Trop J Nat Prod Res, December 2023; 7(12):5606-5610

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Pigments, Phenolics, Antioxidant Activity, Anti-α-Amylase, and In Vitro Anti-Inflammatory Properties of Crude Acetonic Extract from *Viola dalatensis*

Pham M. Tuan[1]*, Nguyen T. Ngan[1], Nguyen X. Ha[2], Hoang V. Trung[3]

[1]*Institute of Biotechnology and Food Technology, Industrial University of Ho Chi Minh City, Ho Chi Minh City, Vietnam*
[2]*Institute of Natural Products Chemistry, Vietnam Academy of Science and Technology (VAST), 18 Hoang Quoc Viet, Cau Giay, Hanoi, Vietnam*
[3]*School of Chemistry, Biology and Environment, Vinh University, 182 Le Duan, Vinh City, Nghean 43000, Vietnam*

ARTICLE INFO

Article history:
Received 01 November 2023
Revised 11 December 2023
Accepted 13 December 2023
Published online 01 January 2024

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ABSTRACT

Viola dalatensis Gagnep. is a member of the Violaceae family. *Viola* plants are often used in traditional medicine. Limited data regarding chemicals and biological activities of this species are found in the literature. This study aimed to identify pigments and phenolics in the plant's aerial parts. Besides, antioxidant, anti-α-amylase, and *in vitro* anti-inflammatory properties of acetonic extract from the plant were assessed. The level of chlorophyll a in the dried *V. dalatensis* aerial parts was twice as much as that of chlorophyll b. Among the phenolics examined, kaempferol was the most abundant compound, with an average concentration of 16.21 mg/100 g of dry weight. The antioxidant activity of the extract determined by ABTS and DPPH scavenging tests was shown to be weaker than that of ascorbic acid. The *in vitro* anti-inflammatory efficacy of the extract assessed by its ability to inhibit albumin denaturation (IC_{50} = 0.17 ± 0.01 mg/mL) was slightly lower than that of diclofenac (IC_{50} = 0.09 ± 0.00 mg/mL). The anti-α-amylase activity of the extract (IC_{50} = 0.80 ± 0.01 mg/mL) was slightly stronger than that of acarbose (IC_{50} = 0.92 ± 0.01 mg/mL). These results give a better understanding of the phytochemical components of *V. dalatensis* and its potential health-endorsing activities.

***Keywords*:** *Viola dalatensis*, kaempferol, antioxidant, amylase, albumin denaturation

Introduction

Viola, a genus belonging to the Violaceae family, encompasses over 500 species widely distributed across the globe.[1] Notably, many *Viola* plants have a rich history of use in traditional medicine. For instance, *V. odorata*, commonly known as wood violet, has been a traditional remedy for coughs, colds and catarrh, skin conditions, rheumatism, and urinary tract infections.[2] Zihua Diding, which is derived from the dried whole plant of *V. philippica*, is often employed as both an antifebrile and detoxifying agent to treat boils, furuncles, and carbuncles.[3] In traditional Uygur medicine, the entire plant of *V. tianshanica* has a history of use as a remedy for alleviating fever, headaches, sore throats, and the treatment of acute pyogenic infections.[4] This plant is valued for its heat-clearing and detoxifying properties. Studies have uncovered that essential oils and extracts from *Viola* species had a diverse range of bioactivities beneficial to human health. Essential oil from *V. diffusa* exerted significant cytotoxic effects on HepG2 and MCF-7 cell lines after 72 h of incubation.[5] Cyclotide-rich extracts of *V. tricolor* whole plant exhibited strong inhibitory activity against HIV-1 infection.[6] In an Alzheimer disease model using *Caenorhabditis elegans* CL4176, phenolic-rich *V.* x *wittrockiana* ethanolic extracts displayed inhibitory activity against acetylcholinesterase and monoamine oxidase A, and also had a protective effect on the paralysis of the roundworm.[7]

