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EFFECTS OF *n*-BUTANOL EXTRACT FROM *VITEX TRIFOLIA* FRUITS ON COGNITIVE FUNCTION DEFICIT IN OVARIECTOMIZED MICE

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Summary

Effects of *n*-Butanol Extract from *Vitex trifolia* Fruits on Cognitive Function Deficit in Ovariectomized Mice

While changes in cognition may occur with the menopausal transition, clinical trial data have not demonstrated that treatment with postmenopausal hormone treatment prevents development of dementia or cognitive impairment. *Vitex trifolia* L. (VT) has been used as a herbal medicine for headaches and anti-depressants in menopausal women. In the present study, we have evaluated the effect of *n*- BuOH extract from the fruit of VT (30 and 60 mg/kg) treatment on cognitive impairment, bone index, and uterine atrophy in ovariectomized (OVX) mice. Five groups of female mice were used; sham group, OVX + water, OVX + VT extracts (30 and 60 mg/kg), and OVX + Progynova® (estradiol valerate, 0.5 mg/kg). Our data revealed that VT *n*-BuOH extract significantly mitigated memory decline and antagonized the atrophy of the uterus in OVX mice. We also found that VT *n*-BuOH extract markedly increased the serum level of estradiol, suggesting that VT *n*-BuOH extract relieves the symptoms of menopause through, at least in part, stimulating the biosynthesis of estrogen in circulation. Taken together, our results demonstrate that *n*-BuOH extract from the fruit of VT might be beneficial for treatment of cognitive deficits in postmenopausal women.

Keywords: *Vitex trifolia* L., *Ovariectomized mice, Cognitive deficit, Uterus-oviduct, Estradiol, Alkaline phosphatase.*

1. Introduction

Postmenopausal hormone therapy is used for controlling menopausal symptoms and it provides relief for uncomfortable symptoms of menopause such as hot flashes and night sweats, reduces bone loss and risk of fractures, and (administered locally) addresses genitourinary syndrome of menopause [1] but has no demonstrable benefit for cognition [2].

Vitex trifolia L. (Verbenaceae) (VT) is a tropical shrub or shrubby plant that is widely distributed in Southeast Asia, Micronesia, Australia, and East Africa [3]. VT has been used as a part of traditional medicine for treatment of a wide range of diseases, including fever, headache, diarrhea, and tuberculosis [4],[5]. Moreover, the results from our previous study revealed that the ethanol extract of the fruits of VT improves learning deficits in ovariectomized mice by using the open field test and Morris water maze task [6]. Interestingly, our recent premilinary data showed that *n*-BuOH extract of VT fruits contains agnuside, which is demonstrated to exert estrogenic activity, with yeild higher than that in ethanol extract (4.05% and 0.37%, respectively). This raises a possibility that VT *n*-BuOH extract might also be beneficial for relieving the uncomfortable symptoms of postmenopause. Therefore, in the present study, we have further evaluated the activity of the *n*-butanol extract from the fruits of VT on the memory function as well as on uterus, bone, and

several serum biochemical markers of OVX mice to explore the potential of VT on treating/preventing cognitive deficit symptoms of postmenopause.

2. Materials and Methods

2.1. Plant materials and extraction

VT was collected in Nghe An, Vietnam and identified by Dr. Pham Thanh Huyen, National Institute of Medicinal Materials (NIMM). A voucher specimen of the herb (no. DL-13720) was deposited in NIMM, Vietnam. VT extract was prepared as follows: 4.0 kg of VT ripe fruits was extracted by reflux extraction processes with 20 L of 70% ethanol, for a period of 2.5 h, then filtered to collect the fluid. The residue was extracted twice more with 70% ethanol at intervals of 2 h and 1.5 h, respectively. The filtrate of 3 extracts was combined, recover ethanol under reduced pressure, then is drained until about 4-5 L remain. This solution was extracted liquid fraction in turn with solvents dichloromethane and *n*-butanol (*n*-BuOH). The *n*-BuOH fraction was then concentrated *in vacuo* at 60 °C to obtain the dried *n*-butanol extract. The yield of the *n*-BuOH from the dried VT was calculated as 3.41% (w/w). The agnuside content in the extract was 4.05% by high-performance liquid chromatographic method.

