two-dimensional (2D) cultures, VAM extract was further investigated stemness-suppressing ability via tumorspheroidal assay. The results indicated that VAM reduced spheroid forming, a unique characteristic of CSCs [14]. According to the report of Tin et al., in 2013, it was also demonstrated that artemisinin, derived from sweet wormwood (*Artemisia annua*), has an inhibitory effect on the tumorspheres derived from breast cancer stem cells [15].

The process of metastasis involves tumor cells moving away from the primary tumor site, with cancer stem cells (CSCs) potentially playing a key role in enabling this movement [16]. Concerning our results, $2xIC_{50}$ of the crude extract might prevent NTERA-2 cell migration compared to the control after 24 h of incubation. Besides, the methanol extract from *A. vulgaris* also demonstrated a reduction in HCT-15 colon cancer cell migration capacity, as stated by G Lian et al., [17]. Additionally, a different study showed that certain plants belonging to the same genus, including *Artemisia dracunculus*, *Artemisia annua*, and *Artemisia absinthium*, could have wound healing potential on HaCat cell line with wound closure rates of 89.65 ± 2.18%, 98.55 ± 0.64%, and 97.84 ± 1.98%, respectively [18].

5. Conclusion

In conclusion, the crude methanol extract of *Artemisia vulgaris* demonstrated anti-cancer potential against NTERA-2 cancer stem cells by inhibiting their survival, reducing stemness characteristics by preventing tumorsphere formation as well as suppressing cell migration *in vitro*.

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ACUTE ORAL TOXICITY AND ANTI-INFLAMMATORY EFFECT OF THE STANDARDIZED EXTRACT FROM *VITEX TRIFOLIA* L. FRUITS

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Summary

Acute Oral Toxicity and Anti-Inflammatory Effect of the Standardized Extract from *Vitex trifolia* L. Fruits

Recent studies suggest that the decline in ovarian function and estrogen levels during menopause cause increased proinflammatory cytokines and oxidative stress associated with these symptoms and perhaps progressive central nervous system (CNS) damage. Our previous studies have shown that a *Vitex trifolia* L. *n*-butanol extract improved cognitive deficit and ameliorated the atrophy of the uterus in ovariectomized mice, as a menopause model. The purpose of this study is to determine acute oral toxicity in mice and evaluate the anti-inflammatory properties of the standardized extract from the fruits of *V. trifolia* (VTE) collected in Vietnam. The acute toxicity study showed that the VTE had an LD_{50} value of 26.0 g/kg BW. Our results indicated that in the carrageenan-induced paw edema rat model, VTE at a dose of 150 mg/kg significantly inhibited paw edema at 4 and 6 hours. In addition, VTE at both doses of 150 mg/kg and 300 mg/kg significantly decreased the volume of inflammatory exudate, the number of leukocytes, and the protein content in the exudate in the carrageenan and formaldehyde-induced peritonitis model. Taken together, these results suggest that VTE exhibits potent acute anti-inflammatory effects. Further research is required to assess the potential for preventing or treating CNS-related inflammatory conditions.

Keywords: *Vitex trifolia fruit extract, Acute oral toxicity, Anti-inflammatory.*

1. Introduction

Vitex trifolia L. is endemic to regions of southeast Asia, China, Japan, Korea, India, Africa, Australia, and other Pacific countries, where it is used in traditional medicines and dietary supplements for a variety of health conditions. The Vietnamese name for *Vitex trifolia* is "Mãn kinh", which means "menopause", for it is often used to treat symptoms experienced during or after menopause, including those associated with the CNS such as anxiety, headache, depression and reduced cognitive function [1],[2],[3],[4]. Recent studies suggest that the decline in ovarian function and estrogen levels during menopause cause increased proinflammatory cytokines and oxidative stress associated with these symptoms and perhaps progressive CNS damage [5],[6],[7].

