Antioxidant Activity of *Acanthopanax trifoliatum*

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Key Words

Acanthopanax trifoliatum • Antioxidant activity •
1,1-Diphenyl-2-picryl-hydrazyl assay • Lipid peroxidation

Abstract

Objective: The aim of this study was to investigate the antioxidant activity of extracts of various parts of *Acanthopanax trifoliatum* obtained by different extraction methods. The total phenolic and flavonoid contents of the extracts were also determined. **Materials and Methods:** The leaves, stems, stem bark, roots and root bark of *A. trifoliatum* were extracted separately using decoction, maceration and refluxing methods. The extracts were analysed for free-radical-scavenging activity using the 1,1-diphenyl-2-picryl-hydrazyl scavenging assay and the thiobarbituric-acid-reactive substance method for the inhibition of lipid peroxidation of a rat brain homogenate. Total phenolic and total flavonoid contents of the extracts were measured by UV spectrophotometry. **Results:** The leaf decoction extracts possessed a significantly stronger antioxidant activity as revealed by both methods. Total phenolic and flavonoid contents of the extracts ranged from 2.16 to 21.79 g% chlorogenic acid equivalent and from 0.37 to 9.61 g% rutin equivalent, respectively. Analysis of the leaf decoction extract, which exhibited the most potent antioxidant activity, by thin-layer chromatography revealed flavonoid and polyphenolic compounds corresponding to rutin and chlorogenic acid. **Conclusion:** The leaf aqueous extracts showed a high level of antioxidative activity and contained

high levels of both phenolic and flavonoid compounds. The magnitude of antioxidant activity corresponded with the level of phenolic and flavonoid compounds.

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Introduction

Acanthopanax is a plant species that possesses ginseng-like activities and is known as a ‘ginseng-like herb’ [1–3]. This genus comprises 18 plant species most of which are widely distributed in Korea, Japan, China and Russia [4, 5]. Ginseng (*Panax ginseng*) root is extensively used for a wide variety of clinical ailments as well as to improve physical and mental well-being of individuals [6]. The roots and stem bark of *Acanthopanax* plants have also been used as tonics and sedatives, as well as in the treatment of rheumatism and diabetes [5, 7]. A Thai traditional plant that belongs to this species is *Acanthopanax trifoliatum* which is called ‘phak pam’ in Thai. It is used in the folk medicines of South-East Asia as a drug with ginseng-like activity [5]. A decoction of the leaves and young shoots is used to treat tuberculosis and lung haemorrhages and as a tonic to improve general weakness [3, 5, 8–10]. The preparation is believed to help in dispersing extravasated blood from bruises, relieving partial paralysis and is applied to ulcers and contusions [3, 7]. In Thailand, the stem bark is used as antifatulence agent, and the decoction or ethanolic extracts have been traditionally used to

treat emaciation and neurosis. Moreover, young leaves and the shoots are popularly consumed as vegetables in northern Thai traditional cuisine [11].

From its ethnomedical uses as a ginseng-like herb, *A. trifoliatum* has potential for the treatment of degenerative diseases and cognitive dysfunction. Moreover, there are some reports of the antioxidant activity of the related plant species *A. senticosus*. Stem bark extracts of *A. senticosus* were reported to inhibit nitric oxide production in murine macrophages in vitro and in vivo [12] while stem extracts possessed antioxidant activity in a carbon-tetrachloride-intoxicated rat model [13]. Park et al. [14] reported that water extracts of *A. senticosus* root showed free radical scavenging activity with protective effects against oxidative DNA damage. However, there has been no report concerning the antioxidant activity of extracts from *A. trifoliatum* or a quantitative analysis of its active components.

Therefore, this study was undertaken to determine the free-radical-scavenging activity and inhibitory effect on lipid peroxidation of rat brain homogenates of extracts from various parts of *A. trifoliatum*. Moreover, the total phenol and flavonoid contents of these extracts were measured, and constituents of the leaf aqueous extract from decoction were separated by chromatography.