*Corresponding author. E mail: phamminhtuan@iuh.edu.vn
Tel: +84-0793-411-337

Citation: Tuan PM, Ngan NT, Ha NX, Trung HV. Pigments, Phenolics, Antioxidant Activity, Anti-α-Amylase, and In Vitro Anti-Inflammatory Properties of Crude Acetonic Extract from *Viola dalatensis*. Trop J Nat Prod Res. 2023; 7(12):5606-5610. http://www.doi.org/10.26538/tjnpr/v7i12.34

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

V. yedoensis n-butanol extract inhibited the production of Hepatitis B surface antigen (HBsAg) and Hepatitis B e antigen (HBeAg) in HepG2.2.15 cells, and hindered the replication of Hepatitis B virus DNA.[8] Additionally, the extract demonstrated protective effects against immunological liver injury in an *in vivo* study. The authors postulated that this hepatoprotection might be associated with coumarin and flavonoid compounds detected in the extract.

Viola dalatensis Gagnep., is a short-stemmed herbaceous perennial with light violet flowers and a 1.5 mm spur. It was first identified and documented by Gagnepain in 1938 and is exclusively found in the southern regions of Vietnam.[9] In the present study, we explored chlorophyll, carotenoids, and phenolics of *V. dalatensis* aerial parts. Besides, the antioxidant activity, anti-α-amylase and *in vitro* anti-inflammatory properties of the acetonic extract of the plant were evaluated. The findings of this study will give a better understanding of the bioactive compounds and potential health-endorsing activities of *V. dalatensis*.

Materials and Methods

Chemicals

Acetone was obtained from VWR Chemicals (France). Ferulic acid and gallic acid were obtained from Sigma-Aldrich (Missouri, USA). Catechin, epicatechin, quercetin, and rutin were procured from Chengdu Biopurify Phytochemicals (Sichuan, China). Chlorogenic acid, kaempferol, and diclofenac were procured from the National Institute of Drug and Quality Control (Hanoi, Vietnam). Acarbose (50 mg tablet, Khapharco Pharmaceuticals Company) was purchased from a drug store in Ho Chi Minh City, Vietnam.

Plant Collection and Identification

Viola dalatensis fresh aerial parts were collected in Lam Dong, Vietnam (12°05'47.6"N; 108°22'35.4"E) on April 15, 2022. The plant species was authenticated by Van-Son Dang at the Institute of Tropical Biology, VAST, Vietnam. The voucher specimen (HieuTT-15042022) was kept in the Department of Chemistry at Vinh University, Nghe

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ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

An, Vietnam. The sample was dried in a convection oven at 45 °C until its moisture reached < 10%, and was ground to a powder.

Determination of chlorophyll and carotenoid
Chlorophyll and carotenoid contents in the sample were determined using the method of Lichtenthaler and Buschmann (2001).[10] In detail, the sample (1 g) was combined with acetone (20 mL) and placed in a tube. After shaking, on a rotating shaker, the tube was subjected to centrifugation (5500 rpm, 15 min), and the resultant supernatant collected was measured at 470, 662, and 645 nm in a spectrophotometer. Chlorophyll and total carotenoid content were determined following the formulae:

Chlorophyll a (CA) = $(11.24 \times A_{662} - 2.04 \times A_{645}) \times V/M$
Chlorophyll b (CB) = $(20.13 \times A_{645} - 4.19 \times A_{662}) \times V/M$
Total carotenoid content = $[1000 \times A_{470} - (1.9 \times CA + 63.14 \times CB)]/214 \times V/M$
where, V and M stand for solvent volume (mL) and sample weight (g).

Acetonic extract preparation
An aliquot of the sample (10 g) was mixed with 150 mL of acetone, and both were placed in a flask. The flask was shaken for 24 h at room temperature, followed by a filter step through filter paper. The solvent in the filtrate was removed under reduced pressure, and the residue was employed for future analyses. The extraction yield of the acetonic extract was 9.5%.