V. trifolia extracts modulate proinflammatory cytokines and oxidative stress through multiple targets and signaling pathways [1],[2],[3]. Our previous studies have shown that a *V. trifolia n*-butanol extract improved cognitive deficit and ameliorate the atrophy of the uterus in ovariectomized mice, as a menopause model [8],[9]. Our recent experiments (unpublished) and studies by others indicate that *V. trifolia* extracts (VTE) exhibit estrogen-like activity [1],[2],[3]. However, virtually all such studies have used VTEs whose components have not been characterized, nor have the plant materials been collected in Vietnam, where they are widely used in medicines and dietary supplements [10],[11].

Fructus Viticis was used in traditional medicines of China, Korea, and Japan and perhaps elsewhere *V. trifolia* may be mixed with *V. trifolia* Steenis subsp. *litoralis* or even *V. rotundifolia* [1],[2],[10],[12],[13],[14]. *V. trifolia* sp. and *V. rotundifolia* are sometimes considered to be synonymous - however, their phenotypes, growth properties, genetic markers and chemical constituents are distinct [2],[13],[15],[16],[17],[18]. Therefore, the present study aims to determine the acute oral toxicity in mice and evaluate the anti-inflammatory properties in the hind paw edema model and peritonitis model in rats of the standardized VTE analyzed by HPLC.

2. Materials and Methods

2.1. Plant materials and extraction

The fruits of *Vitex trifolia* L. were collected in Nghe An, Vietnam, and identified by Dr. Pham Thanh Huyen from the National Institute of Medicinal Materials (NIMM). The voucher specimens of these plants were deposited at the Herbarium of the Medicinal Material Resources Center (NIMM), Vietnam. The standardized extract VTE was prepared as follows: The dried plant sample (1 kg) was coarsely ground and extracted twice by reflex with 50% aqueous ethanol for 3 hours and 1.5 hours, respectively. The extracts were then combined and filtered, and the solvent was evaporated under reduced pressure at 60°C to give a liquid extract. The liquid extract was then vacuum dried at a temperature of 60°C, with a pressure of 50 mmHg. The final extract had a moisture content of less than 5% and a yield of 14%. High Performance Liquid Chromatography (HPLC) chromatograms of VTE and standards are shown in Fig. 1 and indicate agnuside and casticin are major components. According to the analysis method, the content agnuside and casticin in the VTE were not less than 0.7% and 0.2%, respectively.

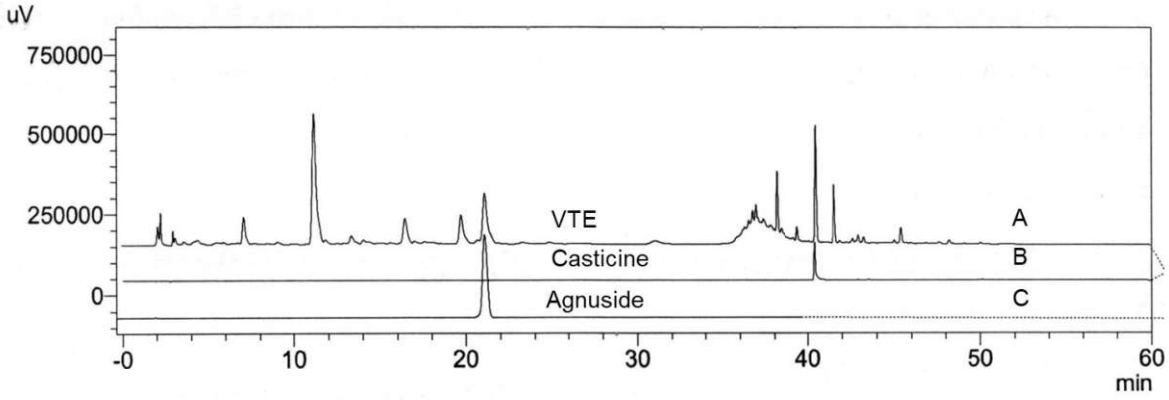

Fig. 1. Characterization of VTE by HPLC.
HPLC/UV profiles of VTE (A), Casticin (B; RT= 40.2 min) and Agnuside (C; RT =20.9 min)

2.2. Animals

For acute oral toxicity studies, male and female *Swiss albino* mice (weight 18-20 g) were purchased from the National Institute of Hygiene and Epidemiology, Hanoi, Vietnam. For anti-inflammatory evaluation, Wistar rats of both sexes (weight 180-200 g) were obtained from the Vietnam Military Medical Academy. The animals were acclimated to the laboratory animal room for at least one week before any procedures were performed. Food and water were available ad libitum. Housing conditions were maintained at 22 ± 1°C with 12-hour light-dark cycles.

