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PHYTOCHEMICAL CHARACTERIZATION AND BIOACTIVITY EVALUATION OF *ARDISIA SYLVESTRIS* LEAVES FROM LAM BINH MOUNTAINOUS DISTRICT, TUYEN QUANG PROVINCE

Nguyen Trung Thanh[1], Dang Hong Duyen[1], Pham Thi Thu Trang[1], Nguyen Thi Thu Minh[1], Nguyen Thi Hai[2], Hua Van Luong[3], Chu Quynh Mai[4], Doan Thi Phuong Ly[4], Nguyen Thi Minh Hue[4], Tran Thi Thanh Van[4], Ngo Thi Thuy Ngan[5], Nguyen Thi Thu Thuy[6], Nguyen Tien Dat[1,*], Do Hoang Giang[1,*]

[1]*Center for Research and Technology Transfer, Vietnam Academy of Science and Technology;*
[2]*Ministry of Education and Training, Vietnam;*
[3]*Lam Binh Forest Protection Office, Tuyen Quang Province;* [4]*Tan Trao University, Vietnam;*
[5]*Department of Fundamental Science, Thai Nguyen University of Medicine and Pharmacy, Vietnam;*
[6]*Joint Vietnam-Russia Tropical Science and Technology Research Center, Vietnam*
*Corresponding author: ntdat@ctctt.vast.vn or dhgiang@ctctt.vast.vn

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Summary

Phytochemical Characterization and Bioactivity Evaluation of *Ardisia sylvestris* Leaves from Lam Binh Mountainous District, Tuyen Quang Province

Ardisia sylvestris Pitard is a common herb which has been used widely for abdominal pain treatment. In this study, several chemical constituents, the antioxidant and anti-inflammatory activities of *A. sylvestris* leaves collected from the Lam Binh mountainous district, Tuyen Quang province, have been evaluated and compared to those of the samples from the domestic traditional medicinal market. The results indicated that the total phenolic contents of *A. sylvestris* samples from the Lam Binh district ranged from 12.61 to 18.96 mg GAE/g, while their total flavonoid contents varied between 7.85 and 13.27 mg QE/g. These were significantly higher than the contents of phenolic and flavonoid compounds of the marketed samples. Total alkaloid and tannin contents of *A. sylvestris* leaves collected from the Lam Binh were 1.39 - 3.94 mg/g and 2.54 - 3.94%, respectively, which were equal to those of the marketed samples. Moreover, extracts of *A. sylvestris* leaves from Lam Binh districts exhibited considerable DPPH radical-scavenging activity with IC_{50} from 22.41 to 41.13 µg/mL, which were mostly higher than those of the positive control (ascorbic acid) and the market samples. Whereas, all samples demonstrated weak NO production inhibition without cytotoxicity in LPS-stimulated RAW264.7 macrophage cells. These were promising results for further investigation and cultivation of *A. sylvestris* in mountainous regions in the Lam Binh district as well as the other northern mountainous areas.

Keywords: *Ardisia sylvestris, Antioxidant, Anti-inflammation.*

1. Introduction

Ardisia sylvestris is a native plant in Vietnam which belongs to the Myrsinaceae family. It's an evergreen shrub 1 - 2 m tall, the leaves are opposite, ovate, 16 - 40 cm long and 6 - 10 cm wide with a sharply serrated margin. The flowers are 10 - 15 cm long with white to pale pink petals. Its fruits are red maturing in spring [1],[2]. *A. sylvestris* distributes mainly in the midland and mountainous regions of Vietnam [1]. Though the plant was used widely as a traditional drug for the treatment of stomachache, abdominal pain, and allergies, until now, little attention has been paid to its chemical constituents and bioactivities [3],[4],[5].

The mountainous region of Tuyen Quang province has a rich and diverse flora with many valuable species. This also has good conditions for the development of medicinal herbs cultivation that not only protects the biological diversity but increases the income of the local population. In this study, for the first time, the total contents of phenolics, flavonoids, tannins and alkaloids, as well as, the antioxidant and anti-inflammatory effects of *A. sylvestris* leaves from Lam Binh district were evaluated and compared to those of similar products in the market. The remarkable phytochemical contents and bioactivities indicated the potential to develop *A. sylvestris* cultivation as a commercial product in Lam Binh district, Tuyen Quang province.