Determination of phenolic contents
To identify and quantify phenolic compounds in the sample, 1 mL of the filter obtained earlier was filtered into a vial through a 0.45 μm membrane before injection into a high performance liquid chromatography (HPLC) system. The system consisted of a Shimadzu LC-2030C HPLC and UV detector, equipped with an Agilent Zorbax Eclipse XDB C18 column (4.6 × 150 mm, 5 μm). The mobile phase included solvent A (0.1% formic acid) and solvent B (100% acetonitrile). The elution was carried out according to a previously developed method.[11] Besides, total phenolic content (TPC) of the acetonic extract was assessed using the method of Singleton et al. (1999).[12]

Antioxidant activity
A solution consisting of 7 mM ABTS and 2.45 mM potassium persulphate in phosphate buffer saline (1:1, v/v) with phosphate-buffered saline was prepared and kept in darkness at room temperature overnight. Subsequently, this solution (3 mL) was mixed with 100 mL of an acetonic extract of *V. dalatensis*. The absorbance at 734 nm was measured using a spectrophotometer.[13, 14]
For the DPPH assay, the diluted *V. dalatensis* extract was mixed with DPPH solution (0.1 mM) at a 1:5 ratio and incubated in darkness at 37 °C for 30 min. The absorbance (517 nm) was determined at 517 nm using a spectrophotometer.[15, 16] The percentage inhibition (%) for ABTS or DPPH was estimated as follows:

Percentage of inhibition = $[1 - AS/AB] \times 100\%$
where, AS and AB represent the absorbance of the sample and blank, respectively. Ascorbic acid served as the reference standard in both tests. The IC_{50} value (mg/mL) was used to predict the ABTS and DPPH radical scavenging properties of the extract.

Inhibition of albumin denaturation
A mixture consisting of 1 mL of a 0.16% bovine albumin, 1 mL of the extract and 2 mL of acetate buffer (pH 5.5) was prepared. This mixture was then incubated at 37 °C for 45 min, followed by heating at 67 °C for 3 min. After the mixture was placed to cool to room temperature, the absorbance at 660 nm was measured. Diclofenac was used as a reference standard. The IC_{50} value (mg/mL) was used to predict the inhibitory activity of the extract.[17]

Inhibition of α-amylase
A diluted extract combined with an α-amylase solution (0.14 U/mL) in phosphate buffer (pH 6.9) was allowed to incubate for 15 min at 37 °C. The enzymatic process was initiated by introducing 15 μL of a 0.25% starch solution, followed by another 15 min incubation at 37 °C. A control sample was prepared using the same steps, except for the omission of α-amylase. To halt the reaction, 50 μL of 1 M HCl was added, followed by 100 μL of KI_3 solution. The absorbance of the mixture was subsequently measured at 595 nm using a spectrophotometer. The IC_{50} value (μg/mL) was calculated to determine the inhibitory effect on α-amylase. Acarbose was used as a reference standard.[18]

Statistical analysis
All the experiments were conducted out three times, and the data were presented as mean ± standard deviation. Statistical comparisons were made through ANOVA followed by Tukey's test. Significance was defined at $p < 0.05$. Minitab 19 (Pennsylvania, USA) was used to perform statistical analyses.

Results and Discussion

Chlorophylls and carotenoids
In the present study, chlorophyll and carotenoids in *V. dalatensis* aerial parts were extracted using acetone and determined by a VIS spectrophotometer. As seen in Table 1, the sample comprised average 1.57 and 0.77 mg of chlorophyll a and chlorophyll b per g of the dried aerial parts. It also contained 0.12 mg of total carotenoids per g. A previous study showed *V. declinata* leaves had about 0.085 mg/g of total carotenoids.[19]
Chlorophyll a is the primary pigment involved in photosynthesis and is essential for capturing light energy, while chlorophyll b plays a supplementary role in capturing light and transferring energy to chlorophyll a. In plants, two types of chlorophyll occur at the ratio of 3:1.[20] However, several factors, such as drying and fertilizers, can affect this ratio as chlorophyll a is more sensitive to heat and light than chlorophyll b. As a result, chlorophyll a is more likely to degrade during the drying process, which can lead to a decrease in the ratio. In the present study, the level of chlorophyll a doubled that of chlorophyll b, and this could stem from the drying process.
To our knowledge, no information about chlorophyll and carotenoids in *V. dalatensis* is available in the literature. These fat-soluble compounds are widely recognized for their role in supporting various aspects of the immune system and detoxification processes.[21]