2.2. Animals

Seven-week-old female *Swiss albino* mice (26-28 g) were purchased from the National Institute of Hygiene and Epidemiology, Hanoi, Vietnam. The animals were habituated to the laboratory animal room for at least 1 week before surgery. Food and water were available *ad libitum*. Housing was thermostatically maintained at 22 ± 1°C and a 12-h light-dark cycle (lights on: 07:00 - 19:00). The behavioral experiments were performed during the light phase from 8:30 to 16:30.

2.3. Surgical operation and treatments

Ovariectomy (OVX) was performed through an abdominal ventral incision under pentobarbital anesthesia (50 mg/kg). The complete surgical removal of the ovaries was corroborated by visual inspection. Sham operation was performed in a similar way without removal of the ovaries. After surgery, females were housed in a community cage with other mice. After 14 days recovery period, the animals were divided into five groups: (1) sham, (2) OVX, (3 and 4) OVX + 30 and 60 mg/kg VT extract (VT 30 and VT 60, respectively), (5) OVX + Progynova® (PRO, estradiol valerate, 0.5 mg/kg). The sham and OVX control groups were oral administered 0.05% Tween 80 for 8 weeks. VT extract and Progynova® were dissolved in 0.05% Tween 80 and oral administered once daily for 8 weeks. After 2 weeks of surgery, all mice received the designed treatment for a week and during the behavioral experiments.

2.4. Behavioral study

2.4.1. Novel Object Recognition Test (ORT):

The ORT relies on rodents' natural proclivity for exploring novelty and is used to test recognition memory [7]. This test was carried out with minor modification on after 3 weeks of treatment. Briefly, 24 hours before the test, mice were individually placed in an examination box (35 × 35 × 50 cm) for 10 minutes. The ORT consisted of two sessions, a 5 min familiarization and a 5 min test that were separated with a 30 min interval. During the familiarization session, two identical objects, object 1 and object 2, were placed separately in the observation box and each mouse was allowed to explore the arena freely for five minutes. During the test session, one of the previously explored objects was replaced with a novel object. The experimental apparatus, including the objects, was cleaned with 70% ethanol to remove odor cues between sessions. The animals' behavior was video-recorded, and the time spent exploring each of the two objects was analyzed using ANY-maze software (ver. 4.99, Stoelting Co., IL, USA). Exploration time was defined by animal nose-point detection within a 2 cm radius around the object.

2.4.2. Modified Y-Maze Test:

Four weeks after treatment, mice were subjected to the modified Y-maze test [8]. The Y-maze apparatus consists of black polypropylene walls with three arms; each arm had a length of 40 cm, a height of 18 cm, a width of 12 cm at the top, and 3 cm at the bottom. Different spatial cues surrounded the maze. The test phase was conducted 30 minutes after the familiarization phase. During the familiarization session, each mouse was individually placed in the maze, where one of the three arms was closed. The mouse was allowed to explore the two arms freely for five minutes. During the test session, all three arms were opened, and the previously closed arm was defined as the novel arm. The animal returned to the maze and allowed to explore the arms freely for five minutes. The entire experimental apparatus was cleaned with 70% ethanol to remove odor cues between sessions. Animal behavior was video-recorded and analyzed using ANY-maze software ver. 4.99 (Stoelting, USA).

2.4.3. Morris Water Maze Test (MWM):

Five weeks after treatment, mice were subjected to the Morris water maze test (MWM). Water maze performance of mice was tested using a 1.1m diameter circular pool according to our previous report with a minor modification [9]. Seven days after the modified Y-maze test, mice were subjected to a hidden platform test for 6 days. Mice received 4 trials each day. Mice were allowed to swim 60 s to find the hidden platform at each trial. When successful, the mice were allowed 10 s to stay on the platform. If the mouse had not found the platform during a 60-s period, it was placed on the platform and was allowed to remain on the platform for 15 s before being removed to a plastic chamber for 60 s. The next trial was then performed. The data for each day were averaged over the 4 trials before being used for statistical analysis. The latency to reach the platform (escape latency), distance covered, and averages of swim speed were recorded to compare the spatial memory and learning ability between groups. One day after the acquisition trials for 6 days, a single 60 s probe trial was run in which the platform was removed from the pool. The amount of time spent in each of the four imaginary quadrants of the pool was video-recorded and analyzed using ANY-maze software ver. 4.99 (Stoelting, USA).