2. Experimental

2.1. Samples

Mature leaves of *Ardisia sylvestris* were collected in several cultivating areas in Lam Binh district, Tuyen Quang province (6 samples, ASLB 1-6) in June 2022. At the same time, other *A. sylvestris* leaves samples (11 samples, ASM 1-11) were purchased in traditional medicine stores in Hanoi or ordered from other provinces by online purchasing (Table 1). All of the samples were identified by Dr. Nguyen The Cuong,

Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology. The samples were cleaned under a water tap, dried at 60°C, and powdered.

Table 1. List of *A. sylvestris* leaves samples

No.	Code	Origin
1	ASLB 1	Collected in Lam Binh district, Tuyen Quang province
2	ASLB 2	
3	ASLB 3	
4	ASLB 4	
5	ASLB 5	
6	ASLB 6	
7	ASM 1	Traditional medicine store in Ha Noi
8	ASM 2	Traditional medicine store in Ha Noi
9	ASM 3	Medicine manufacture in Ha Noi
10	ASM 4	Online purchasing from Hoa Binh
11	ASM 5	Online purchasing from Hoa Binh
12	ASM 6	Online purchasing from Bac Giang
13	ASM 7	Online purchasing from Bac Giang
14	ASM 8	Online purchasing from Ha Giang
15	ASM 9	Online purchasing from Hai Duong
16	ASM 10	Online purchasing from Hai Duong
17	ASM 11	Online purchasing from Ha Giang

2.2. Instruments and chemicals

UV-Vis absorbance was measured on a BioTek Synergy HTX multimode reader, Agilent, US. The incubator and the sonication bath were supplied by Daihan Scientific, Korea. Micropipette and pipette tips were supplied by Isolab, Germany. The solvents such as hexane, methanol (MeOH), dichloromethane (DCM), ethyl acetate (EA), DMSO, water, together chemicals, such as HCl (36%), NaOH, $NaNO_2$, $AlCl_3$, Na_2CO_3, NaCl (over 98% of purity) were purchased from Daihan Scientific, Korea. Folin-Ciocalteu reagent, gallic acid, lipopolysaccharides from *Escherichia coli* O26:B6 (LPS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), cardamonin, Griess reagent, and quercetin were supplied by Merck, Germany.

2.3. Extraction of the samples for bioassay

To prepare the extract for bioassays, 50 g of each sample was extracted in triplicate with 1 L of methanol in a sonication bath at 60°C. The solution was filtered and evaporated under a vacuum to a constant weight to yield the total extract. Moisture contents of the extracts were controlled under 10%.

2.4. Total phenolic assay

The total phenolic contents of the samples were evaluated by modifying a reported method [6]. The external calibration was done with different concentrations of gallic acid (10-400 µg/mL). The analytes were prepared by extracting in triplicate 2 g of the dry sample in 15 mL of methanol in a sonication bath at 60°C for 20 minutes. The solutions were filtered, combined and made the volume up to 50 mL. Each 100 µL of the sample or standard solution was mixed with 400 µL of Folin - Ciocalteu 10% and 500 µL of Na_2CO_3 6% and incubated for 15 min at 40°C. Its absorbance was measured with a UV-Vis. spectrophotometer at 750 nm. The total phenolic content was calculated as mg gallic acid equivalent (mg GAE/g) by using the gallic acid calibration curve.

2.5. Total flavonoid assay

The total flavonoid contents of the samples were examined using a previous method [6]. The external calibration was done with different concentrations of quercetin (10-200 µg/mL). The analytes were prepared by the same process as the total phenolic assay. Add 100 µL of $NaNO_2$ 5% to 500 µL sample or standard solution mix at room temperature for 5 min, then add 100 µL of $AlCl_3$ 10% and 500 µL NaOH 1M to the mixture. Keep the mixture at room temperature for 15 min. Its absorbance was measured with a UV-Vis. spectrophotometer at 510 nm. The total flavonoid content was calculated as mg quercetin equivalent (mg QE/g) by using the quercetin calibration curve.

2.6. Total alkaloid assay

The total alkaloid contents of the samples were quantified by a previous method [7]. The total extracts of *A. sylvestris* leaves were prepared by the same process for bioassay as described above. Then, 5 grams of each extract was suspended in 50 mL of water and acidified to pH 2 by HCl 1N and partitioned with EA (75 mL × 3 times). The organic phase was removed and the water phase was basified by 1N NaOH to pH 11 and then extracted with CH_2Cl_2 (4 times × 200 mL). The combined CH_2Cl_2 extracts were evaporated under reduced pressure to give total alkaloid mixtures. Total alkaloid content was measured by the weight of the alkaloid mixture in a gram of the total extract (mg/g).

