MHz, methanol-d4)

Figure S1.7 - CD spectrum of compound 3 (methanol, 40 µM)
### Figure S1.8 - HR-ESIMS of compound 3

![HR-ESIMS Image]

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### Figure 2.1 - Biological control: A large necrotic tissues and a large number of degenerated polymorphonuclear leukocytes in the center (HE x 400).

![Biological Control Image]
**Figure 2.2** - Biological control: Necrotic tissues and a large number of degenerated polymorphonuclear leukocytes septum with a large number of fibroblasts and neutrophils (HE x 400).

**Figure 2.3** - The *V. negundo* leaves at the dose of 9.6 g/kg. Necrotic tissues and a large number of degenerated neutrophils in the center (HE x 400).
**Figure 2.4** - The *V. negundo* leaves at the dose of 9.6 g/kg: Blood vessels, fibroblasts, some neutrophils and lymphocytes in the septum (HE x 400).

**Figure 2.5** - The *V. negundo* leaves at the dose of 9.6 g/kg: Abscess shell has some lymphocytes and plasmocytes (HE x 400).
Figure 2.6 - The V. negundo leaves at the dose of 28.8 g/kg: Necrotic tissues and degenerated neutrophils at the center of abscess (HE x 400).

Figure 2.7 - The V. negundo leaves at the dose of 28.8 g/kg: A large number of fibroblasts, blood vessels and neutrophils, very few lymphocytes (HE x 400).
Figure 2.8 - The V. negundo leaves at the dose of 28.8 g/kg: A few lymphocytes and cytoplasms at the outer of the abscess shell (HE x 400).