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Influence of Green Solvent Extraction on Phytochemicals, Potential Antidiabetic and *In Vitro* Anti-Inflammatory Activities of *Pseuderanthemum palatiferum* (Nees.) Radlk. Leaves

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ABSTRACT

Pseuderanthemum palatiferum (Nees.) Radlk., belonging to the Acanthaceae family, is commonly cultivated in Vietnam and Thailand for food and medicinal uses. The aim of the study was to compare phenolic composition, antioxidant, potential anti-inflammatory and antidiabetic activities of different extracts obtained from ethanol, 80% ethanol and water. The use of these solvents significantly affected the extractability of phytochemical classes, phenolic contents and bioactivities in the extracts. The extract obtained from 80% ethanol contained the highest amount of phenolics (113.74 ± 26.17 mg GAE/g). It also appeared to be more suitable for recovering phenolic constituents in *P. palatiferum* leaves. The extracts obtained from ethanol and 80% ethanol showed significantly higher antioxidant potential and inhibition of α-glucosidase compared to water. Regarding inhibition of bovine albumin denaturation, no differences were observed for the extracts at the examined concentration. The research helps better understand phytochemical contents and potential health benefits of *P. palatiferum*, which could be used for development of new plant-derived agents for disease prevention and treatment.

Keywords: *Pseuderanthemum palatiferum*, phenolics, antioxidant, glucosidase, anti-inflammatory.

Introduction

Pseuderanthemum palatiferum (Nees.) Radlk., also known as Hoan Ngoc or Xuan Hoa in Vietnamese, is a tree species in the Acanthaceae family. This plant is widely cultivated in Vietnam and Thailand for food and medicinal uses.[1] Leaves of *P. palatiferum* have been investigated and traditionally used in folk medicine as a treatment for ailments, such as trauma, stomachache, headache, colitis, nephritis and diarrhea since 1980s.[1,2] One of the commercialized products from leaves of the plant is Hoan Ngoc herbal tea which is commonly consumed in Vietnam.[3] Studies have shown that *P. palatiferum* exerts a variety of bioactivities, such as antioxidant activity, anti-inflammatory, analgesic, hypotensive, antidiabetic and antiproliferative effects. For example, oral administration of fresh leaf aqueous extracts (0.6 g/kg) considerably suppressed paw edema induced by carrageenan (57%) and arachidonic acid (47%) in rats.[4] An oral glucose tolerance test in streptozotocin-nicotinamide-induced diabetic rats showed that leaf aqueous extract (0.25 g/kg and 0.50 g/kg) exerted hypoglycemic effects.[5] Leaf aqueous extracts also displayed anticancer activity by inhibiting cell viability and induce ROS-mediated mitochondrial related apoptosis in A549 cells.[6] Ethanolic extracts of fresh leaves stimulated human breast cancer MDA-MB-231 programed cell death through intrinsic (caspase-9) and extrinsic (caspase-8) pathways.[7]

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Previously, one study by Mai *et al*[8] identified different classes of phytochemicals in the plant, such as lignans (palatiferin A and palatiferin B) and triterpenoids (betulin and lupeol). According to these authors, the lignans exerted moderate cytotoxicity against KB and HepG2 cells. Additionally, the two triterpenoids were shown to have inhibitory effects on MCF7 cell line. It is widely known that plants are often composed of a diverse mixture of large and small molecules, such as proteins, polysaccharides, fatty acids, phytosterols, saponins, polyphenols and pigments. Extraction of these constituents plays an important role in recovery of compounds of interest in food, nutraceutical and cosmeceutical industries. Several solvents with different polarity that have often been used include methanol, ethyl acetate, hexane and chloroform. Despite their effectiveness, these extractants can be toxic for users and have environmental impacts. In this study, ethanol and water as safer, environmentally friendly solvents to extract bioactive constituents were used. The aim was to explore phenolic composition, antioxidant, potential antidiabetic and anti-inflammatory properties of *P. palatiferum* leaves. The results will hopefully give a better understanding of how this class of compounds make contributions to health-promoting activities of *P. palatiferum* and open a new application of the plant in food and nutraceutical products.

Matherials and Methods

Plant collection

P. palatiferum was cultivated in Ho Chi Minh city, Vietnam and was harvested in January 2022. It was authenticated with a voucher specimen (CV472022) by the Institute of Ecology (Southern Vietnam). The leaves were collected, thoroughly washed to remove soil and dirt under running tap water. Afterwards, the leaf sample was air-dried until dryness (moisture < 8%). The dried leaf sample was kept in ziploc bags, and stored in a refrigerator (4°C, 65% relative humidity) until analysis.