2.3. Acute oral toxicity in mice and determination of LD_{50}

For the acute oral toxicity test [19], mice were fasted for approximately 12 hours, with free access to water, prior to oral administration (0.2 ml/10 g body weight) of VTE (up to 42.5 g/kg, po.). Behavioral expressions and signs of toxicity were observed in the animals two hours post-treatment. Mortality within the first 24 hours was recorded for each group, and surviving animals were monitored for an additional seven days for signs of delayed toxicity. The median lethal dose (LD_{50}) was estimated using the Behrens-Karber method and calculated as following:

$$LD_{50} = LD_{100} - \frac{\sum(d \times z)}{n}$$

Where:

LD_{50}: the dose that causes the death of 50% of the test animals

LD_{100}: the lowest dose that causes the death of 100% of the test animals

d: the difference between two consecutive doses

z: the average number of mice dead between two consecutive doses

n: the number of test mice per group

2.4. Evaluation of anti-inflammatory activities in rats

2.4.1. Hind paw edema induced by carrageenan:

For the carrageenan-induced hind paw edema test [20],[21], rats were placed into 4 groups (n=10

rats/group). The control group (#1) and the reference group (#2) received normal water and ibuprofen (30 mg/kg, Traphaco JSC, Vietnam), respectively, and the test groups (#3 and #4) were treated with 150 and 300 mg/kg body weight of VTE. Water, VTE, and ibuprofen were administered orally (1 mL/100 g body weight) for five consecutive days. On day 5, one hour after treatment, each rat received subcutaneously a subplantar injection of 0.05 mL freshly prepared 1% carrageenan solution (BDH Chemicals Ltd., Poole, England) suspended in sterile physiological saline into the right hind paw. Paw volume was measured by a digital water plethysmometer (Ugo Basile, model 7140, Italy), before the treatment (V_0) and 2, 4, 6, and 24 h after carrageenan injection (V_t). The increase in volume of edema was determined for each rat.

The increase in paw volume for each rat was calculated using the following equation:

$$\Delta V\% = \frac{V_t - V_0}{V_0} \times 100$$

Where:

V_0 is the paw volume before inducing inflammation;

V_t is the paw volume after inducing inflammation **at *t* h.**

The anti-inflammatory effect is evaluated by the ability to inhibit edema (I%)

$$I\% = \frac{\Delta\bar{V}_c\% - \Delta\bar{V}_t\%}{\Delta\bar{V}_c\%} \times 100$$

Where:

$\Delta\bar{V}_c\%$ is the average increase in paw volume in the control group;

$\Delta\bar{V}_t\%$ is the average increase of paw volume in the treated group **at *t* h.**

2.4.2. Peritonitis induced by carrageenan and formaldehyde:

Rats were placed into 4 groups and administered water, extracts, or ibuprofen for five days as described above. On day 5, rats were injected with carrageenan (0.5% w/v) and formaldehyde (1.5% v/v) suspended in sterile physiological saline with a volume of 1 mL/100 g body weight [22]. Twenty-four hours later the rats were sacrificed and the peritoneal cavity of each was opened to collect exudate, whose volume and protein concentration and leucocytes count were determined. Protein concentration was determined using a colorimetric method based on the Biuret reaction principle. In brief, the exudates were centrifuged to separate the supernatants, which were then transferred into specific cuvettes and analyzed using an Atellica® CH Analyzer (Siemens Healthcare Diagnostics Inc., USA) following the manufacturer's instructions. Simultaneously, the leukocyte count was assessed with an XN-2000™ Automated Hematology Analyzer (Sysmex, Japan).

2.5. Statistical analysis

Statistical analyses were conducted using IBM SPSS Statistics version 23.0 software, and data are presented as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) or a t-test, where only two conditions were compared, was used to examine the significant differences among groups. Differences of $p < 0.05$ were considered significant.