Materials and Methods

Materials

The leaves, stems and roots of *A. trifoliatum* were collected from Sunpathong district, Chiang Mai province, Thailand. The plant samples were identified by Mr. Winai Somprasong, Botanist, Bangkok Herbarium, Department of Agriculture. The voucher specimens were deposited at the same place (BK63947). 1,1-Diphenyl-2-picryl hydrazyl (DPPH), chlorogenic acid and thiobarbituric acid were obtained from Sigma (St. Louis, Mo., USA), rutin from Sigma-Aldrich Co. (Steinheim, Germany) and sodium dodecyl sulphate from Merck (Germany).

Sample Preparation

Extracts from *A. trifoliatum*. The leaves, stems, stem bark, roots and root bark of *A. trifoliatum* were separately cleaned, dried in a hot air oven (60°C) for 6 h and powdered with an electronic mill (20-mesh sieve). Each sample was extracted using various solvents and extraction conditions derived from the ethnomedical procedures [5, 11] and Chinese pharmacopoeia [15].

Decoction. The leaves, stems, stem bark, roots and root bark were separately boiled with distilled water (1:10 w/v) for 3 h and then filtered. The filtrate was taken lyophilized to yield dried leaf, stem, stem bark, root and root bark decoction extracts.

Maceration. The leaves, stems, stem bark, roots and root bark were separately macerated with 95% ethanol (1 h sonication, filtered; repeated 3 times). The filtrate was collected and evaporated

under reduced pressure to dryness. These dried leaf, stem, stem bark, root and root bark 95% ethanolic extracts were then used for assessments.

Refluxing. The leaves, stems, stem bark, roots and root bark were refluxed (80°C) with 75% ethanol for 3 h and then filtered. The filtrate was collected and evaporated under reduced pressure.

Determination of Antioxidant Activity by DPPH Scavenging Method

The free-radical-scavenging effect of *A. trifoliatum* extracts as well as standard ascorbic acid, chlorogenic acid and rutin corresponding to the quenching ability of DPPH was carried out as described by Sithisarn et al. [16]. Each sample was assayed in triplicate, and the average of the 50% effective concentration (EC₅₀) value was calculated.

Inhibition of Lipid Peroxidation of Rat Brain Homogenate by Thiobarbituric-Acid-Reactive Substance Assay

Preparation of Rat Brain Homogenate. Rats weighing 200–250 g were sacrificed by carbon monoxide inhalation, and whole brains were dissected out and homogenized using an Art-micra D-8 homogenizer (Germany). A suspension of 100 mg/ml containing ice-cold phosphate buffer (0.1 M, pH 7.4) was used as a source of polyunsaturated fatty acids for determining lipid peroxidation. The tissue homogenate was centrifuged at 800 g for 15 min at 4°C, and the supernatant was divided into 1-ml aliquots and preserved at –80°C until used.

Thiobarbituric-Acid-Reactive Substance Assay. Adapted from Auddy et al. [17] and Singh et al. [18], 50 µl of rat brain homogenate and 25 µl of various concentrations (2–60 µg/ml) of the plant extracts, or standard trolox (water-soluble vitamin E derivative), dissolved in phosphate buffer were taken. In the case of the control experiment, volume adjustments were done with phosphate buffer. Peroxidation was initiated by adding 30 µl of 0.2 mM ferric chloride. After incubation in a shaking water bath at 37°C for 40 min, the reactions were stopped by adding 350 µl of 20% (v/v) acetic acid followed by 600 µl of 0.5% thiobarbituric acid in 20% (v/v) acetic acid (pH 3.5). The mixtures were incubated at 85°C for 60 min. After cooling, 50 µl of 10% sodium dodecyl sulfate was added, centrifuged at 3,000 g for 10 min and the absorbance of the supernatant determined at 532 nm [19] using a microplate reader (Tecan, USA). EC₅₀ values for plant extracts were determined from a calibration curve. All experiments were done in triplicate. Results are reported as means of EC₅₀ ± SD.