2.5. Analysis of serum and tissues

Eight weeks after treatment, mice were sacrificed by decapitation. Blood samples were collected and the serum was separated by centrifugation at 3500 rpm for 15 min. The serum levels of estradiol (E2 - Cobas E411, Roche, USA) and alkaline phosphatase (ALP - Cobas IFCC, Roche, USA) was measured according to the manufacturer's instructions.

The uterus-oviduct was excised and weighed. The weight of these tissues was represented in grams (g) and mg/100g body weight, respectively.

Equal numbers of uterine tissues (3 per group) were fixed in 10% neutral buffered formalin for 24 h. Specimens were embedded in paraffin and 5 μm sections were prepared and stained with hematoxylin and eosin (H&E). Histopathologic examination was examined under a light microscope. The observation of uterine endometrium thickness and the number of uterine glands was performed on a selected two slides in each animal.

2.5. Statistical analysis

All statistical analyses were conducted using IBM SPSS Statistics version 23.0 software, and data are presented as the mean ± standard error of the mean (S.E.M). One-way analysis of variance (ANOVA) or t-test, where only two conditions were made, were used to examine the significant difference among groups. Differences of $p < 0.05$ were considered significant.

3. Results

3.1. Effect of VT n-BuOH extract on short-term non-spatial memory OVX mice

The ORT were conducted to examine the short-term non-spatial memory of OVX mice. The results from ORT shown that the sham group and Progynova®-treated OVX group significantly spent more time exploring the new object than the familiar one ($p < 0.05$), whereas the vehicle-treated OVX group exhibited no preference for exploring the novel object. These data suggest that OVX causes impairment of short-term non-spatial memory in a manner that can be reversed by the daily administration of Progynova®. Similarly, the daily administration of VT *n*-BuOH extract (30 and 60 mg/kg) increased the time OVX animals spent exploring the novel object (Fig. 1).

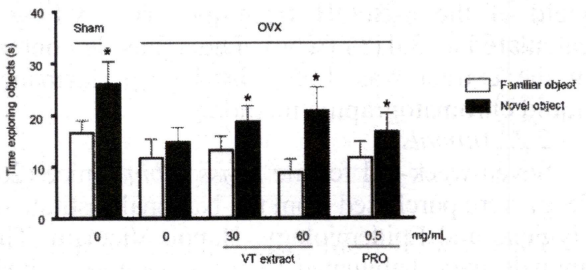

Fig. 1. Treatment of VT *n*-BuOH extract improves object recognition memory in OVX mice. On the day 21 after treatment, ORT was performed to evaluate the effect of VT *n*-BuOH extract (30 and 60 mg/kg) or PRO (estradiol valerate 0.5 mg/kg) on cognitive impairment in OVX mice. Time exploring object of animals from ORT are recorded and analyzed; *n = 10-12/group.* $^{*}p < 0.05$ *versus time spent exploring a familiar object (paired t-test).*

3.2. Effect of VT n-BuOH extract on spatial working memory OVX mice

Using a modified Y-maze test, we examined the effect of *n*-BuOH VT on spatial working memory in OVX animals. As shown in Fig. 2, the vehicle-treated OVX group spent significantly less time in the novel arm than that of the sham group, indicating an impairment of spatial working memory. The administration of VT *n*-BuOH extract (30 and 60 mg/kg) significantly attenuated OVX-induced deficits in spatial working memory in a dose-dependent manner. Also, PRO treatment (0.5 mg/kg) significantly ameliorated the impairment of spatial working memory of OVX mice (Fig. 2).

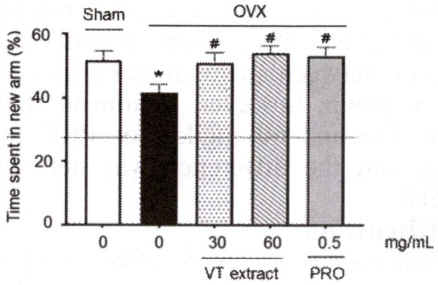

Fig. 2. Effects of VT *n*-BuOH extract on OVX-induced spatial working memory deficit. The modified Y-maze test were conducted at days 28 after treatment. % time animals spent exploring the novel arm in the test trial of modified Y-maze test are recorded and analyzed; *n = 10-12/group.* $^{*}p < 0.05$ *versus sham group,* $^{\#}p < 0.05$ *versus vehicle-treated OVX group.*

3.3. Effect of VT n-BuOH extract on long-term memory of OVX mice

Table 1 and Fig. 3 shows the effects VT *n*-BuOH extract and Progynova® on water maze performance of OVX mice.