3. Results

3.1. Acute toxicity

Following the oral administration of varying doses of VTE ranging from 17.41 to 42.5 g/kg (BW) to mice, the mortality and dose response were documented, as outlined in Table 1. Accordingly, the LD_{50} value was estimated at 26.0 g/kg BW based on the Behrens-Karber's method.

Table 1. Mortality percentage after 24 hours from oral administration of various doses of VTE

Group	Dose (g/kg)	n	Mortality	d	z	d x z
1	17.41	10	0			
2	21.76	10	1	4.4	0.5	2.18
3	27.20	10	8	5.4	4.5	24.48
4	34.00	10	9	6.8	8.5	57.80
5	42.50	10	10	8.5	9.5	80.75
∑ (d x z)						**165.21**

3.2. Anti-inflammatory effect of VTE on rat paw edema assay

Carrageenan injection induced inflammation in the rat's hind paw, which increased paw diameter and reached a maximum by 4 hours. Administration of ibuprofen significantly reduced inflammation in the hind paw at almost all time points, including at 2, 4, and 6 hours. At 24 hours, ibuprofen tended to decrease the rat paw thickness but not significantly compared to the control group. While VTE at a dose of 150 mg/kg also significantly inhibited paw edema at 4 and 6 hours, VTE at a dose of 300 mg/kg decreased the rat paw thickness only at 4 hours. At other time points, VTE at doses of 150 and 300 mg/kg slightly inhibited inflammation in the hind paw but not significantly compared to the control group. Collectively, these results suggest that VTE possesses relatively strong acute anti-inflammatory activity in the rat paw edema assay.

Table 2. Effect of VTE and ibuprofen on rat paw edema induced by carrageenan

Group	V_2		V_4		V_6		V_{24}	
	% Edema compared to V_0	*Edema inhibition (%)*	*% Edema compared to V_0*	*Edema inhibition (%)*	*% Edema compared to V_0*	*Edema inhibition (%)*	*% Edema compared to V_0*	*Edema inhibition (%)*
1	39.33 ± 11.58	-	54.35 ± 20.22	-	52.48 ± 17.33	-	3.73 ± 4.60	-
2	**21.22 ± 9.54***	46.05	**36.98 ± 8.90***	31.96	**36.74 ± 9.48***	29.99	2.70 ± 6.63	27.61
3	32.42 ± 15.68	17.47	**36.33 ± 14.36***	33.16	**34.57 ± 17.31***	34.13	2.86 ± 2.72	23.32
4	41.92 ± 9.02	-6.58	**33.90 ± 16.33***	37.63	38.70 ± 14.80	26.26	1.66 ± 1.39	55.50

*Group 1: Control, Group 2: Ibuprofen 30 mg/kg, Group 3: VTE 150 mg/kg, Group 4: VTE 300 mg/kg. *$p < 0.05$ compared to Group 1 (Control group)*

3.3. Anti-inflammatory effect of VTE on peritonitis induced by carrageenan and formaldehyde

Rats were treated with saline, VTE, or ibuprofen for five consecutive days before peritonitis induction. The exudate volume, protein concentration, and leucocyte count in exudate were determined and the results are presented in **Table 3** and **Fig. 2**.

Table 3. Effect of VTE and ibuprofen on exudate volume in peritonitis model induced by carrageenan and formaldehyde

Group	Exudate volume (mL/100g)
Group 1: Control	3,92 ± 1,01
Group 2: Ibuprofen 30 mg/kg	**3,04 ± 0,70***
Group 3: VTE 150 mg/kg	**3,00 ± 0,71****
Group 4: VTE 300 mg/kg	**1,42 ± 0,43****,$^{\Delta}$**

*, ** $p < 0.05$; 0.01 compared to Group 1, respectively, $^{\Delta}p < 0.05$ compared to Group 2*