Determination of Total Phenolic Content

Using the method applied by Naithani et al. [20], plant extract solutions were oxidized with Folin-Ciocalteu reagent, and the reactions were neutralized by sodium carbonate solution. The absorbance of the resulting blue-coloured solution was measured at 765 nm after 120 min. Each sample was done in duplicate. Total phenolic content was expressed as grams chlorogenic acid equivalent in 100 g extract (g%).

Determination of Total Flavonoid Content

Total flavonoid content was investigated using the colorimetric method previously described [21]. Plant sample solutions were reacted with aluminium chloride solution in the same volume. Absorbances were read at 415 nm after 10 min, and flavonoid con-

Table 1. Free-radical-scavenging activity and lipid-peroxidation-inhibitory potential of extracts from *A. trifoliatum* tested by DPPH assay and TBARS method

Sample	Yield %	DPPH assay EC ₅₀ μg/ml	TBARS method EC ₅₀ μg/ml
<i>Leaves</i>			
LM	21.85	100.81 ± 5.05 ^{a, b}	23.90 ± 3.07 ^{a, b}
LR	25.70	72.20 ± 9.61 ^{a, c}	21.11 ± 1.09 ^{a, b, c}
LD	21.15	14.50 ± 1.04 ^d	16.11 ± 0.29 ^{c, d, e}
<i>Stem bark</i>			
SBM	10.10	118.60 ± 5.50 ^b	17.31 ± 0.69 ^{c, d}
SBR	10.25	37.85 ± 0.85 ^{d, e, f}	35.09 ± 6.08 ^f
SBD	14.45	63.39 ± 1.84 ^{c, e}	51.31 ± 9.29 ^g
<i>Stems</i>			
SM	8.85	686.09 ± 56.02 ^g	39.02 ± 2.04 ^f
SR	6.14	41.66 ± 1.11 ^{e, f}	33.85 ± 2.46 ^f
SD	8.70	54.71 ± 2.18 ^{c, e, f}	75.35 ± 7.52 ^h
<i>Root bark</i>			
RBM	8.05	263.34 ± 9.12 ^h	20.55 ± 1.20 ^{a, d}
RBR	13.40	34.51 ± 2.74 ^{d, f}	26.48 ± 0.33 ^b
RBD	14.20	34.24 ± 5.01 ^{d, f}	25.72 ± 3.61 ^{a, b}
<i>Roots</i>			
RM	5.75	338.55 ± 17.44 ⁱ	61.00 ± 0.86 ⁱ
RR	7.45	44.62 ± 0.74 ^{e, f}	20.21 ± 0.75 ^{a, d}
RD	8.75	54.24 ± 3.07 ^{c, e, f}	11.18 ± 2.60 ^e
<i>Standards used</i>			
Ascorbic acid	–	3.05 ± 0.11	–
Chlorogenic acid	–	18.87 ± 0.76	–
Rutin	–	8.04 ± 0.10	–
Trolox	–	–	331.37 ± 7.41 × 10 ⁻³

Percent yield assessed by grams extract per gram dry plant. TBARS = Thiobarbituric-acid-reactive substances; LM = leaf maceration extract; LR = leaf refluxing extract; LD = leaf decoction extract; SBM = stem bark maceration extract; SBR = stem bark refluxing extract; SBD = stem bark decoction extract; SM = stem maceration extract; SR = stem refluxing extract; SD = stem decoction extract; RBM = root bark maceration extract; RBR = root bark refluxing extract; RBD = root bark decoction extract; RM = root maceration extract; RR = root refluxing extract; RD = root decoction extract. Values in each column with different superscript letters are significantly different ($p < 0.05$).

tent was expressed as grams rutin equivalent in 100 g of plant extracts (g%).