Phenolics
The results revealed the presence of five phenolic acids and two flavonoids in the acetonic extract of *V. dalatensis* aerial parts (Table 2). Kaempferol was the most abundant phenolic compound detected in the sample, with the concentration averaging 16.21 mg/100g DW (dry weight). Two catechins (i.e., catechin and epicatechin) were found in the sample, with concentrations of 1.09 and 9.94 mg/100g, respectively. Quercetin was identified as a minor compound while its rutinoside form (i.e., rutin) was present at a much higher level in the sample (0.69 mg/100g). Chlorogenic acid and ferulic acid are two phenolic acids that were detected in this study. Their concentrations were 0.10 and 0.93 mg/100g of dried aerial parts. It is noted that numerous phenolics in other *Viola* species have been documented.[1] Quercetin, rutin, and chlorogenic acid were previously found in *V. yedoensis* while catechin was detected in *V. odorata.*[22,23] Recently, we have identified multiple phenolics in various *V. dalatensis* extracts.[11] Research has provided evidence that flavonoids may play a role in various biological functions within *Viola* plants, including antioxidant, antimicrobial, hepatoprotective and antidepressant-like effects.[24-27]
The results also showed that the average TPC of the *V. dalatensis* extract was 10.72 mg GAE/g. This indicated that the acetonic extract was not as rich in phenolics as those obtained by methanol, ethanol, and ethyl acetate reported previously.[11] Prior studies have demonstrated TPC of extracts from *Viola* species broadly ranged between 0.77 and 48.18 mg GAE/g.[23, 28-31] Generally, TPC of the *V. dalatensis* acetonic extract fell within the reported range.

Antioxidant activity
The antioxidant potential of the *V. dalatensis* acetonic extract was evaluated through DPPH and ABTS assays, and the results are

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ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

displayed in Table 3. As per the DPPH method, the extract exhibited an average IC_{50} value of 6.09 mg/mL, while through the ABTS method, the IC_{50} value was determined to be 4.46 mg/mL. Notably, these values were comparatively lower than the positive control, ascorbic acid. When comparing the antioxidant activity with other extracts of *V. dalatensis* explored in a prior study—methanol, water, ethyl acetate, and ethanol—it was found that the acetonic extract showcased lower antioxidant potential.[11] Additionally, in comparison with previously studied species, *V. canescens* displayed antioxidant activity with DPPH and ABTS IC_{50} values ranging from 0.24 to 1.36 mg/mL and 0.06 to 1.09 mg/mL, respectively. For *V. pilosa*, IC_{50} values were recorded at 0.3 – 0.64 mg/mL (DPPH) and 0.1 – 0.92 mg/mL (ABTS).[32]

Albumin denaturation inhibition

To assess *in vitro* anti-inflammatory effect of the *V. dalatensis* acetonic extract, we carried out bovine albumin denaturation inhibition assay. Table 4 illustrates the results, indicating that the extract could exert a slightly lower inhibitory activity against albumin denaturation (IC_{50} value of 0.17 ± 0.01 mg/mL) compared to diclofenac as a standard (IC_{50} = 0.09 ± 0.00 mg/mL). Previous research has also demonstrated the potential inhibitory effectiveness of water extracts from this species, with an IC_{50} value of 90.39 ± 8.415 µg/mL.[11] Research has shown that extracts of *Viola* species may possess anti-inflammatory activity. One study by Jeong et al. (2016) reported that extracts of *V. yedoensis* presented anti-inflammatory activity through inhibiting the release of proinflammatory cytokines and suppressing the activation of HO-1, NF-κB, and MAPK signaling pathways in RAW 264.7 cells.[33] The study also suggested apigenin derivatives in the extract could contribute to the activity.