Chemicals
Phenolic acid reference standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). Flavonoids were obtained from Chengdu Biopurify Phytochemicals Ltd. (Sichuan, China). Diclofenac sodium (> 99%) was obtained from National Institute of Drug Quality Control (Ha Noi, Vietnam). Ascorbic acid (99.75%) was purchased from Duchefa Biochimie (Haarlem, The Netherlands). Ethanol and water (99.5% ACS grade) were obtained from Fisher Scientific (Pittsburg, Pennsylvania, USA).

Determination of chlorophylls
To extract chlorophylls, ground leaves of *P. palatiferum* were mixed with acetone at a ratio of 1:10 (g/mL) in a screw-capped tube. After 24-hour shaking, the tube was centrifuged in an ordinary centrifuge at a speed of 5500 rpm. After 10 min, the supernatant obtained was spectrophotometrically determined at 645 and 662 nm.[9] Calculation of chlorophylls was based on the formula:

$$\text{Chlorophyll a} = (11.24 \times A - 2.04 \times B) \times V/W$$

$$\text{Chlorophyll b} = (20.13 \times B - 4.19 \times A) \times V/W$$

where A and B stand for the absorbance measured at 662 and 645 nm, respectively. V is the volume (mL) of the solvent while W is the amount (g) of the dried leaf sample.

Crude extract preparation
Ten gram of the ground sample was mixed with 100 mL of 100% ethanol or 80% ethanol in water. The mixture was shaken for 24 hours and underwent centrifugation at 5500 rpm.[10] After 15 min, the supernatant was obtained and evaporated to remove solvents in a rotary evaporator. The crude extract was obtained and used to determine phenolic and flavonoid contents and predict bioactivities.

Phytochemical screening
Phytochemicals, such as tannins, saponins, anthraquinones and alkaloids, present in the crude extracts were qualitatively analyzed.[11]

Determination of phenolics
Total phenolic content (TPC) in the extracts of *P. palatiferum* leaves were estimated using the method of Vu (2022).[10] To quantitatively identify individual phenolics, a liquid chromatograph connected to a diode-array detector (HPLC-DAD) was used. The chromatographic separation was performed using a VertiSep GES C18 reverse-phase column (250 × 4.6 mm, 5.0 µm particle size) set at 40 °C. The mobile phase was a mixture of 100% methanol (solvent A) and 1% formic acid in water (solvent B). Elution of phenolics was performed on a flow rate of 0.8 mL/min with elution gradient previously performed by Vu et al. (2022).[12] Detection wavelengths were set at 295 nm and 340 nm for phenolic acids and flavonoids, respectively.

Antioxidant activity
ABTS assay. A mixture of 7 mM ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) and 2.45 mM $K_2S_2O_8$ in phosphate buffered saline (1:1, v/v) was incubated for 12 hours at ambient temperature in the dark. Afterwards, 3 mL of the mixture combined with 100 µL of a diluted extract or ascorbic acid solution (a reference standard) in a tube were well mixed and spectrophotometrically measured at 734 nm.[12] The calibration curve constructed for ascorbic acid was $y = 0.006x - 0.0035$, with $R^2 = 0.99$.
DPPH assay. A mixture of 40 µg/mL DPPH (2,2-diphenyl-1-picrylhydrazyl) solution in 80% methanol (300 µL) and a diluted extract (200 µL) or ascorbic acid solution (a reference standard) underwent an incubation at 37°C under a constant dark condition overnight. The change in absorbance was measured at 517 nm in a spectrophotometer.[13] The calibration curve constructed for ascorbic acid was $y = 0.0456x + 0.0295$, with $R^2 = 0.98$.
Reducing power assay. The reaction mixture containing the diluted extract (0.2 mL), 1% potassium ferricyanide (0.5 mL) and phosphate buffer (pH 6.0; 0.5 mL) was well vortexed and then underwent 20 min incubation at 50°C. Afterwards, trichloroacetic acid (0.5 mL, 10%) was pipetted into the mixture, followed by 1 min shaking. The supernatant, 1% ferric chloride and distilled water (2:1:5, v/v) were mixed and measured at 700 nm.[13] The calibration curve constructed for ascorbic acid (a reference standard) was $y = 0.0018x + 0.0567$ ($R^2 = 0.99$).