Table 1. Time to the platform of OVX mice in MWM test

Group	Time to the platform (s)					
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Sham	44.0 ± 6.0	30.7 ± 2.9	30.8 ± 4.5	33.0 ± 3.6	20.3 ± 2.6	19.0 ± 2.4
OVX	44.5 ± 5.6	35.8 ± 4.0	29.7 ± 4.6	23.5 ± 3.3	22.1 ± 2.5	29.4 ± 2.6*
OVX + VT (30 mg/kg)	48.6 ± 4.6	33.6 ± 3.4	27.1 ± 4.2	23.0 ± 4.9	24.5 ± 4.1	22.3 ± 3.1
OVX + VT (60 mg/kg)	39.2 ± 5.9	35.9 ± 4.1	24.6 ± 5.2	26.6 ± 3.9	21.1 ± 2.5	20.8 ± 2.9#
OVX + PRO (0.5 mg/kg)	43.4 ± 1.1	33.7 ± 5.8	21.7 ± 5.5	22.4 ± 3.3	22.2 ± 6.3	20.6 ± 3.1#

$^{*}p < 0.05$ versus sham group, $^{\#}p < 0.05$ versus vehicle-treated OVX group. (n = 10-12/group).

The escape latencies of OVX control were also reduced by training, but this animal group needed significantly longer time to find the hidden platform than the sham group ($p < 0.05$ at day 6). The escape latencies of OVX-VT at dose of 60 mg/kg, but not 30 mg/kg, and OVX-PRO mice were also shortened from the day by day practice ($p < 0.05$ at day 6) (Table 1).

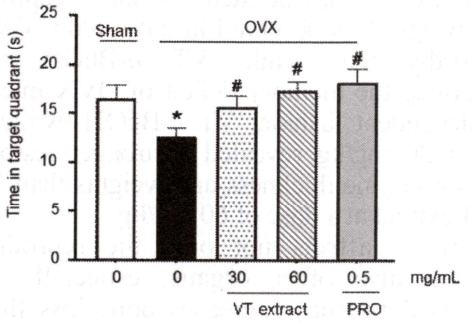

Fig. 3. VT *n*-BuOH extract prevent OVX-induced cognitive impairment of water maze performance in mice. *n = 10-12/group.* $^{*}p < 0.05$ *versus sham group,* $^{\#}p < 0.05$ *versus vehicle-treated OVX group.*

In the probe test conducted on the day after 6 days of training (Fig.3), swimming time of the OVX control mice in the target quadrant where the platform had been located during training was significantly shorter than that of the Sham group ($p < 0.05$). Treatment of OVX mice with Progynova® or VT *n*-BuOH extract (30 and 60 mg/kg) significantly prolonged swimming time in the target quadrant ($p < 0.05$).

Taken together, the results obtained from the ORT, modified Y-maze, and MWM test clearly demonstrated that treatment of VT *n*-BuOH extract ameliorated cognitive deficits in OVX mice.

3.4. Effect of VT n-BuOH extract on uterus of OVX mice

The effects of VT *n*-BuOH extract on weight and histology of the uterus in OVX mice were assessed. The uterus-oviduct weight of the vehicle-treated OVX group was significantly less than that of the sham group ($p < 0.05$). PRO treatment markedly increased the weight of the uterus compared to the OVX group ($p < 0.05$). Similarly, administration of VT *n*-BuOH extract (30 and 60 mg/kg) significantly reversed the OVX-induced uterus decrease (Table 2).

Table 2. Effect of VT *n*-BuOH extract on uterus-oviduct weights of OVX mice

Group	Uterus-oviduct weights (mg%)	% increasing
Sham	60.74 ± 7.37	-
OVX	15.30 ± 1.03 ***	-
OVX + VT (30 mg/kg)	27.69 ± 3.13##	80.93
OVX + VT (60 mg/kg)	19.08 ± 1.30#	24.68
OVX + PRO (0.5 mg/kg)	109.79 ± 19.95##	617.46

$^{***}p < 0.001$ versus sham group, $^{\#, \#\#}p < 0.05; 0.01$ versus vehicle-treated OVX group. (n = 10-12/group).

