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).

8a-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]− (caclcd. for C26H39O12 543.2447).

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

| Position | δH | mult., J | δC | type |
|----------|-----|----------|-----|------|
| 1        | 5.49 d, 2.0 | 93.4 | d    |
| 3        | 7.22 d, 0.8 | 149.8 | d    |
| 4        | -    | -       | 112.6 | s    |
| 5        | 2.94 m   | 29.6 | d    |
| 6        | 1.27 m   | 35.5 | t    |
| 7        | 1.57 m   | 40.2 | t    |
| 8        | -    | -       | 78.3 | s    |
| 9        | 2.24 m   | 51.1 | d    |
| 10       | 1.25 s   | 22.8 | q    |
| 11       | -    | -       | 168.7 | s    |
| 1'       | 4.84 d, 7.6 | 96.3 | d    |
| 2'       | 4.77 dd, 8.0, 9.6 | 73.4 | d    |
| 3'       | 3.58 m   | 74.5 | d    |
| 4'       | 3.36 m   | 70.4 | d    |
| 5'       | 3.37 m   | 77.1 | d    |
| 6'       | 3.69 m, 3.91 | 61.3 | t    |
| 1"       | -    | -       | 167.2 | s    |
| 2"       | -    | -       | 126.9 | s    |
| 3"       | 6.77 dt, 1.6, 7.6 | 143.2 | d    |
| 4"       | 2.21 m   | 25.9 | t    |
| 5"       | 1.27 m, 1.47 | 35.5 | t    |
| 6"       | 1.61 m   | 29.1 | d    |
| 7"       | 1.36 m, 1.59 | 39.2 | t    |
| 8"       | 3.61 m   | 59.6 | t    |
| 9"       | 1.79 s   | 11.1 | q    |
| 10"      | 0.95 d, 6.4 | 18.4 | q    |
**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 4 u 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

\[
\Delta V\% = \frac{V_1 - V_0}{V_0} \times 100
\]

\(V_0\): rat paw volume before inducing rat paw edema
\(V_1\): 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
Phytochemical investigation on Vitex negundo leaves and their anti-inflammatory and analgesic activities

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 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:

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

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 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-dihydrofamethonyl)-β-D-glucopyranoside (3), and 3,5-dicafeoylquinic acid (4). Compound 3 was identified as a new natural product.

**FIGURE 1** - Structures of compounds isolated from V. negundo leaves.
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 (cacl. for C$_{26}$H$_{39}$O$_{12}$ 543.2447) in the HR-ESI mass spectrum. The $^1$H 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 anomic 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-9/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-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-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

| Parameter / Compound | 1 |
|----------------------|---|
| Regression equation  | $y = 30167x - 81.807$ |
| $\sigma_{rel}$ of slope | 1.38 |
| $R^2$                | 0.9999 |
| Range (µg/mL)        | 1055-32.7 |
| LOD (µg/mL)          | 0.045 |
| LOQ (µg/mL)          | 0.135 |
| Accuracy$^1$         |  |
| High spike           | 96.58 |
| Medium spike         | 101.86 |
| Low spike            | 98.94 |
| Precision            |  |
| Intra-day$^2$        | 2.50 |
| Inter-day            | 0.76 |

$^1$expressed as recovery rates in percent.
$^2$maximum relative standard deviation (peak area) within one and three consecutive days (n = 3).

Abbreviations: $y$ = peak area, $x$ = concentration (mg/mL), $\sigma_{rel}$ = relative standard deviation, $R^2$ = 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)
Anti-inflammatory activity

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)
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%. 

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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)

**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. Aguuside (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). 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, aguside (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<
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,
Vinuchakkaravartthy’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 (Vinuchakkaravartthy 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.

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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**

Ankier SI. New hot plate tests to quantify antinociceptive and narcotic antagonist activities. Eur J Pharmacol. 1974;27(1):1-4.

Baghdikian B, Lanfers MC, Fleurentin J, Ollivier E, Maillard C, Balansard G, et al. An analytical study, anti-inflammatory and analgesic effects of *Harpagophyutm procumbens* and *Harpagophyutm zeyheri*. Planta Med. 1997;63(2):171-176.

Chattopadhyay P, Hazarika S, Dhiman S, Upadhyay A, Pandey A, Karakar S, et al. *Vitex negundo* inhibits cyclooxygenase-2 inflammatory cytokine-mediated inflammation on carrageenan-induced rat hind paw edema. Pharmacognosy Res. 2012;4(3):134-137.

Chen J, Fan CL, Wang Y, Ye WC. A new triterpenoid glycoside from *Vitex negundo*. Chin J Nat Med. 2014;12(3):218-221.

Dharmasiri MG, Jayakody JRAC, Galhena G, Liyanage SSP, Ratnasooriya WD. Anti-inflammatory and analgesic activities of mature fresh leaves of *Vitex negundo*. J Ethnopharmacol. 2003;87(2):199-206.

Gill BS, Mehr R, Naveet, Kumar S. *Vitex negundo* and its medicinal value. Mol Biol Rep. 2018;45(6):2925-2934.

Gupta RK, Tandon VR. Antinociceptive activity of *Vitex negundo* Linn leaf extract. Indian J Physiol Pharmacol. 2005;49(2):163-170.