Inhibition of α-glucosidase
A reaction mixture consisting of 50 µL of a diluted extract in 5% DMSO and 40 µL of α-glucosidase (0.05 U) in phosphate buffer (pH 6.8) underwent a 20 min incubation at 37°C. Afterwards, 40 µL of 5 mM 4-nitrophenyl-β-D-glucopyranoside was added in, and the mixture was incubated for 20 min at 37°C. A solution of sodium carbonate solution (130 µL, 0.2 M) was added to halt the reaction. The absorbance was measured at 405 nm in a spectrophotometer.[14] In the assay, acarbose was employed as a reference standard. The activity was predicted using the calibration curve ($y = 0.000059x + 0.001952$; $R^2 = 0.99$) plotted for this standard. The inhibitory effect was presented as mg acarbose equivalents per gram of extract (mg ACAE/g).

In vitro anti-inflammatory activity
In this assay, the extracts of *P. palatiferum* leaves were evaluated for their ability to inhibit albumin denaturation according to a method previously used by Uttra et al. (2019) with minor modifications.[15] One hundred milliliters of each extract diluted in 5% DMSO were mixed with 100 µL of 0.16% bovine serum albumin solution and 200 µL of sodium acetate buffer (pH 5.5), followed by a 45 min incubation at 37 °C then heated for 3 min at 67 °C. After cooling down to ambient temperature, the absorbance was determined at 660 nm using a UV-VIS spectrophotometer. The analytical reference standard used in the assay was diclofenac sodium, a nonsteroid anti-inflammatory drug. The percentage inhibition of protein denaturation was predicted as follows:

$$\%I = (A_b - A_s)/A_b \times 100\%$$

where, A_b and A_s are absorbance of the blank and sample/standard.

Statistical analysis
Phenolic concentrations were expressed as mean of duplicate measurements while all the other experimental data were carried out in triplicate and shown as mean ± standard deviation. One-way analysis of variance (ANOVA) and Tukey's HSD test were employed to evaluate the means. Differences were regarded as significance at $p < 0.05$. All the statistical analyses were carried out using XLSTAT 2016 software package (Addinsoft, France).

Results and Discussion

Phytochemical screening
In this study, we screened different classes of phytochemicals, including sterols, triterpenoids, saponins and alkaloids. As shown in Table 1, these classes were all detected in the 80%-ethanol extract (EW), with sterols particularly showing an abundant presence. These were also present in the 100%-ethanol extract (ET), with the exception of alkaloids. In contrast, the aqueous extract (AQ) tested positive only for sterols, indicating low extractability of water on alkaloids, saponins and triterpenoids. Previously, 80% ethanol extract of *P. palatiferum* leaves was put under a screen test for steroids and the detection of this chemical class was confirmed.[16] In a prior study, leaves of *P. palatiferum* were shown to contain a considerable amount of β-sitosterol, stigmasterol and their glycoside derivatives,[17-19] which are phytosterols commonly found in fruits, vegetables, nuts and seeds. Other than that, scarce information about phytochemicals in leaves of *P. palatiferum* is available in the literature.

Chlorophylls
Compositional data about these pigments have been very limited in the literature. In this section, quantification of chlorophylls in fresh leaves of *P. palatiferum* was carried out using acetone and VIS spectrophotometry. The results showed that each gram of the leaves contained 588.05 ± 7.70 µg of chlorophyll a and 312.48 ± 6.69 µg of

chlorophyll b. Chlorophylls are recognized constituents that may have capacity to enhance functions of essential detoxification pathways, antioxidant, anti-inflammatory and chemoprotective agents.[20]

Phenolics

The results indicated that EW consisted of the highest amount of phenolics among the extracts. Table 2 shows TPC of EW (113.74 ± 26.17 mg GAE/g) was 67% higher compared to that of ET. The aqueous extract of the plant was found to be poor in phenolics, with its average TPC reaching 7.59 mg GAE/g. These values were comparable with those reported previously, in which ethanolic and aqueous extracts of *P. palatiferum* leaves contained average 62.86 and 33.12 mg GAE/g.[21] However, a prior study by Sittisart and Chitsomboon[22] observed much higher TPC for ethanolic and aqueous extracts of the leaves. This discrepancy could be due to extraction or geographic and/or seasonal differences in sample collection and handling. In addition to total phenolic content, six individual phenolics were quantified using HPLC-DAD and the results were presented in Table 2. All these compounds were found to be present in EW while only ferulic acid was detected in AQ. Except quercetin, all the others were found at quantifiable levels in ET. In general, most of the compounds had higher levels in EW compared to ET. Particularly, the average concentration of chlorogenic acid in EW was twice as much as that in ET. Similarly, rutin and p-coumaric acid are two phenolics which were found to have much higher levels in EW than in ET as presented in Table 2. Quercetin appeared to be present less abundant in leaves of the plant as this compound was detected only in EW at a very low concentration (0.81 µg/g). To our knowledge, individual phenolic acids and flavonoids in *P. palatiferum* have scarcely been reported in the literature. Previously, some of these compounds were quantified in Hoan Ngoc herbal tea purchased in Vietnam.[3] It was shown that concentrations of rutin and quercetin in the tea were comparable to those revealed in the present study. In addition, the presence of chlorogenic, caffeic, p-coumaric and ferulic acids were reported in the tea, demonstrating that Hoan ngoc leaves were a rich source of phenolics.

