ANTI-INFLAMMATORY EFFECTS OF THE EXTRACTS FROM *ARDISIA SILVESTRIS* PITARD LEAVES IN EXPERIMENT

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This research aimed to explore the anti-inflammatory effects of n-hexane, ethyl acetate (EtOAc), and n-butanol extracts from the leaves of Ardisia silvestris Pitard, both in vitro and in vivo, specifically focusing on the EtOAc extract. The in vitro assessment of anti-inflammatory activity involved measuring the inhibition of nitric oxide (NO) production in LPS-stimulated RAW 264.7 macrophage cells. The findings revealed that the EtOAc extract exhibited the highest potency, with an IC50 value of 52.10 ± 2.07 µg/mL, effectively inhibiting NO production without causing cellular damage. In vivo, the EtOAc extract's anti-inflammatory properties were evaluated using carrageenan-induced paw edema in rats and the cotton pellet-induced granuloma test in mice. At both tested dosages, the EtOAc extract significantly reduced paw edema induced by carrageenan in rats. Additionally, it effectively inhibited exudate and granuloma formation following cotton pellet implantation at both doses, indicating its anti-inflammatory activity during the subacute phase of inflammation. Overall, these results strongly suggest that the ethyl acetate extract of Ardisia silvestris Pitard possesses significant anti-inflammatory properties and may be beneficial in traditionally treating various inflammatory conditions.

Keywords: Anti-inflammatory, RAW 264.7, carrageenan, cotton pellet, *Ardisia silvestris*.

I. INTRODUCTION

Elevated concentrations of free radicals, specifically reactive oxygen species (ROS) and reactive nitrogen species (RNS), play a significant role in inflammatory processes, typically arising from oxidative stress.[1,2] Nitric oxide (NO) is produced by nitric oxide synthase (NOS) enzymes during inflammatory responses, with its levels often heightened as a component of the immune reaction. Additionally, an imbalance characterized by an overabundance of free radicals and a deficiency of antioxidants can lead to oxidative stress.[3] In such oxidative stress conditions, NO may engage with free radicals, resulting in complex reactions, including peroxynitrite (ONOO-) formation, which contributes to inflammatory processes.[4]

When inflammation becomes excessive, it can lead to significant health issues such as infections, cardiovascular diseases, cancer, and diabetes. While synthetic medications can address various ailments, they often come with serious side effects for both humans and animals. The adverse effects associated with chemical drugs are well established, including allergic reactions, teratogenic effects, and long-term genetic damage that heightens the risk of cancer development.[5] Consequently, many individuals turn to herbal remedies each year, believing that these natural treatments are

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devoid of harmful side effects. According to the World Health Organization, approximately 80% of people in developing nations depend on traditional medicine for their primary healthcare, with around 85% of these practices involving plant extracts. This indicates that roughly 3.5 to 4 billion individuals worldwide utilize plants as sources of medication.[6,7] However, the absence of systematic research indicates that most herbal practices globally, including those in Vietnam, remain unsupported by full scientific evidence.

Ardisia silvestris Pitard, commonly referred to as "khoi tia" in Vietnamese, is a plant species predominantly located in Southeast Asia, especially in Vietnam. It is well-known for its traditional medicinal applications, with research indicating its potential antioxidant, antibacterial, and anti-photoaging effects attributed to the compounds found in its leaves and extracts.[8,9] Up to now, the knowledge surrounding the efficacy of *A. silvestris* leaf extract (ALE), particularly concerning its anti-inflammatory properties, has been largely based on traditional medicine and historical practices. Therefore, this study aims to assess the anti-inflammatory effects of ALE both *in vitro* and *in vivo*, compared to a standard pharmaceutical treatment.

II. MATERIALS AND METHODS

1. Subjects

Plant collection and extraction

Ardisia silvestris Pitard leaves were collected in Lai Chau province, Vietnam, in October 2024 and identified by botanist Dr. Ngo Xuan Phuong at the Vietnam Institute of Traditional Medicine. A voucher specimen (AG-OIL) was deposited at the Faculty of Pharmacy, University of Medicine and Pharmacy, Vietnam National University, Hanoi.

Briefly, dried *A. silvestris* leaves (5.0kg) were macerated four times with 10.0L of ethanol overnight to give the EtOH extract. After partitioning with organic solvents, the following extracts were obtained: n-hexane (50.22g), ethyl acetate (EtOAc) (187.07g), n-butanol (87.31g), and aqueous (500.14g) fractions.