2.7. Total tannin assay

Tannin contents were determined by the titrimetric method. Indigo carmine solution and $KMnO_4$ 0.1N were the main reagents used for tannin measurement [7]. 1 gram of samples was added to 50 mL of distilled water and heated at 80°C for 15 minutes. The mixture was cooled

and filtered through filter paper before being centrifuged at 4000 rpm for 10 minutes. Next, 25 mL of *A. sylvestris* leaves extract was mixed with 25 mL indigo carmine solution in a 1 L conical flask. Then 750 mL of distilled water was added to the mixture. The mixtures were titrated with standardized $KMnO_4$ until the blue colour of the mixture changed into green colour. Then, a few drops were added until the solution becomes golden yellow. The blank test was carried out by titration a mixture of 25 mL indigo carmine solution and 750 mL distilled water. All samples were analyzed in triplicates. The tannin concentration (T%) in the sample was calculated as follows:

$$T(\%) = \frac{(V-V_0)\times 0.004157 \times 50 \times 100}{m \times 25}$$

V is the volume of 0.1 N $KMnO_4$ for titration of the sample (mL), V_0 is the volume of 0.1 N $KMnO_4$ for titration of the blank sample (mL), 0.004157 is tannins equivalent in 1 mL of 0.1 N $KMnO_4$, m is mass of the sample taken for analysis (gram), 25 is the volume of sample, 50 volume of extraction solvent for sample.

2.8. DPPH radical-scavenging assay

DPPH assay was used to evaluate the antioxidant activity of extracts. DPPH radical-scavenging activity was conducted by modifying a previous method [8]. Briefly, each sample (20 µL) was mixed with 380 µl of DPPH in methanol and then dark incubated at 37°C for 20 minutes. The absorbance was measured at 517 nm. Ascorbic acid was used as a positive control. The IC_{50} value of each extract was measured based on experiments in several concentrations. The statistically significant difference in IC_{50} values was evaluated by one-way ANOVA and Tukey's HSD posthoc analysis.

2.9. Assay for inhibition of NO production

The effects of extracts on the NO production in LPS-stimulated RAW264.7 macrophage cells were examined as a reported method [9]. The cells (ATCC, MD, US) were cultivated in 96-well plates at 2×10^5 cells/well and incubated for 18 h. The plates were treated with the extracts (from 0,1 µg/mL to 100 µg/mL) for 30 min and then incubated for another 24 h with or without 1 µg/mL LPS. 100 µL of the culture supernatant was transferred to another 96-well plate, and 100 µL of Griess reagent was added. The absorbance of the reaction solution was read at the wavelength 570 nm. The remaining cell solutions in a cultured 96-well plate were used to evaluate cell viability by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [10]. Cardamonin, a well-known NO production inhibitor [11], was used as a positive control.

2.10. Statistics

One-way ANOVA, Tukey's HSD posthoc analysis, and correlation analysis were performed in R Package. Values of $p < 0.05$ were considered significant.

3. Results and discussion

3.1. Total phenolic, flavonoid, alkaloid, and tannin contents of the A. sylvestris leaves

Phenolic, flavonoids, tannins and alkaloids were reported as common secondary metabolites in *A. sylvestris* [3-5]. In this study, the contents of these compositions in *A. sylvestris* leaves were quantified. Moreover, we compared those of *A. sylvestris* leaves which were collected in the Lam Binh mountainous region to those of the plant leaves in the market. The data were shown in 0. As can be seen, the total phenolic contents (TPC) in *A. sylvestris* dry leaves from Lam Binh mountainous region ranged from 12.61 to 18.96 mg GAE/g. Meanwhile, the TPC of marketed samples fluctuated between 3.96 and 12.37 mg GAE/g, which were significantly lower ($p < 0.05$) than the data of samples from the Lam Binh district. Considerably, the total flavonoid contents (TFC) of *A. sylvestris* dry leaves from the Lam Binh mountainous district (from 7.85 to 13.27 mg QE/g), were significantly higher ($p < 0.01$) than those of market *A. sylvestris* dry leaves (3.64-10.36 mg QE/g). Whereas, alkaloids and tannins contents of both two groups of samples have no statistically significant difference. While the total alkaloid contents of these samples ranged from 1.39 to 4.63 mg/g, the total contents of tannin varied slightly from 1.27% to 3.94% among samples.