Antioxidant activity

In this study, antioxidant activity of the extracts was assessed using DPPH, ABTS radical scavenging assays and reducing power capacity test as described earlier. The concentrations of the extracts used for the assessment were 1 mg/mL (DPPH and ABTS) and 4 mg/mL (reducing power). As seen in Table 3, ABTS antioxidant potential of the samples varied significantly ($p < 0.05$) and ET seemed to exert the strongest activity compared to the others, followed by EW. At the test concentration, ET and EW scavenged ABTS free radicals by 22.51 ± 2.04 and 14.33 ± 0.80%, respectively. Regarding the DPPH assay, no significant differences in the capacity between these two extracts were noted. However, the use of reducing power assay demonstrated that EW displayed the strongest antioxidant activity. The assay was performed to predict the capacity to transform Fe^{3+} to Fe^{2+} in the presence of the extracts. This assay is often used to evaluate antioxidant activity of phenolics-rich samples. This could explain why EW, which was identified as having the highest TPC, possessed the strongest reducing power activity. The results also indicated that the aqueous extracts showed no inhibition in DPPH and reducing power assays. One study reported that ethanolic leaf extract of *P. palatiferum* exhibited significantly higher ability to suppress the formation of DPPH free radicals compared to aqueous extract.[21]

Table 1: Phytochemical screening of the extracts of *P. palatiferum* leaves

Phytochemicals	Indicator		
	ET	**EW**	**AQ**
Sterols	+	+	+
Triterpenoids	+	+	–
Saponins	+	+	–
Alkaloids	–	+	–

+: present; –: absent.

Table 2: Phenolic contents of the *P. palatiferum extracts*

	ET	**EW**	**AQ**
Chlorogenic acid	410.05	844.44	nd
Caffeic acid	130.20	130.43	nd
p-Coumaric acid	172.01	277.87	nd
Ferulic acid	14.44	11.18	1.18
Rutin	67.78	185.96	nd
Quercetin	nd	0.81	nd
TPC	67.85 ± 5.55 b	113.74 ± 26.17 a	7.59 ± 0.89 c

Concentrations of phenolics were expressed as µg/g FW. TPC was expressed as mg GAE/g extract. Results of individual phenolic compounds are presented as means of duplicate measurements.

Table 3: Antioxidant activity (mg AAE/g extract) of the *P. palatiferum* extracts

	ET	**EW**	**AQ**
ABTS	25.81 ± 2.46 a	16.64 ± 1.06 b	7.67 ± 0.36 c
DPPH	9.78 ± 0.62	10.56 ± 0.07	nd
Reducing power	6.85 ± 0.77 b	12.49 ± 0.29 a	nd

Data are presented as mean ± standard deviation, nd: not determined
Different lowercased letters for the same activity indicated significant differences among the extracts ($p < 0.05$).

It also recorded a similar result for the two extracts via reducing power assay. However, in an article investigating intracellular reactive oxygen species scavenging activity of the fresh leaves, Sittisart and Chitsomboon[22] revealed that aqueous extract was better at trapping DPPH radicals.

Inhibition of α-glucosidase

The three extracts of *P. palatiferum* leaves were evaluated for antidiabetic potential via their ability to inhibit α-glucosidase. At the concentration of 4 mg/mL, EW displayed the highest activity among the extracts, with its inhibitory effect expressed as acarbose equivalents amounting to twice as much as those of ET and AQ (Figure 1). The results also showed no significant differences in inhibition of α-glucosidase between ET and AQ (295.40 ± 6.47 and 296.81 ± 52.92 mg ACAE/g, respectively). Prior research revealed ethanolic extracts of the leaves may exert powerful inhibitory action against α-amylase and α-glucosidase.[19] The inhibitory properties were shown to be much higher than those of stigmasterol and sitogluside isolated from the extracts. Furthermore, in an oral starch tolerance test, the extracts were demonstrated to reduced remarkably blood glucose levels. In animal studies, extracts of the leaves had a capacity to lower fasting plasma glucose levels in streptozotocin-diabetic rats.[23] Evidence has strongly suggested that *P. palatiferum* may act as promising modulators of enzymes associated with carbohydrate digestion. Therefore, these findings provide a scientific support for medicinal uses of *P. palatiferum* leaves in the prevention and treatment of diabetes.