Thin-Layer Chromatographic Fingerprints

Thin-layer chromatography of *A. trifoliatum* leaf decoction extracts was performed on a thin-layer chromatography precoated silica gel 60 GF₂₅₄ plate measuring 10 × 10 cm using ethyl acetate/acetic acid/formic acid/water (137:11:11:26; solvent A), chloro-

form/methanol/water (70:30:3; solvent B) and *n*-butanol/acetic acid/water (4:1:5; solvent C) as mobile phases. Flavonoids separated on the thin-layer chromatography plate were detected under UV 366 nm using a natural product/polyethylene glycol spray reagent and polyphenolic compounds by 10% ferric chloride spray.

Statistical Analysis

All data are reported as means ± standard deviation of triplicates in antioxidant activity determinations and duplicates in phenolic content analysis. Least significant difference was used to compare means ($p < 0.05$). Correlation coefficients *R* to determine the relationship between two variables were calculated using Pearson's *R* test. All analyses were performed using SPSS for Windows, version 16.0 (SPSS Inc., USA).

Results

The leaves of *A. trifoliatum* gave the highest yield of crude extracts (more than 20% w/w) from every method of extraction (table 1). Other extracts gave lower yields ranging from 5 (root maceration) to 14% w/w (stem bark decoction and root bark decoction). The *A. trifoliatum* extracts showed free-radical-scavenging activity with EC₅₀ ranging from 14.50 ± 1.04 (decoction of leaves) to 686.09 ± 56.02 μg/ml (stem maceration). The extracts obtained by refluxing with 75% ethanol or the decoction method ($p < 0.05$) showed significantly higher antioxidant activities tested by the DPPH scavenging assay while extracts from maceration with 95% ethanol showed lower activities. Leaf decoction extracts exhibited a significantly higher free-radical-scavenging effect with EC₅₀ of 14.50 ± 1.04 μg/ml which was similar to values obtained for chlorogenic acid and rutin, which were 18.87 ± 0.76 and 8.04 ± 0.10 μg/ml, respectively, but 5 times less than those obtained for ascorbic acid (EC₅₀ of 3.05 ± 1.04 μg/ml). Inhibitory effects on lipid peroxidation of rat brain homogenate of tested samples ranged from EC₅₀ of 11.18 ± 2.60 to 75.35 ± 7.52 μg/ml. Root and leaf decoction extracts showed significantly higher effects than others (EC₅₀ of 11.18 ± 2.60 and 16.11 ± 0.29 μg/ml, respectively) which was 34 and 49 times lower than the value obtained for trolox (EC₅₀ 331.37 ± 7.41 × 10⁻³ μg/ml).

Total phenolic and flavonoid contents of each extract are given in table 2; stem bark refluxing extracts showed a significantly higher phenolic content (21.79 ± 0.23 g% chlorogenic acid equivalent). Root bark decoction extracts, stem and root refluxing extracts and leaf decoction extracts also exhibited significantly higher phenolic contents (20.74 ± 0.44, 19.17 ± 0.58, 17.51 ± 0.07 and 16.26 g% chlorogenic acid equivalent, respectively). Leaf maceration extracts contained significantly higher

Table 2. Total phenolic and total flavonoid contents of extracts from *A. trifoliatum*