Chemicals and reagents

Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were sourced from Life Technologies, Inc. (Gaithersburg, MD, USA). Lipopolysaccharides (LPS), sodium nitrite, sulfanilamide, *N*-1-naphthyl ethylenediamine dihydrochloride, and dimethyl sulfoxide (DMSO), carrageenan were obtained from Sigma Aldrich (St. Louis, MO, USA). Prednisolone and diclofenac were of the highest commercially available grade.

Animals

Male *Sprague Dawley* rats (180 - 200g) and male *Swiss* albino mice (18 - 25g) were obtained from the Laboratory Animal of Pharmacology Department, Hanoi University of Pharmacy, Vietnam. The animals were kept in polyacrylic cages containing six animals per cage and maintained under standard housing conditions (room temperature 24 - 27°C and humidity 60 - 65%) with a 12-h light and dark cycle. Food, in the form of dry pellets, and water were available ad libitum. Animals were acclimatized at least 1 week before the experiments were started.

2. Methods

In vitro anti-inflammatory assay

The anti-inflammatory property of ALE *in vitro* was examined by nitric oxide assay.[10] Complete DMEM media supplemented with 10% v/v FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin was utilized to culture RAW 264.7 macrophage cells. The cultures were maintained in a humidified incubator at 5% CO_2 and 37°C, with the culture media being replaced daily. Cells were plated at a density

of 2 × 10^5 cells per well in a 96-well microtiter plate. Following overnight incubation, LPS (1 µg/mL) was introduced, either alone or in combination with various extracts from *A. silvestris*, which were serially diluted from 2.65 to 100 µg/mL, and the incubation continued for an additional 24 hours. Subsequently, 100 µL of Griess reagent was mixed with 100µL of the cell culture supernatant. This mixture was allowed to incubate at room temperature for 15 minutes, after which the absorbance was measured at 540nm using a BioTek Eko800 spectrometer. The IC50 value, representing 50% inhibition of NO production, was calculated using TableCurve 2D4 software. Dexamethasone served as a positive control, while untreated cells were designated as the blank. Cells treated with LPS alone, without any extracts, were classified as the negative control group.

In vivo anti-inflammatory assay

Carrageenan-induced paw edema in rats

The anti-inflammatory effect was evaluated by carrageenan-induced rat paw edema as described by Winter et al.[11] Animals that had been fasted overnight were categorized into five groups (n = 10 each). Group I was administered a vehicle (distilled water orally) and served as the control group; Group II received diclofenac at a dosage of 10 mg/kg orally and was designated as the positive control. Groups III and IV were given *A. silvestris* EtOAc extracts at doses of 1.2 g/kg and 3.6 g/kg, respectively, also orally. One hour after the extract or vehicle administration, paw edema was induced by subplantar injection of 0.1 ml of 1% carrageenan (dissolved in normal saline) into the left hind paw of each animal. The measurement of paw edema was conducted using a digital plethysmometer (Ugo Basile, Italy) at baseline (before carrageenan administration) and at 1, 2, 3, and 4 hours after carrageenan administration.

Edema was expressed as the increase in paw volume (mL) after carrageenan injection relative to the preinjection value for each animal. The degree of swelling was evaluated by measuring the increase in paw volume.

The increase in paw volume was calculated using the following formula:

Increase paw volume (%) = [(Ct − C0) / C0] × 100

(Where: Ct = thickness of paw after carrageenan injection; C0 = thickness of paw before carrageenan injection).

Cotton pellet-induced granuloma formation in mice

The cotton pellet-induced granuloma in mice was evaluated as specified by Kumar R et al. (2016).[12] A total of thirty-six *Swiss* mice participated in the study, which were categorized into four groups, each consisting of nine mice. The first group acted as the vehicle control and was administered distilled water orally. The second group received prednisolone at a dosage of 2.5 mg/kg orally, serving as the standard treatment. Groups three and four were given EtOAc extract of *A. silvestris* leaves at doses of 2.4 g/kg and 7.2 g/kg body weight orally, respectively.

Thirty minutes following the initial administration of the drug or vehicle, the animals were anesthetized. A sterile cotton pellet weighing 20 ± 2mg, saturated with normal saline, was then implanted subcutaneously on both sides beneath the axilla of the neck. Treatment with the drug or vehicle continued for an additional six days. On the eighth day, the animals were anesthetized again, and the pellets were meticulously extracted, ensuring they were free from any surrounding tissues.