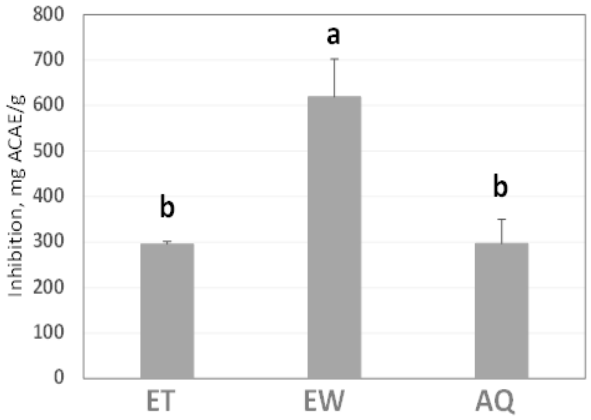

Figure 1: Inhibitory effect of the extracts on α-glucosidase. Different lowercased letters indicated significant differences among the extracts ($p < 0.05$).

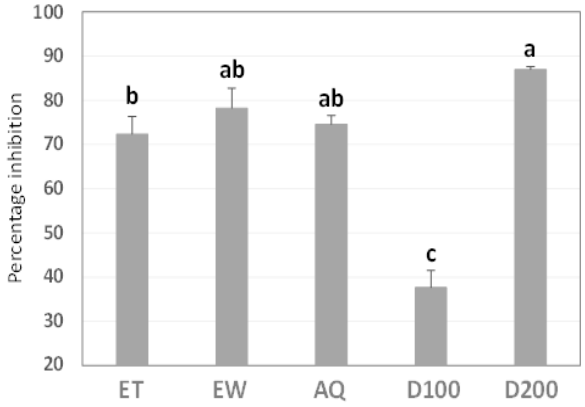

Figure 2: Inhibitory effect of the extracts on albumin denaturation. D100 and D200 stand for the diclofenac solutions at 100 and 200 μg/mL. Different lowercased letters showed significant differences among the extracts ($p < 0.05$).

Inhibition of bovine albumin denaturation

Protein denaturation may occur due to exposure to chemicals, heat or environmental stresses, resulting in loss of their functions. Tissue protein denaturation is among the well-known causes of inflammatory diseases.[24] In this study, albumin denaturation was determined to estimate *in vitro* anti-inflammatory potential of the *P. palatiferum* leaf extracts at the concentration level of 4 mg/mL. As illustrated in Figure 2, no significant differences in percentage inhibitions of albumin denaturation among the three extracts were observed ($p > 0.05$). In comparison with diclofenac solutions used as a positive control, EW and AQ may be on a par with that of 200 μg/mL diclofenac solution (86.9%). The results also indicated that all the extracts had a higher effect on protecting albumin from denaturation compared to 100 μg/mL diclofenac solution. It is unclear about the mechanism by which the extracts prevented albumin from heat-induced denaturation. However, an explanation could be related to interactions between their phenolics and aliphatic regions located around lysine residue on the albumin molecules.[25]

Many attempts to better understand anti-inflammatory properties of *P. palatiferum* have been made. For example, one study demonstrated that ethanolic and aqueous extracts of the plant leaves were able to inhibit nitric oxide (NO) release and expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) protein levels in lipopolysaccharide plus interferon-γ-induced RAW264.7 cells.[22] In an animal model, it was shown that oral administration of aqueous extract of the plant leaves (600 mg/kg) considerably suppressed paw edema induced by carrageenan and arachidonic acid.[4] Despite limited data about in vivo anti-inflammatory effects and human trials of the plant are available, the findings from these studies above altogether highlighted that *P. palatiferum* may be suitable for developing plant-derived anti-inflammatory drugs.

Conclusion

Pigments, phenolic constituents, antioxidant activity, and inhibition of albumin denaturation and α-glucosidase of *P. palatiferum* fresh leaf extracts were determined. The use of environmentally friendly solvents for extraction influenced the presence of phenolics and other phytochemicals as well as bioactivities in the extracts obtained. The research provides a better understanding of phytochemical contents and potential health benefits of *P. palatiferum*, which may contribute to development of plant-derived drugs for disease prevention and treatment. Future investigations should be focused on bioassay-guided purification to predict potential health endorsing properties of bioactive constituents in the plant.

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.

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