Histological analysis of uterine sections also showed atrophy in the uterus in OVX mice compared to those in the sham group, evidenced by the significant reduction in the thickness of the uterus. PRO treatment dramatically increased the thickness of the uterus in OVX mice. Similarly, VT *n*-BuOH extract (30 and 60 mg/kg) ameliorated OVX-induced uterine atrophy. Intriguingly, VT *n*-BuOH extract at a dose of 30 mg/kg revealed a stronger restoration effect on uterine thickness than VT *n*-BuOH extract at a dose of 60 mg/kg (Fig. 4B).

In addition, the endometrium of the uterus in OVX mice was composed of single-layered columnar epithelial cells, and mitotic activity was not visualized in these cells. The number of glands of the uterus in OVX mice was also lower than that in the sham group. Administration of PRO and VT *n*-BuOH extract stimulated endometrial

epithelium to become multilayered as well as enhanced the mitotic activity of endometrial cells and increased the number of glands in the uterus (Fig. 4A). Treatment of VT *n*-BuOH extract substantially restored the OVX-induced atrophy of the uterus, suggesting that VT *n*-BuOH extract possess estrogenic activity in the uterus. Notably, VT *n*-BuOH extract at a dose of 30 mg/kg showed a more powerful effect on uterus atrophy of OVX mice than a dose of 60 mg/kg.

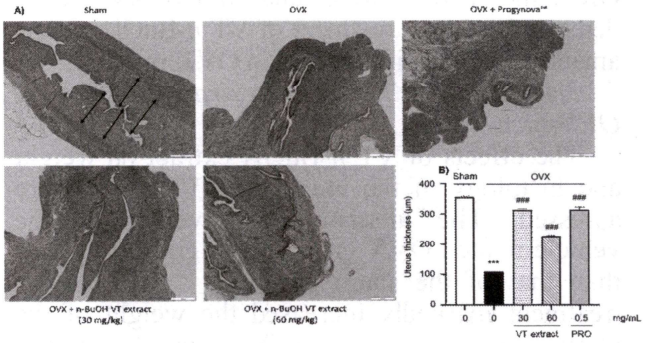

Fig. 4. The effect of VT *n*-BuOH extract on uterus in OVX mice. After 8 weeks treatment, mice were sacrificed to collect uterus. The uterus was subjected to histological analysis to visualize to morphology of uterus (A) and measure uterus thickness (B) and; n = 3/group. $^{***}p < 0.001$ compared with sham group, $^{###}p < 0.001$ compared with vehicle-treated OVX group. Black arrow indicates uterine thickness.

3.5. Effect of VT n-BuOH extract on serum biochemical markers

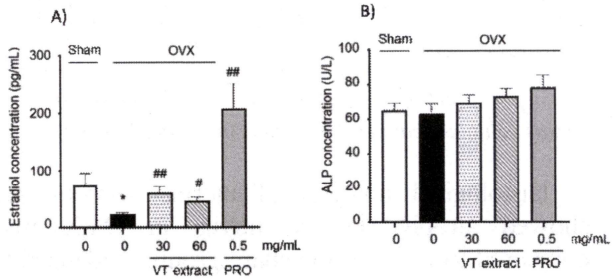

Fig. 5. The effect of VT *n*-BuOH extract on serum biochemical markers of OVX mice. (A) Serum levels of estradiol (E2) and (B) alkaline phosphatase (ALP); n = 10 - 12/group. $^{*}p < 0.05$ compared with sham group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with vehicle-treated OVX group.

Several serum biochemical markers, including estradiol and alkaline phosphatase (ALP) were measured. The serum level of estradiol in the OVX group was significantly lower than that in the sham group. The administration of PRO markedly reversed the OVX-induced decrease in serum estradiol concentration. Importantly, supplementation with 30 and 60 mg/kg VT *n*-BuOH extract significantly increased the level of estradiol in OVX mice (Fig. 5A). The results of ALP revealed that there is no significant difference between sham animal group compared to OVX group; however, treatment of both VT extracts (30 and 60 mg/kg) or PRO tended to increase but the difference was not significant (Fig. 5B).