The pellets were initially weighed to determine their wet weight and subsequently placed in an incubator at 60ºC for 24 hours

to achieve a stable dry weight, ensuring that all exudates were thoroughly dried. Following this process, the dried pellets were weighed again. The weight of the exudate, measured in milligrams, was determined by subtracting the constant dry weight of the pellet from its initial wet weight. The formation of granulation tissue, represented by the dry weight of the granuloma, was calculated by subtracting the weight of a cotton pellet (20mg) from the constant dry weight of the pellet, serving as an indicator of granuloma tissue development.

The percent inhibitions of exudate and granuloma tissue formation were determined using the following formular:

Exudate inhibition (%) = $(1 – WE_t/WE_c) \times 100$

Granuloma inhibition (%) = $(1\text{-}WG_t/WG_c) \times 100$.

Where: WEt, WEc: Weight of exudate in mg of the treated and control groups, respectively; WGt, WGc: Dry weight of granuloma in mg of the treated and control group, respectively.

Statistical analysis

The data are expressed as mean ± standard deviation. The statistical analysis was performed using GraphPad Prism 9.3.1 (San Diego, CA, USA). $p < 0.05$ was considered significant.

III. RESULTS

1. Nitric oxide assay

Table 1. IC_{50} values for the inhibition of NO production of the different extracts

Extracts	IC_{50}	% Cell viability at 100 µg/mL
n-Hexane	NA	18.21 ± 2.04
EtOAc	52.10 ± 2.07	81.58 ± 1.91
n-BuOH	> 100	77.42 ± 3.21
Dexamethasone*	12.48 ± 1.17	93.98 ± 0.12

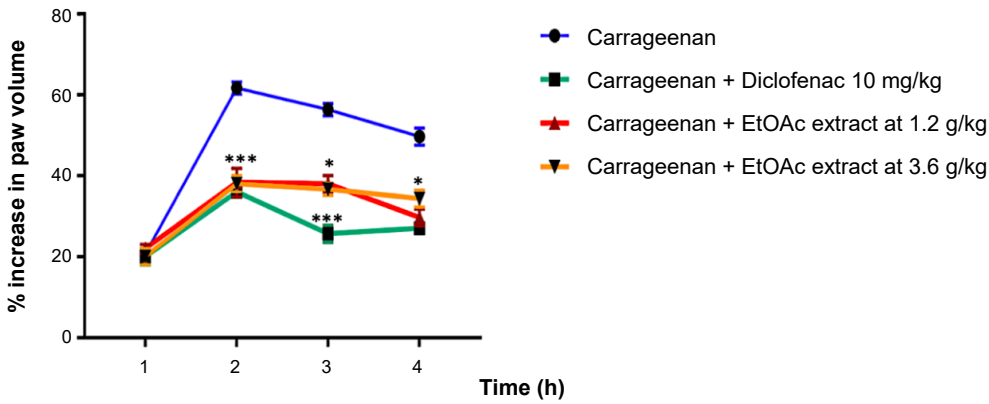
**Data were presented as the mean ± SD (n = 3); *Positive control (µM); Extracts (µg/mL); NA: Not available due to cell death*

The impact of various leaf extracts from *A. silvestris* on nitric oxide (NO) production in LPS-stimulated RAW 267.4 cells is summarized in Table 1. The EtOAc extract demonstrated significant inhibitory effects on NO generation, with an IC_{50} value of 52.10 ± 2.07 µg/mL, while maintaining a cell survival rate exceeding 80%, indicating no cytotoxicity. Conversely, the n-hexane extract exhibited detrimental effects on cell viability, resulting in less than 80% survival of RAW 264.7 cells. Consequently, the EtOAc extract from *A. silvestris* leaves was selected for further investigation of its anti-inflammatory properties *in vivo*.

2. Carrageenan-Induced Paw Edema Test

The administration of carrageenan resulted in a sustained increase in paw edema throughout the observation period. The peak edema was recorded at 2 hours following carrageenan administration in all subjects (Chart 1). The standard treatment, diclofenac, demonstrated a notable reduction in paw edema at 2, 3, and 4 hours after carrageenan administration. Additionally, the extract-treated groups exhibited a decreasing trend in paw edema compared to the control group, with this reduction being statistically significant across all tested extract doses starting from 2 hours

post-carrageenan administration. The edema inhibition observed with the EtOAc extract (1.2 and 3.6 g/kg, p.o.) at 4 hours post-induction was comparable to that achieved with diclofenac (10 mg/kg, p.o.).