The data indicate that the administration of ibuprofen (30 mg/kg) significantly decreased the leukocyte migrations (13.91 ± 3.78 leukocytes/mL), exudate volume (3.04 ± 0.70 mL/100 g), and protein content (4.66 ± 0.17 mg/mL) compared with the control group (23.08 ± 5.78 leukocytes/mL, 3.92 ± 1.01 mL/100 g, and 7.73 ± 2.46 mg/mL for leukocyte count, exudate volume, and protein content, respectively). Administration of VTE also significantly affected these parameters. Specifically, the results for groups pretreated with VTE at doses of 150 mg/kg and 300 mg/kg were 17.24 ± 3.23 and 16.58 ± 3.47 leukocytes/mL, 3.00 ± 0.71 mL/100 g and 1.42 ± 0.43 mL/100 g, and 5.40 ± 0.73 mg/mL and 4.90 ± 0.20 mg/mL for leukocyte count, exudate volume, and protein content, respectively. Intriguingly, the exudate volume in the group administered 300 mg/kg of VTE was even significantly lower than that in the group treated with ibuprofen, indicating the potent anti-inflammatory effect of VTE in the peritonitis model.

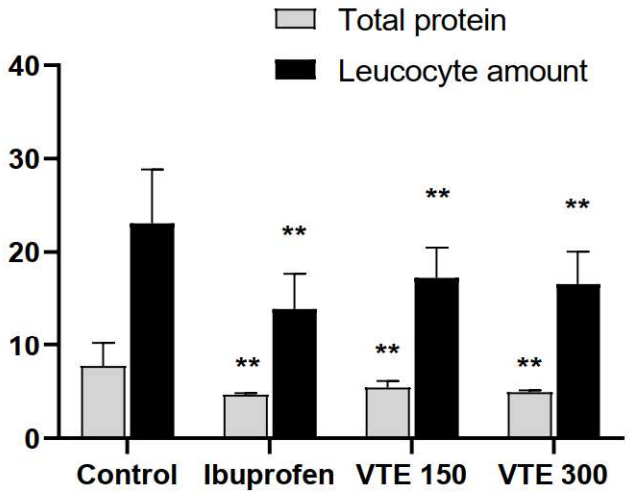

Fig. 2. Effect of VTE and ibuprofen on leukocytes/mL and protein content (mg/mL) in the exudate in the carrageenan and formaldehyde-induced peritonitis model
*** p < 0.01 compared to Group 1 (control group)*

4. Discussion

Vitex trifolia L. has long been used in traditional medicines and dietary supplements, however, no prior studies have evaluated the composition, safety, and anti-inflammatory properties of VTE from Vietnamese sources. In this study, the acute anti-inflammatory effects of VTE were evaluated by the carrageenan-induced paw edema model [23],[24] and the peritonitis model. In both models, ibuprofen decreased paw edema at 2, 4, and 6 hours in the paw edema model, and significantly reduced indicators (leukocyte count, protein content) in the peritonitis model compared to the control group. In the present study, VTE at both doses of 150 mg/kg and 300 mg/kg significantly reduced the paw edema volume at the time points of 4 hours and 6 hours post-inflammation induction. In addition, VTE at both doses of 150 mg/kg and 300 mg/kg also significantly decreased the volume of inflammatory exudate, the number of leukocytes, and the protein content in the exudate in the peritonitis model.

Our findings resemble previous studies regarding the anti-inflammatory activities *in vivo* of extracts of leaves of *V. trifolia*. According to Parvathi Annamalai et al. (2022), the methanol extract of *V. trifolia* leaves at doses of 100 mg/kg and 200 mg/kg significantly inhibited carrageenan-induced paw edema and effectively reduced leukocyte infiltration. Moreover, *V. trifolia* leaves significantly inhibited the tissue levels of interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α induced by carrageenan, as well as the levels of cytokine-induced neutrophil chemoattractants CINC-2/C-X-C (CXCL3) and CINC-3/CXCL2 in tissue and serum, while significantly upregulating anti-inflammatory cytokine IL-10 levels. Additionally, *V. trifolia* leaves significantly reduced carrageenan-induced IκBα degradation and NF-κB p65 nuclear translocation, providing insights into its molecular mechanism mediated through the downregulation of the NF-κB signaling pathway [1],[2],[3],[25].