4. Discussion

Postmenopausal women suffer a wide range of cognitive symptoms due to estrogen deficiency, including anxiety and depression, memory impairment [10]. The ovariectomy rodent model, in which ovaries are surgically removed resulting in depletion of the female hormone estrogen, has often been used as the model of cognitive decline, anxiety following ovarian hormone deprivation [11],[12]. Using this model, we investigated the potential of VT *n*-BuOH extract on improving symptoms of postmenopause. Our data revealed that VT *n*-BuOH extract significantly improved OVX-induced cognitive decline, evidenced by increasing time spent in new arms in the modified Y-maze test and time spent in novel object in ORT as well as time in the target quadrant in Morris water test. It is noteworthy that the results from our previous study demonstrated that the ethanol extract of the fruits of VT possesses learning deficits in OVX mice by using Morris water maze task [6].

Diminished estrogen also affects the reproductive tract, such as uterine and vaginal tissues, and causes atrophy in these organs. Previous studies have reported that uterine weight loss is related to the reduction in uterine epithelial height, uterine myometrial thickness, and uterine stromal expansion [13]. In the present study, we observed uterine atrophy in OVX mice, clearly demonstrating estrogen insufficiency. Indeed, the weight and thickness of the uterus of the vehicle-treated OVX group were significantly less than those of the sham group, confirming the successful OVX model. Importantly, administration of VT *n*-BuOH extract ameliorated uterine atrophy and tissue weight loss observed in untreated OVX. It is noteworthy that while VT *n*-BuOH extract ameliorated the memory deficit of OVX mice in a dose-dependent fashion, VT *n*-BuOH extract at a dose of 30 mg/kg revealed a stronger restoration effect on uterine thickness and weights than VT *n*-BuOH extract at a dose of 60 mg/kg.

Estrogen affects not only the reproductive tract but also other organs, especially bone. Estrogen deficiency increases bone loss through the activation of osteoclast differentiation and leads to a higher incidence of osteoporosis in postmenopausal women [14]. Therefore, we analyzed the ALP concentration. Treatment of

Progynova® and VT *n*-BuOH extract tended to increase the bone formation marker ALP level in, suggesting that both VT *n*-BuOH extract and Progynova® might beneficial for preventing osteoporosis in OVX mice.

We hypothesize that VT *n*-BuOH extract improves memory function and increased uterine weight *via* up-regulating biosynthesis of estrogens. To test this hypothesis, serum level of estrogen was measured. Indeed, the serum level of estradiol in the OVX group was significantly lower than that in the sham group. Expectedly, the administration both of Progynova™ and VT *n*-BuOH extract significantly reversed the OVX-induced decrease in serum estradiol concentration. As estrogens are synthesized majorly in the ovary or testis and adrenal gland, and fat cells with small amounts, our data suggest that VT *n*-BuOH extract promoted the release of estrogens from other tissues [15].

It is also noteworthy that a recent phytochemical study has reported that the fruits of VT compose terpenoids, flavonoids, and polyphenols, which are similar to the components of other species of the genus *Vitex.* such as *V.agnus-castus* and *V.rotundifolia* [16]. Intriguingly, both *V.agnus-castus* and *V.rotundifolia* have been demonstrated to exert estrogenic activity. Indeed, previous *in vitro* studies reported that the methanol extract of fruits of *V.agnus-castus* significantly induces the transcription of estrogen-responsive genes such as *progesterone receptor* and *presenelin-2* in Ishikawa cells [17],[18]. Moreover, an *in vivo* study showed that the ethanol extract of fruits of this plant markedly increases the uterine weight, plasma progesterone, and total estrogen levels of ovariectomized female rats [19]. Clinical studies have also demonstrated that *V.agnus-castus* supplement might be beneficial for women suffering from menopausal problems [20]. Similarly, *V. rotundifolia* has been also demonstrated to exert estrogenic activity due to its stimulating effect on the proliferation of MCF-7 cells as well as up-regulation of ER-dependent genes. Besides, agnuside, an iridoid glycoside isolated from VT and also found in *V. rotundifolia* and *V. agnus castus*, has been demonstrated to exert estrogenic activity in MCF-7 cells, evidenced by stimulating the proliferation and up-regulating the levels of *PR* and *pS2* mRNA in this cell line [21].