***p < 0.01; ***p < 0.001 as a significant difference compared with the control group (Group I)*

Chart 1. Effect of EtOAc extract of *A. silvestris* leaves on the carrageenan-induced paw edema test

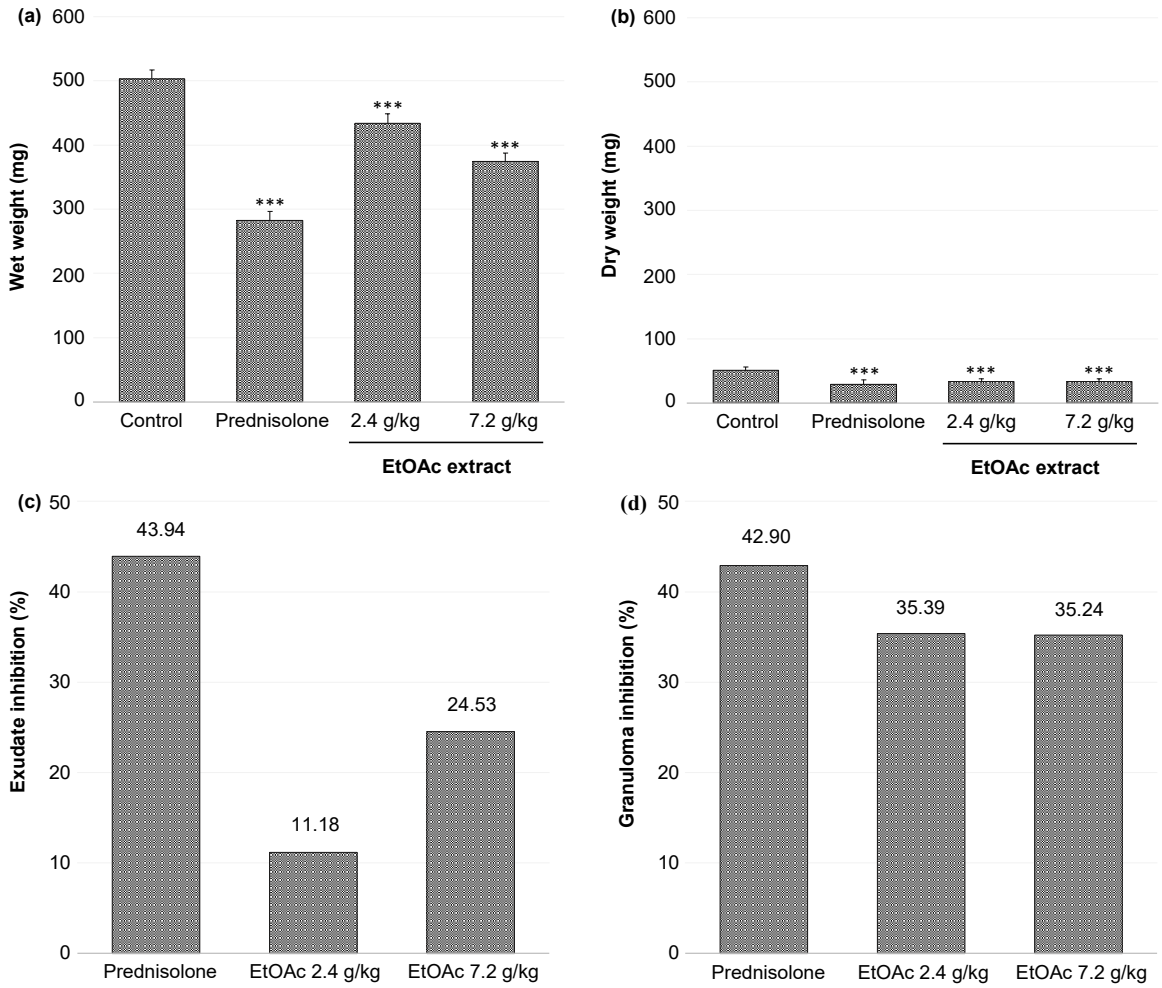
3. Cotton Pellet-Induced Granuloma Test

The plant extract, along with the reference medication prednisolone, exhibited significant reductions in the wet and dry weights of granulomas (see Charts 2a and 2b). Compared to prednisolone, the plant extract showed a less marked effect on granuloma development. Specifically, the wet and dry weights of granulomas were diminished by 13.64% and 35.39% ($p < 0.001$), and 25.62% and 35.24% ($p < 0.001$), respectively, at doses of 2.4 and 7.2 mg/kg of the EtOAc extract, whereas prednisolone (at 2.5 mg/kg) resulted in a more substantial inhibition of 43.83% and 42.90% ($p < 0.001$).