Sample	Total phenolic content		Total flavonoid content	
	g CAE in 100 g extract	mg CAE in 100 g dried plant	g RE in 100 g extract	mg RE in 100 g dried plant
<i>Leaves</i>				
LM	12.86 ± 0.04 ^a	2,808.82 ± 7.73 ^a	9.61 ± 0.52 ^a	2,098.74 ± 113.97 ^a
LR	10.13 ± 0.49 ^b	2,603.41 ± 127.21 ^b	3.51 ± 0.08 ^b	902.50 ± 20.77 ^b
LD	16.26 ± 0.00 ^c	3,437.93 ± 0.00 ^c	1.31 ± 0.01 ^c	277.97 ± 3.11 ^c
<i>Stem bark</i>				
SBM	7.64 ± 0.09 ^d	771.89 ± 8.93 ^d	1.90 ± 0.01 ^d	191.51 ± 0.74 ^d
SBR	21.79 ± 0.23 ^e	2,233.73 ± 23.56 ^e	0.95 ± 0.00 ^e	96.91 ± 0.00 ^e
SBD	12.13 ± 0.39 ^{a, f}	1,752.79 ± 56.20 ^f	0.59 ± 0.04 ^{f, g}	84.82 ± 5.31 ^e
<i>Stems</i>				
SM	2.16 ± 0.07 ^g	190.72 ± 6.26 ^g	0.37 ± 0.00 ^f	32.87 ± 0.33 ^e
SR	19.17 ± 0.58 ^h	1,176.88 ± 35.82 ^h	0.64 ± 0.01 ^{e, f, g}	39.55 ± 0.90 ^e
SD	11.19 ± 0.55 ^f	973.75 ± 47.68 ⁱ	0.89 ± 0.05 ^{e, g}	77.74 ± 4.47 ^e
<i>Root bark</i>				
RBM	7.83 ± 0.07 ^d	630.32 ± 5.69 ^j	0.64 ± 0.02 ^{e, f, g}	51.44 ± 1.77 ^e
RBR	11.32 ± 0.34 ^f	1,516.55 ± 45.01 ^k	0.65 ± 0.01 ^{e, f, g}	87.01 ± 0.98 ^e
RBD	20.74 ± 0.44 ⁱ	2,945.44 ± 62.76 ^l	0.67 ± 0.03 ^{e, f, g}	95.53 ± 4.69 ^e
<i>Roots</i>				
RM	9.46 ± 0.28 ^b	543.66 ± 16.26 ^j	0.74 ± 0.00 ^{e, g}	42.56 ± 0.21 ^e
RR	17.51 ± 0.07 ^j	1,304.12 ± 5.27 ^m	0.68 ± 0.03 ^{e, f, g}	50.31 ± 2.19 ^e
RD	15.82 ± 1.29 ^c	1,384.03 ± 112.92 ^m	2.43 ± 0.24 ^h	212.73 ± 20.57 ^d

LM = Leaf maceration extract; LR = leaf refluxing extract; LD = leaf decoction extract; SBM = stem bark maceration extract; SBR = stem bark refluxing extract; SBD = stem bark decoction extract; SM = stem maceration extract; SR = stem refluxing extract; SD = stem decoction extract; RBM = root bark maceration extract; RBR = root bark refluxing extract; RBD = root bark decoction extract; RM = root maceration extract; RR = root refluxing extract; RD = root decoction extract; CAE = chlorogenic acid equivalent; RE = rutin equivalent. Values in each column with different superscript letters are significantly different ($p < 0.05$).

amounts of flavonoids than other tested extracts (9.61 ± 0.52 g% rutin equivalent).

A thin-layer chromatogram of decoction extract of the leaves (fig. 1) contained components that corresponded to chlorogenic acid and rutin which were used as standards for measuring DPPH scavenging effects (table 1).

Discussion

This work was conducted following the work previously reported on the evaluation of antioxidative effects. The first step suggested is quantification and identification of the phenolic compounds in the samples. The second step concerns quantification of the radical-scavenging activity of the samples. The final step was the evaluation

of the ability of the samples to inhibit or halt lipid oxidation in relevant model systems [22, 23]. Of the antioxidant activity tested by two models, the DPPH scavenging assay and thiobarbituric-acid-reactive substance method of lipid peroxidation of rat brain homogenate, leaf decoction extracts yielded significantly higher antioxidant activities with higher phenolic and flavonoid content than other extracts. Extraction procedures were significantly associated ($p < 0.05$) with DPPH scavenging activity ($R = 0.765$, high correlation) and phenolic content in extracts ($R = 0.480$, moderate correlation) and in plant material ($R = 0.405$, moderate correlation). From the results, refluxing with 75% ethanol and decoction are methods using high-polarity solvents with high temperatures promoting the extracts with a high phenolic content which then provide the DPPH scavenging activity