In general, *A. sylvestris* samples from Lam Binh have higher contents of phenolics and flavonoids than those of marketed samples, while the contents of alkaloids and tannins were similar between the two groups.

Table 2. Total phenolic (TPC), flavonoid (TFC), alkaloid (TAC), and tannin (TTC) contents of A. sylvestris dry leaves from Lam Binh district (ASLB) and from the medicinal market (ASM).

Sample	TPC (mg GAE/g)	TFC (mg QE/g)	TAC (mg/g)	TTC (%)
ASLB 1	12.61 ± 0.71	7.85 ± 0.55	2.08 ± 0.22	2.54 ± 0.40
ASLB 2	16.75 ± 1.47	13.07 ± 1.25	3.94 ± 0.38	2.66 ± 0.23
ASLB 3	14.54 ± 1.05	8.87 ± 0.54	2.78 ± 0.21	3.94 ± 0.22
ASLB 4	18.96 ± 1.55	13.27 ± 0.96	1.39 ± 0.14	3.70 ± 0.31
ASLB 5	15.10 ± 0.95	10.57 ± 0.72	3.13 ± 0.26	3.01 ± 0.26
ASLB 6	13.63 ± 0.82	10.90 ± 1.00	2.55 ± 0.19	2.66 ± 0.29
ASM 1	14.92 ± 0.96	7.43 ± 1.32	4.63 ± 0.47	2.88 ± 0.76
ASM 2	10.37 ± 2.57	5.82 ± 0.64	4.52 ± 0.33	2.72 ± 0.75
ASM 3	3.96 ± 0.48	3.64 ± 0.43	1.97 ± 0.12	2.62 ± 0.16
ASM 4	5.84 ± 0.37	3.85 ± 0.40	3.59 ± 0.23	1.74 ± 0.15
ASM 5	7.21 ± 0.40	9.41 ± 0.57	2.66 ± 0.16	3.01 ± 0.22
ASM 6	11.02 ± 0.89	6.83 ± 0.50	4.28 ± 0.48	3.13 ± 0.23
ASM 7	10.43 ± 0.76	10.36 ± 0.59	3.01 ± 0.32	2.66 ± 0.22
ASM 8	6.78 ± 0.75	5.02 ± 0.40	4.63 ± 0.29	1.85 ± 0.24
ASM 9	8.29 ± 0.84	6.47 ± 0.67	4.28 ± 0.33	2.89 ± 0.21
ASM 10	7.40 ± 0.57	5.62 ± 0.61	3.70 ± 0.38	1.27 ± 0.10
ASM 11	12.37 ± 1.26	9.9 ± 1.09	3.47 0.35	2.66 ± 0.23

3.2. DPPH radical-scavenging and NO inhibitory effects of A. sylvestris leaves extracts

Free radicals are molecules or atoms that carry one or some unpaired electrons. They are highly reactive and very unstable that can easily attack closet stable molecules. In a healthy body condition, there is a balance between reactive free radicals and endogenous antioxidant defence mechanisms. However, if this equilibrium is disturbed, it can lead to oxidative stress and associated damage which can affect DNA and protein, or lead to cell death. As a result, this can cause numerous diseases which include diabetes, cardiovascular diseases, inflammation, cancer, etc. [12]. In this study, we evaluated the DPPH radical-scavenging activity and NO production inhibition which represented the antioxidant and anti-inhibitory effects of the *A. sylvestris* leaves extracts.

The DPPH radical-scavenging activities of *A. sylvestris* leaves extracts were evaluated in terms of IC_{50} values (0). Most extracts of *A. sylvestris* leaves from the Lam Binh mountainous region demonstrated better antioxidant effects than ascorbic acid, the positive control (IC_{50} 30.62 µg/mL). The samples ASLB 4 and 2 exhibited the strongest antioxidant effects that the IC_{50} values at 22.41 and 25.09 µg/mL. Whereas, extracts of the marketed samples showed lower activities than the positive control. ASM 1 and 11 showed the best DPPH radical-scavenging activities among samples of this group, which IC_{50} at 34.53 and 38.07 µg/mL. As described, the samples from Lam Binh had significantly higher contents of phenolics and flavonoids than those of the marketed samples. These could deeply affect the stronger free radical scavenging activity of the samples from Lam Binh than the others. Also, the results were in agreement with the previous study on *Ardisia gigantifolia*, that the leaf extracts exhibited considerable antioxidant effects with a certain amount of tannins and phenolics [13].