Other *in vitro* studies have also provided evidence regarding the anti-inflammatory effect of *V. trifolia*. Specifically, Hai-Ning Wee et al. (2020) investigated the effects of various extracts of *V. trifolia* leaves on the production of cytokines TNF-α and IL-1β using enzyme-linked immunosorbent assay (ELISA) in lipopolysaccharide-stimulated human U937 macrophages. Among the 14 different leaf extracts studied, the extracts processed by ultrasonic extraction in dichloromethane and ethanol soaking exhibited the highest activity in inhibiting TNF-α and IL-1β production in human U937 macrophages [26]. Additionally, Mariko Matsui et al. (2012) studied the effects of *V. trifolia* leaf extract on lipopolysaccharide (LPS)-induced inflammatory gene expression, focusing on the

regulation of chemokine C-X-C motif 10 (CXCL-10) and C-C motif ligand 3 (CCL-3) and cyclooxygenase (COX)-2. Their results showed that *V. trifolia* extract at a concentration of 5000 µg/ml significantly inhibited the expression of multiple LPS-induced inflammatory genes in RAW 264.7 cells after 8 hours of incubation. TransAM assays indicated that *V. trifolia* extract also inhibited LPS-induced NF-κB translocation at 250 µg/mL [27].

Of the prevalent metabolites in VTE and extracts of other *Vitex* species, agnuside and casticin are the best characterized for their anti-inflammatory activities[28],[29],[30],[31]. While pharmacokinetic evidence indicates agnuside crosses the blood brain barrier [32], only indirect evidence is available for casticin [33]. Additional studies are required to document their anti-inflammatory activities in the CNS.

Our study provides evidence regarding the anti-inflammatory effects of VTE and the knowledge from extensive traditional uses for menopausal symptoms support its potential for prevention or treatment of inflammation-related postmenopausal CNS conditions. Given the evidence that hormone replacement therapies containing estrogen receptor modulators may cause dementia and related neurocognitive conditions [34], alternative treatments are needed. Further studies are needed to evaluate the anti-inflammatory and potential estrogenic effects of compounds isolated from *V. trifolia* fruits as well as to investigate the underlying mechanisms of action.

5. Conclusion

The results from the acute oral toxicity test showed that the VTE has an LD_{50} of 26.0 g/kg BW. In the carrageenan-induced paw edema rat model, VTE at both doses of 150 mg/kg and 300 mg/kg significantly reduced the paw edema volume at 4 hours and 6 hours post-inflammatory induction. In addition, VTE at both doses of 150 mg/kg and 300 mg/kg also significantly decreased the volume of inflammatory exudate, the number of leukocytes, and the protein content in the exudate in the carrageenan and formaldehyde-induced peritonitis model. Taken together, these results indicated that VTE has significant anti-inflammatory effects.

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BINARY ETHOSOMES FOR ENHANCING SKIN DELIVERY OF RUTIN BY SOLVENT INJECTION

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Summary

Binary Ethosomes for Enhancing Skin Delivery of Rutin by Solvent Injection

Rutin, a natural extract from buckwheat, tea, and apple, has powerful pharmacological properties, especially antioxidant effects. However, the bioactivity of rutin is limited since it has low solubility and poor permeability. Nanovesicles, especially ethosomes, have been a non-invasive drug carrier for skin delivery with high entrapment efficiency and stability. In this research, the simple solvent injection was used to fabricate classical ethosomes, and binary ethosomes. Various polyols, like propylene glycol, isopropyl alcohol, and glycerol at different ratios were investigated in rutin binary ethosome preparation. Then, rutin ethosomes were characterized by physical properties, loading efficiency, drug release, and short-term stability. The optimum binary ethosomes were made from the mixture of ethanol and glycerol at the ratio of 3:7. Rutin binary ethosomes had a lower size than classical ethosomes and better stability during 30 days. Rutin ethosomes posed sustained release through dialysis membrane and mouse skin and significantly improved the drug release in the Franz cell model. Therefore, binary ethosomes could be considered as potential nanovesicles to deliver rutin via the skin route.

Keywords: *Ethosomes, Classical ethosomes, Binary ethosomes, Skin delivery, Rutin.*