5. Conclusion

In summary, the present study demonstrated that *Vitex trifolia* L. *n*-BuOH extract improved cognitive deficit and ameliorated the atrophy of the uterus in OVX mice by stimulating the biosynthesis of estrogen in circulation. These data not merely confirmed but also provided supportive information about the estrogenic property of VT. Therefore, VT might be beneficial for treating or preventing symptoms of postmenopausal cognitive deficits.

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ANTI-INFLAMMATORY EFFECTS OF ETHANOL EXTRACT FROM *HOMALOMENA PIERREANA* RHIZOME

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Summary

Anti-Inflammatory Effects of Ethanol Extract from *Homalomena pierreana* Rhizome

Homalomena has been used in traditional medicine as pain relief in rheumatic diseases. *Homalomena pierreana* (HP) is a rare species of the Araceae family found in Vietnam. This study aimed to investigate the anti-inflammatory effects of ethanol extract from HP rhizome by using lipopolysaccharide (LPS)-induced nitric oxide (NO) production in murine macrophage cell line RAW 264.7 and a carrageenan-induced paw edema test in mice. The results revealed that HP extract inhibited NO production (up to 68.7%) at 100 µg/mL in a concentration-dependent manner in LPS-stimulated RAW 264.7 macrophages. The NO production inhibitory activity of HP extract was equivalent to a positive control, dexamethasone, at the concentration of 50 µg/mL. Furthermore, the extract at doses of 390 mg/kg and 780 mg/kg (equivalent to 1.25 and 2.5 g raw materials) attenuated 25.9% to 35.6% of paw inflammation after 3 hours of carrageenan injection as compared to control. In conclusion, this is a first report to determine the anti-inflammatory effects of ethanol extract from HP rhizome. The follow-up studies should aim at elucidating the anti-inflammatory mechanisms of HP rhizome to achieve beneficial effects of this plant for medicine usages.

Keywords: *Homalomena pierreana, Lipopolysaccharide-induced NO production, RAW 264.7 macrophages, Carrageenan-induced mouse paw edema.*

1. Introduction

Homalomena has been used in traditional medicine as a bone tonic, rheumatic remedy and pain relief [1]. *Homalomena pierreana* Engl. (HP) [Vietnamese name: Than phuc, another scientific name: *Homalomena griffithii* (Schott) Hook.f.] is a rare species of Araceae family in Vietnam with little research on chemical composition till now. HP has been reported distributed in Bach Ma National Park (Thua Thien-Hue province) and southern Vietnam located in Phu Quoc National Park (Kien Giang province) and identified by DNA barcode sequence (trnL intron and trnL-trnF IGS sequences) [2],[3]. Successful clonal propagation of HP has made this species more attractive to study for their chemical composition as well as potential for medicine usages [4].

Nonetheless, there are no exclusive scientific investigations which have been carried out to search for anti-rheumatic activity of this plant. As rheumatoid arthritis is the most common form of inflammatory arthritis, medications that suppress joint inflammation such as non-steroidal anti-inflammatory drugs and glucocorticoids are frequently used as first-line therapeutic agents for treatment of the symptoms. Therefore, this study has been designed to explore *in vitro* and *in vivo* anti-inflammatory effects of HP in order to provide a scientific evidence for benefit use of this plant.

2. Materials and Methods

2.1. Plant materials and extraction

HP rhizome was collected in Phu Quoc National Park, Kien Giang province on December, 2022. The plant was identified and scientifically named by Mr. Cao Ngọc Giang of the Research Center of Ginseng and Medicinal Materials in Ho Chi Minh City (sample code: ĐD 042023). Dried powdered material was extracted (The ratio is 1: 15 w/v) with 45% ethanol at room temperature for 48 h in a percolator apparatus. The extract was collected at a rate of 1 mL/min and concentrated using a rotary evaporator at 60°C under reduced pressure to obtain crude condensed extract (HP extract) with moisture content of 18.1%. The yield of extraction was 31%. HP extract at oral doses of 390 mg/kg and 780 mg/kg (equivalent to 1.25 and 2.5 g raw materials) were selected for *in vivo* study.

2.2. Animals

Six-week-old male *Swiss albino* mice (25 ± 2 g) were purchased from the Institute of Vaccines and Medical Biologicals, Nha Trang, Vietnam.