Table 3. IC_{50} (µg/mL) values for DPPH radical-scavenging effects and NO production inhibition of A. sylvestris leaves extracts

Sample	DPPH (µg/mL)	NO inhibition IC_{50} (µg/mL)
ASLB 1	41.13 ± 4.07	49.79 ± 3.25
ASLB 2	25.09 ± 1.96	40.89 ± 4.47
ASLB 3	30.28 ± 3.33	36.48 ± 3.69
ASLB 4	22.41 ± 2.13	41.03 ± 4.18
ASLB 5	28.97 ± 2.49	46.26 ± 5.13
ASLB 6	34.53 ± 3.8	45.65 ± 3.23
ASM 1	36.01 ± 3.49	41.21 ± 3.60
ASM 2	44.34 ± 4.88	51.42 ± 3.28
ASM 3	60.46 ± 6.29	54.07 ± 4.21
ASM 4	61.59 ± 4.8	45.88 ± 3.64
ASM 5	59.67 ± 5.31	45.39 ± 4.20
ASM 6	43.38 ± 4.47	49.90 ± 3.07
ASM 7	60.01 ± 6.36	49.36 ± 3.98
ASM 8	55.13 ± 5.51	51.25 ± 4.86
ASM 9	49.81 ± 4.93	50.31 ± 2.71
ASM 10	51.36 ± 5.39	50.29 ± 3.01
ASM 11	38.07 ± 4.19	43.63 ± 3.59
Ascorbic acid *	30.62 ± 3.34	
Cardamonin #		3.64 ± 0.38

*, # Positive controls.

On the other hand, the anti-inflammatory effects of the *A. sylvestris* leaves extracts were evaluated via the inhibition of NO production in LPS-stimulated RAW264.7 macrophage cells. As shown (0), all extracts exhibited weak NO production inhibitions than cardamonin, the

positive control. IC_{50} of the Lam Binh samples ranged from 36.48 to 49.79 µg/mL while the data for marketed samples varied between 41.21 and 54.07 µg/mL. No significant difference between the NO inhibitions of the two sampling groups could be observed ($p > 0.05$). The MTT assay indicated that all of the extracts had no cytotoxicity to the cells (data not shown).

4. Conclusion

Results of chemicals quantification and bioassays indicated that *A. sylvestris* leaves had a number of secondary metabolites with considerable free radical-scavenging and anti-inflammatory effects. The *A. sylvestris* samples from the Lam Binh have the phenolics, and flavonoids contents at 12.61 - 18.96 mg GAE/g and 7.85 - 13.27 mg QE/g, respectively, which were significantly higher than those of current commercial samples in the market. Meanwhile, tannins and alkaloid contents of the Lam Binh *A. sylvestris* samples leaves were at 2.54% - 3.94% and 1.39 - 3.94 mg/g, respectively, which were similar to the marketed samples. The DPPH radical-scavenging activity of *A. sylvestris* leaves extracts was deeply affected by phenolics and flavonoids contents while the anti-inflammatory composition of the plant should be further investigated. These could be promising results for the further development of *A. sylvestris* cultivation in the region.

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QUANTITATIVE DETERMINATION OF MIMOSINE IN THE MEDICINAL HERB *MIMOSA PUDICA* MIMOSACEAE BY HPLC-PDA

Nguyen Thi Thanh Van[1], Vo Quoc Ngu[2], Huynh Lam Diem My[2], Duong Hong To Quyen[3], Phan Van Ho Nam[1], Hua Hoang Oanh[1],*

[1]University of Medicine and Pharmacy, Ho Chi Minh City, Vietnam;

[2]University of Natural Sciences, Vietnam National University, Ho Chi Minh City, Vietnam;

[3]Hospital of Traditional Medicine, Ho Chi Minh City, Vietnam

*Corresponding author: hhoanh@ump.edu.vn

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Summary

Quantitative Determination of Mimosine in the Medicinal Herb *Mimosa pudica* Mimosaceae by HPLC-PDA

An HPLC method was proposed for quantification of mimosine in the aerial parts of *Mimosa pudica*. The chromatography was established as: using the C_{18} (250 mm × 4.6 mm, 5 µm) column; the PDA detector was set at 275 nm; the mobile phase consisted of methanol and pH 2.0 buffer with ion-pairing agent sodium hexane-1-sulfonate, gradient