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Contributed Paper

Phytochemical Investigation and Antioxidant Properties of Different Extracts from *Artabotrys harmandii* Finet & Gagnep

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ABSTRACT

Oxidative stress is important cause of pathogenesis of various diseases. Nowadays, antioxidants derived from plants are important substances which have the property to prevent oxidative-related diseases. Thus, we aimed to study phytochemicals and antioxidant efficacy of *Artabotrys harmandii* Finet & Gagnep. Antioxidant parameters including total antioxidant, 2,2-azino-bis-(3-ethyl-benzothiazoline-6-sulfonic acid ($ABTS^{\bullet+}$) radical scavenging activity, 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) radical scavenging activity, reducing power and anti-lipid peroxidation (anti-LPO) were determined in different extracts (petroleum ether (AH-PET), ethyl acetate (AH-EtOAc), ethanol (AH-EtOH) and aqueous extracts (AH-DW)) from the stems of *A. harmandii*. Total phenolics, total flavonoids, ascorbic acid, alkaloids, tannins, and saponins were also investigated. The highest contents of total phenolics, ascorbic acid and alkaloids were found in the AH-EtOH extract while the highest contents of flavonoids and tannins were found in the AH-EtOAc. The AH-EtOAc exhibited the strongest total antioxidant, reducing power, anti-LPO and scavenging activity against $DPPH^{\bullet}$ radicals. The order of antioxidant ability of *A. harmandii* extracts was as follows: AH-EtOAc > AH-EtOH > AH-DW > AH-PET. The strongest scavenging activity against $ABTS^{\bullet+}$ was found in the AH-EtOH extract. From our results, the ethyl acetate and ethanolic extracts from *A. harmandii* stems had high amounts of bioactive compounds and could be a powerful source for preventing degenerative disorder or promoting health.

Keywords: antioxidant, anti-lipid peroxidation, *Artabotrys harmandii* Finet & Gagnep, free radicals, phytochemicals

1. INTRODUCTION

Reactive oxygen species (ROS) such as hydrogen peroxide radical, hydroxyl radical and superoxide anion are important free radicals formed in normal metabolism. Over generation of ROS leads to oxidative stress. This condition has been encouraged to be main factors leading to the development of aging and various degenerative diseases [1].

Antioxidants or radical scavengers are important substances which have the property to prevent or inhibit the body from damage resulting from free radicals. Although a variety of synthetic antioxidants is popularly used in promoting health, the long-term use of synthetic antioxidants at high doses has been proven to have toxicity and carcinogenicity [2]. Thus, interest has greatly increased to find powerful antioxidants from natural sources. Natural compounds derived from various plants have the ability to reduce oxidative stress by acting as antioxidants [3]. These compounds may prevent the progression of diseases via an antioxidant defense mechanism.

Artabotrys harmandii Finet & Gagnep is the plant in the Annonaceae family. This plant is distributed in tropical areas, especially Asia and Africa. It is commonly known in Thailand as nom ngua. Various plants in the Annonaceae family have been used by local Thai people as folk medicines for the treatment of fever, urological disorders, prostatic diseases and intestinal ulcers [4-5]. In addition, *A. harmandii* has been found to possess biological properties such as anti-microbial, anti-cancer and anti-estrogenic efficacies [6]. Nevertheless, there is no scientific report available in the literature on its phytochemicals and antioxidant property. Thus, this research was to determine phytochemical constituents and antioxidant efficacy of various extracts from the stems of *A. harmandii*.

2. MATERIALS AND METHODS

2.1 Chemicals

All chemicals used in this study were analytical grade. 2,2-azino-bis-(3-ethyl-benzothiazoline-6-sulfonic acid (ABTS), 2, 4-dinitrophenyl hydrazine, 2,2-diphenyl-1-picrylhydrazyl (DPPH), aluminium chloride ($AlCl_3$), ammonium hydroxide (NH_4OH), ammonium molybdate, ascorbic acid, butylated hydroxytoluene (BHT), ferric chloride ($FeCl_3$), ferrous sulfate ($FeSO_4$), gallic acid, potassium ferricyanide ($K_4Fe(CN)_6.3H_2O$), quercetin, sodium carbonate (Na_2CO_3), sodium chloride (NaCl), thiobarbituric acid (TCA) and trichloroacetic acid (TBA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Diethyl ether, ethanol, Folin-Ciocalteu reagent, methanol, n-butanol and sulfuric acid were obtained from Union Science Co, Ltd. (Thailand).

2.2 Plant Materials and Collection

A. harmandii stems were collected from Chiang Mai Province, Thailand. The plant materials were identified in the Queen Sirikit Botanical Garden, Thailand, where a voucher specimen under a reference number of QSBG No. 78881 was deposited.