α-Amylase inhibition

The percentage inhibition of the acetonic extract (at different concentrations) on *α*-amylase was presented in Table 5. Increase in extract concentrations resulted in remarkable inhibition ability against *α*-amylase, and this followed in a dose-dependent manner. The extract had a lower IC_{50} value (0.80 ± 0.01 mg/mL) compared to acarbose (IC_{50} = 0.92 ± 0.01 mg/mL), hinting at its higher activity. No reports on anti-*α*-amylase activity of *V. dalatensis* have been found in the literature. However, extracts of some *Viola* species have been documented for their potential to inhibit the enzyme. For instance, methanolic and acetonic extracts of *V. mandshurica* (0.5 mg/mL) inhibited more than 90% of *α*-amylase activity.[34] A methanol soluble fraction obtained from a methanolic crude extract of *V. odorata* showed a moderate inhibitory effect on *α*-amylase (42%).[35]

Conclusion

In summary, chlorophyll, total carotenoid content, and phenolics of *V. dalatensis* dried aerial parts were analyzed. Kaempferol was the most abundant compound among the monitored phenolics. Besides, antioxidant, anti-*α*-amylase and *in vitro* anti-inflammatory properties of the acetonic extract of the plant were determined. Antioxidant activity and *in vitro* anti-inflammatory effect of the extract were lower than ascorbic acid and diclofenac, respectively. Its anti-*α*-amylase activity was higher than acarbose. The research results offer a better understanding of the chemical composition and potential health benefits of the plant. This insight could lead to the development of plant-derived drugs for disease prevention and treatment.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Table 1: Chlorophyll and carotenoid contents in *V. dalatensis* aerial parts

	Concentrations, mg/g DW
Chlorophyll a	1.57 ± 0.02
Chlorophyll b	0.77 ± 0.01
Total carotenoid content	0.12 ± 0.00

Table 2: Phenolic content in *V. dalatensis* aerial parts

		Concentrations
Chlorogenic acid		0.10 ± 0.01
Ferulic acid		0.93 ± 0.01
Catechin		1.09 ± 0.30
Epicatechin	mg/100g DW*	9.94 ± 0.13
Rutin		0.69 ± 0.01
Quercetin		0.03 ± 0.00
Kaempferol		16.21 ± 0.11
TPC	mg GAE/g**	10.72 ± 0.06

*: based on dried aerial parts
**: based on dried extract

Table 3. Antioxidant activity of the *V. dalatensis* extract

	ABTS	DPPH
	IC_{50}, mg/mL	
Extract	4.46 ± 0.16 a	6.09 ± 0.56 a
Ascorbic acid	0.06 ± 0.00 b	0.03 ± 0.00 b

Lowercase letters (a, b) show significant differences in antioxidant activity between the extract and ascorbic acid ($p < 0.05$).

Table 4: Inhibitory activity of the *V. dalatensis* acetonic extract on albumin denaturation

Samples	Percentage inhibition*, %				IC_{50} (mg/mL)
	0.125	0.25	0.5	1.0	
Extract	32.6 ± 1.2	73.6 ± 1.4	94.9 ± 1.1	97.0 ± 1.1	0.17 ± 0.01 a
Diclofenac	-	-	-	-	0.09 ± 0.00 b

*: the percentage inhibition was determined at the different extract concentrations (0.125 – 1.0 mg/mL).
Lowercase letters (a, b) show significant differences in antioxidant activity between the extract and ascorbic acid ($p < 0.05$).

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ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Table 5: Inhibitory effect of the *Viola dalatensis* aerial part extract on α-amylase

Samples	Percentage inhibition*, %				IC_{50} (mg/mL)
	0.125	0.25	0.5	1.0	
Extract	2.1 ± 1.2	10.6 ± 0.3	34.5 ± 0.6	61.1 ± 1.2	0.80 ± 0.01 b
Acarbose	-	-	-	-	0.92 ± 0.01 a

*: the percentage inhibition was estimated at the different extract concentrations (0.125 – 1.0 mg/mL)
Lowercase letters (a, b) show significant differences in antioxidant activity between the extract and ascorbic acid ($p < 0.05$).

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