As illustrated in Charts 2c and 2d, prednisolone exhibited the most significant efficacy in inhibiting exudate and granuloma formation across all experimental groups. The EtOAc extract at doses of 2.4 and 7.2 g/kg (administered orally) significantly ($p < 0.001$) reduced both exudate and granuloma formation compared to the vehicle control. The higher dose of the extract resulted in a greater reduction in exudate (24.53%) compared to the lower dose (11.18%) ($p < 0.001$). However, no significant difference was observed in granuloma inhibition between the groups treated with the extract.

IV. DISCUSSION

Nonsteroidal anti-inflammatory drugs (NSAIDs) have traditionally been the preferred option for the rapid management of inflammatory disorders. Nevertheless, their regular consumption can lead to significant adverse effects, leading researchers, traditional medicine practitioners, and patients to explore alternative treatments using herbs, rhizomes, and wild plants known for their anti-inflammatory properties. This study examined the impact of ethyl acetate extract from the leaves of *A. silvestris* on paw edema induced by carrageenan. The findings revealed that the plant extract (administered at doses of 1.2 and 3.6 g/kg orally) markedly reduced the carrageenan-induced paw edema, suggesting a potential inhibition of the release and/or production of inflammatory mediators during



**Data were presented as the mean ± SD (n = 9); ***p < 0.001 as a significant difference compared with the vehicle control (Group I)*

Chart 2. Effects of EtOAc extract of *A. silvestris* leaves on the granuloma wet (a) and dry (b) weight, and exudate (c) and granuloma (d) inhibition

the acute phase of inflammation.

The carrageenan-induced paw edema model is commonly utilized to assess the acute anti-inflammatory effects of new compounds due to its excellent reproducibility. This model exhibits a biphasic response: the initial phase, occurring from 0 to 2 hours post-carrageenan injection, is characterized by the release of histamine, serotonin, and 5-hydroxytryptamine. The subsequent phase, from 3 to 5 hours after injection, involves bradykinin-mediated release of kinins and prostaglandin-like substances, along with an increase in cyclooxygenase activity.[13] In our research, the administration of EtOAc extract demonstrated a significant reduction in paw edema starting at 2 hours, comparable to the effects of diclofenac (10 mg/kg, p.o), which served as the control drug. These findings suggest that the EtOAc extract may influence COX enzymes or the synthesis of prostaglandins from arachidonic acid, operating through a mechanism akin to that of diclofenac.

The onset of edema during carrageenan-induced acute inflammation is linked to the

activation of various nitric oxide synthase (NOS) isoforms, including endothelial, neuronal, and inducible NOS. The soluble nitric oxide (NO) present in the bloodstream serves as a strong vasodilator and, when combined with NOS, contributes to tissue damage and heightened pain sensitivity.[14] Notably, the EtOAc extract has demonstrated the ability to inhibit NO production in LPS-stimulated RAW 264.7 cells *in vitro* without exhibiting cytotoxic effects. Our findings indicate that the EtOAc extract significantly reduces paw edema in the carrageenan-induced paw edema model, implying its potential to suppress NOS expression *in vivo*. Further investigation into the impact of the EtOAc extract on different NOS isoforms *in vivo* is advisable to substantiate these observations.

The inflammatory granuloma is a characteristic aspect of subacute inflammatory responses.[15,16] The cotton pellet granuloma technique has been extensively utilized to evaluate the transudative, exudative, and proliferative phases of subacute inflammation. The amount of fluid absorbed by the pellet significantly affects the wet weight of the granuloma, while the dry weight is closely associated with the quantity of granulomatous tissue developed.[16,17] Steroidal medications demonstrate a marked reduction in granuloma formation. In this study, various doses of a test extract were found to significantly inhibit the wet weight of the granuloma in a dose-dependent manner, indicating that the compounds may reduce vascular permeability. Furthermore, when examining the dry weight of the granuloma, which reflects the impact of the test substances on the proliferative phase of inflammation, it was observed that two doses of the extract effectively inhibited granuloma formation. Based on the findings of our study, we propose that the anti-granuloma properties of the EtOAc extract may be linked to its inhibitory effects on macrophage activation, infiltration, and aggregation.

V. CONCLUSION

The ethyl acetate extract derived from the leaves of *Ardisia silvestris* Pitard (p.o.) demonstrated notable anti-inflammatory properties in the carrageenan-induced paw edema model, likely by modulating the activities of COX and NOS during both the initial and subsequent phases of the test. The outcomes of our *in vivo* anti-inflammatory investigation align with the findings from the *in vitro* nitric oxide assay. Furthermore, the plant extract, across all administered doses, significantly inhibited the subacute phase of inflammation, as evidenced by results from the cotton pellet-induced granuloma method. These results support the traditional application of “khôi tía” in treating acute and subacute inflammatory conditions.

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