2.3 Extract Preparation

The stems of *A. harmandii* were washed, chopped, air dried and ground into powder. One hundred grams of the powdered stems were extracted with 1,000 ml of petroleum ether, ethyl acetate, 95% ethyl alcohol, and distilled water using a Soxhlet apparatus. Each extracts was filtered through filter paper No. 1. The solvents were eliminated from the filtrates using a rotary evaporator and they were dried using a hot-air oven. The yields of the extracts were 0.65, 1.11, 5.37 and 1.19 % for the petroleum ether (AH-PET), ethyl acetate (AH-EtOAc), ethanol (AH-EtOH) and aqueous (AH-DW) extracts respectively.

2.4 Preliminary Phytochemical Screening

The standard procedures described by Edeoga et al. (2005) [7] were used to detect the phytochemical constituents in each extract. Phytochemical compounds such as

phenolics, flavonoids, tannins, phlobatannins, terpenoids, alkaloids, sterols, saponins and cardiac glycosides were detected.

2.5 Quantitative Phytochemical Analysis

2.5.1 Total phenolic content (TPC)

The TPC was investigated following the procedure described by Wolfe et al. (2003) [8]. Each extract was mixed with 2.0 ml of Na_2CO_3 solution and 2.0 ml of Folin-Ciocalteu solution. After 20 min of incubation at 25 °C, the absorbance was recorded at 760 nm using a spectrophotometer. The standard calibration curve was created using gallic acid. The TPC was exhibited as mg of gallic acid equivalent (mgGAE) per gram of extract.

2.5.2 Total flavonoid content (TFC)

The TFC in different extracts was investigated [9]. Different extracts of *A. harmandii* were incubated with 0.5 ml of $AlCl_3$ solution for 60 min. The absorbance was read at 420 nm. The calibration of quercetin was plotted. TFC was exhibited as mg of quercetin equivalent (mgQE) per gram of extract.

2.5.3 Ascorbic acid content (AAC)

The amount of AAC in different extracts was determined by a 2,4-dinitrophenylhydrazine (DNPH) reagent [10]. Each extract was mixed with DNPH reagent, 0.2 ml of TCA (13.3 % w/v) and 0.2 ml of distilled water. After 3 hr of incubation at 37 °C, 1.0 ml of 65 % v/v of sulfuric acid was mixed .The absorbance was recorded at 520 nm. The standard calibration curve was made from ascorbic acid. AAC in each extract was exhibited as mg of ascorbic acid (mgAAC) per gram of an extract.

2.5.4 Tannins

Determination of tannins in the extracts was done using the method of Tamilselvi et al. (2012) [11]. Each extract was added to 5.0 ml of Folin-Ciocalteu reaction (7.5 % v/v) and 1.0 mL of Na_2CO_3 solution (35 % w/v). The reaction mixture was mixed and incubated for 30 min. The absorbance was read at 725 nm. The calibration curve of tannic acid was plotted. The content of tannins in each extract was exhibited as mg of tannic acid equivalent (mgTAE) per gram of an extract.

2.5.5 Alkaloids

Determination of alkaloids in various extracts was carried out [12]. In brief, each extract was added to 10 ml of acetic acid (10 % v/v) and incubated for 4 hr. After incubation, NH_4OH solution was added to the reaction mixture until it developed precipitate. The NH_4OH solution was used to wash precipitate. The alkaloid solution was filtered and dried. The determination was done in triplicate. The alkaloids content in each extract was exhibited as mg of alkaloids per gram of extract.

2.5.6 Saponins

The content of saponins was determined following the standard procedure [13]. Each extract was dissolved in aqueous ethanol (20 % v/v), shaken and heated at 55 °C for 90 min. The mixture was filtered. The residue was re-dissolved in aqueous ethanol and mixed with diethyl ether. The aqueous layer was discarded. The saponins were shaken with n-butanol and washed with sodium chloride. The saponins extract was evaporated to dryness. The content of saponins was exhibited as mg of saponins per gram of extract.

2.6 Measurement of Antioxidant Efficacy

2.6.1 Total antioxidant assay (TTA)

The TTA of various extracts was investigated [14]. Each extract was mixed with the reagent solution containing ammonium molybdate in acid solution. After incubation for 90 min at 95 °C, the degree of green-yellow color was recorded at 695 nm. The standard calibration curve was made from gallic acid. The TTA was expressed as mg of gallic acid equivalent (mgGAE) per gram of an extract.