Hu P, Li DH, Hu X, Li SG, Sai CM, Sun XC, et al. Lignans and triterpenoids from *Vitex negundo* var. heterophylla and their biological evaluation. Fitoterapia. 2016;111:147-153.

Hu P, Li DH, Jia CC, Liu Q, Wang XF, Li ZL, et al. Bioactive constituents from *Vitex negundo* var. heterophylla and their antioxidant and α-glucosidase inhibitory activities. J Funct Foods. 2017;35:236-244.

International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, I., ICH Harmonised Tripartite Guideline: Validation of Analytical Procedures: Text and Methodology Q2(R1). Place, Published. 2005.

Koster R, Anderson M, de Beer E. Acid acetic for analgesic screening. Fed Proc. 1952;18:412.

Kulkarni RR, Virkar DA, Priscilla D’Mello. Antioxidant and antiinflammatory activity of *Vitex negundo*. Indian J Pharm Sci. 2008;70(6):838-840.

Lanfers MC, Fleurentin J, Mortier F, Vinche A, Younos C. Anti-inflammatory and analgesic effects of an aqueous extract of *Harpagophyutm procumbens*. Planta Med. 1992;58(2):117-123.

Li MM, Su XQ, Sun J, Gu YF, Huang Z, Zeng KW, et al. Anti-inflammatory ursane- and oleanane-type triterpenoids from *Vitex negundo* var. *cannabifolia*. J Nat Prod. 2014;77(10):2248-2254.
Phytochemical investigation on Vitex negundo leaves and their anti-inflammatory and analgesic activities

National Institute of Medicinal Materials, Medicinal plants and animals in Vietnam. Place, Published Science and Technics Publishing House. 2006.

Okuyama E, Fujimori S, Yamazaki M, Deyama T. Pharmacologically active components of *Vitex fructus* (*Vitex rotundifolia*). II. The component having analgesic effects. Chem Pharm Bull. 1998;46(4):655-662.

Pandey A, Bani S, Satti NK, Gupta BD, Suri KA. Anti-arthritis activity of agnuside mediated through the down-regulation of inflammatory mediators and cytokines. Inflamm Res 2012;61(4):293-304.

Ryu KH, Rhee HI, Kim JH, Yoo H, Lee BY, Um KA, et al. Anti-inflammatory and analgesic activities of SKLJJ, a highly purified and injectable herbal extract of *Lonicera japonica*. Biosci Biotechnol Biochem. 2010;74(10):2022-2028.

Singh DD, Chitra G, Singh IP, Bhutani KK. Immunostimulatory compounds from *Vitex negundo*. Indian J Chem B. 2005;44:1288-1290.

Suksamrarn A, Kumpun S, Kirtikara K, Yingyongnarongkul B, Suksamrarn S. Iridoids with anti-inflammatory activity from *Vitex peduncularis*. Planta Med. 2002;68(1):72-73.

Vinuchakkaravarthy T, Kumaravel KP, Ravichandran S, Velmurugan D. Active compound from the leaves of Vitex negundo L. shows anti-inflammatory activity with evidence of inhibition for secretory Phospholipase A(2) through molecular docking. Bioinformation 2011;7(4):199-206.

Vogel HG. Analgesic, Anti-Inflammatory, and Anti-Pyretic Activity. In: Drug discovery and evaluation: pharmacological assays. 3rd completely rev., updated, and enl. ed. Berlin New York: Springer. 2008:984-1116.

Vu PX. Vietnam flora. Place, Published Science and Technics Publishing House. 2007.

Wang LY, Chen MH, Wu J, Sun H, Liu W, Qu YH, et al. Bioactive glycosides from the twigs of *Litsea cubeba*. J Nat Prod. 2017;80(6):1808-1818.

Xiong H, Zheng Y, Yang G, Wang H, Mei Z. Triterpene saponins with anti-inflammatory activity from the stems of *Entada phaseoloides*. Fitoterapia. 2015;103:33-45.

Xu JM, Hu BC, Yuan L, Wu YL, Luan SS, Yuan T, et al. Labdanes and megastigmanes from *Vitex negundo* var. *heterophylla*. Fitoterapia. 2019;137:104265-104265.

Yang LR, Xiong J, Tan NH, Chu HB, Xu L, Li MY. Chemical constituents of *Pedicularis tricolor* (Scrophulariaceae). Acta Botanica Yunnanica. 2006;28(5):553-557.

Zheng CJ, Li HQ, Ren SC, Xu CL, Rahman K, Qin LP, et al. Phytochemical and pharmacological profile of *Vitex negundo*. Phytother Res. 2015;29(5):633-647.

Zheng Y, Yin X, Huo F, Xiong H, Mei Z. Analgesic effects and possible mechanisms of iridoid glycosides from *Lamiophlomis rotata* (Benth.) Kudo in rats with spared nerve injury. J Ethnopharmacol. 2015;173:204-211.

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SUPPLEMENTARY DATA

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

Figure S1.2 - ^1^C NMR spectrum of compound 3 (100 MHz, methanol-d_4).

Necrotic tissues
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 - 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

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