Fig. 1. Thin-layer chromatogram of leaf decoction extract of *A. trifoliatum*. Lane 1 = *A. trifoliatum* leaf decoction extract; lane 2 = standard chlorogenic acid; lane 3 = standard rutin. **A** Solvent

system A. **B** Solvent system B. **C** Solvent system C. **A1, B1, C1** Natural product/polyethylene glycol spraying reagent + UV 366 nm. **A2, B2, C2** 10% FeCl_3 spraying reagent.

ity by proton donating. The correlated results were similar to previous reports of Hosein and Zinab [24], i.e. that extraction methods have significant effects on phenolic compound and antioxidant activity of henna extracts, as well as a report from Sithisarn et al. [16] that decoction was the most suitable method for the extraction of active free-radical-scavenging extracts of Siamese neem tree leaves. Extraction procedures did not significantly affect the inhibition of lipid peroxidation of rat brain homogenate tested by the thiobarbituric-acid-reactive substance method, as well as flavonoid content in the extracts and plant material ($p > 0.05$). In nature, flavonoids can be found in plants both in the form that binds to one or more sugar molecules, which are known as flavonoid glycosides and include rutin, quercitrin and isoquercetin, and those that are not bound to a sugar molecule which are called aglycones, which include quercetin and kaempferol [25]. Flavonoid glycosides have a higher polarity than aglycones or some derivatives such as methylated and acetylated forms. Therefore, different methods and solvents could affect the characteristic and ratio of flavonoids in the resultant extracts depending on their chemical structures and polarities. This could explain the low correlation ($R = 0.327$) between the method of extraction and total flavonoid content. Moreover, in plant extracts, antioxidants are present as mixtures and therefore there is a possibility of synergistic interactions of antioxidants and between mechanisms of such inter-

actions, including chelation of metal catalysts, scavenging of initiating radicals, chain-breaking reactions and reductions in the concentration of reactive oxygen species [26]. This suggests that in lipid peroxidation of the polyunsaturated fatty acids of rat brain homogenate, there could have been synergistic effects between polyphenolic and flavonoid compounds with other antioxidants in plant samples such as ascorbic acid, carotenoids, tocopherols, chlorophylls and metal-chelating compounds [27, 28]. These antioxidant compounds remain in different ratios in extracts from different methods of extraction depending on their chemical characteristics and polarities. Therefore, there was a low correlation ($R = 0.040$) between inhibition of lipid peroxidation and extraction methods. There was no simple correlation ($R = 0.026$) between phenolic and flavonoid contents in the extracts ($p > 0.05$) in this experiment. However, leaf and root bark decoction extracts and stem bark refluxing extracts had a high phenolic content and exhibited strong DPPH scavenging activity. Therefore, the phenolic content could play an important role in free-radical-scavenging activity. Moreover, the leaves contained significantly higher amounts of flavonoids than other samples. Since the leaf decoction extracts resulted in the highest antioxidant activity and phenolic content, constituent analysis by thin-layer chromatography was conducted using three different solvent systems and two spray reagents for the detection of phenolic compounds

[29]. However, chlorogenic acid was reported to be found in the leaves of another *Acanthopanax* species, *A. senticosus* or Siberian ginseng [30].

Conclusion

Of the different *A. trifoliatum* extracts obtained from varying of plant part and solvent and extracting condition, the leaf decoction exhibited the strongest DPPH scavenging activity and inhibition of lipid peroxidation corresponding to its high phenolic and flavonoid content. Refluxing with 75% ethanol and decoction were found to be suitable methods for the extraction of active radical-

scavenging compounds. The results showed that extracts from the leaves, stem bark, stems, root bark and roots of *A. trifoliatum* possessed antioxidant activity and contained some phenolics and flavonoids which confirmed the ethnomedical uses of this plant as tonic vegetables.

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