2.6.2 $ABTS^{\bullet+}$ radical scavenging activity

The scavenging activity of each extract against the $ABTS^{\bullet+}$ radical was determined [15]. The $ABTS^{\bullet+}$ solution (4.9 mM) was diluted using deionized water to produce an absorbance of 0.70 ± 0.02 at 734 nm. Then, 0.01 ml of each extract was added to 1.0 ml of the $ABTS^{\bullet+}$ solution. After 1 min, the degree of decolorization of the ABTS radicals from blue-green to clear color was recorded at 734 nm. The following formula was used to calculate the percentage inhibition:

$$\% \text{ inhibition} = [A_0 - A_1/A_0] \times 100$$

A_0 and A_1 were absorbances at 734 nm at 0 min and 1 min respectively. The concentration value of the sample which reduces 50 % of the $ABTS^{\bullet+}$ or the IC_{50} value was calculated from the percentage of inhibition. The concentration value of the sample reduces 50 % of the $ABTS^{\bullet+}$. Gallic acid was used as a standard reference.

2.6.3 $DPPH^{\bullet}$ radical scavenging activity

The antioxidant in various extracts to scavenge $DPPH^{\bullet}$ radical was assessed [16]. Briefly, 0.1 ml of each extract at different concentrations was added to 2.0 ml of methanol solution of $DPPH^{\bullet}$ (0.13 mM). The absorbance was immediately recorded at 517 nm, and this served as A_0. After 30 min of incubation in the dark, the absorbance was recorded, and this served as A_{30}. The analysis was performed in triplicate. Gallic acid was used as reference compound. The percentage inhibition was calculated according to the formula:

$$\% \text{ inhibition} = [A_0 - A_{30}/A_0] \times 100$$

A_0 and A_{30} were absorbances at 517 nm at 0 min and 30 min respectively. The percentage of inhibition was used to calculate the IC_{50} value.

2.6.4 Reducing power assay

Reducing power of various extracts of *A. harmandii* was estimated [17]. A volume of 0.1 ml of each extract was mixed with 2.0 ml of phosphate buffer and 2.0 ml of $K_4Fe(CN)_6.3H_2O$ solution. After incubating the mixture for 20 min at 50 °C, the TCA solution was added to the reaction solution and mixed. Then, 2.0 ml of the mixture was taken and mixed with 0.4 ml of $FeCl_3$ solution (0.1% w/v) and 2.0 ml of distilled water. After 10 min of incubation, the absorbance was recorded at 700 nm. Gallic acid was considered as a standard reference. The reducing power was expressed as a haft maximal effective concentration (EC_{50}) value.

2.6.5 Anti-lipid peroxidation (Anti-LPO) assay

The investigation of anti-LPO efficacy of the extracts was done [18]. The study procedures for the animal care and experimentation were approved by the Institutional Animal Care and Use Committee of the Biology Department, Faculty of Science, Chiang Mai University (ID: Re. 004/13). The liver of the healthy rats were excised

and minced with 1.0 ml of phosphate buffer, pH 7.2. The homogenate was centrifuged at 3,500 rpm for 10 min, 4 °C to obtain the supernatant. To investigate the level of (MDA), which is the end product of LPO, 3.0 ml of the liver homogenate was incubated with 0.1 ml of each extract and 0.1 ml of $FeSO_4$ solution (15 mM) at 37 °C for 30 min. After incubation, the TCA solution (10% w/v) was added and the mixture was incubated for 10 min, and then centrifuged at 3,500 rpm for 10 min. The collected supernatant was mixed with 1.5 ml of TBA solution (0.67% w/v) and boiled for 30 min. The analysis was performed in triplicate. The BHT was used as a reference standard. The degree of MDA was measured at 532 nm. The following formula was used to calculate the percentage inhibition:

$$\% \text{ inhibition} = [A_C - A_T/A_C] \times 100$$

A_C was an absorbance at 532 nm of the reagent blank, while A_T was an absorbance of the reaction mixture in the presence of the extract or standard.

2.7 Data Analysis

All graphs and data were analyzed using GraphPad Prism version 7.0 for Windows. All assays were performed in triplicate. All data was exhibited as mean ± S.E.M. The IC_{50} and EC_{50} values were calculated by linear regression.

3. RESULTS

3.1 Phytochemical Analysis

Phytochemical screening of various extracts from *A. harmandii* revealed the different phytochemical constituents in each extract. The AH-EtOH extract possessed secondary metabolites such as phenolics, flavonoids, tannins, phlobatannins, terpenoids, sterols, saponins and cardiac glycosides. Most of the phytochemical constituents investigated in this study except for sterols and cardiac glycosides could be detected in the AH-DW extract (Table 1).

Table 1. Preliminary phytochemical constituents of various extracts from *A. harmandii*.

Phytochemicals	*A. harmandii* extracts			
	PET	EtOAc	EtOH	DW
Phenolics	+	+	+	+
Flavonoids	+	+	+	+
Tannins	+	-	+	+
Phlobatannins	-	-	+	+
Terpenoids	-	+	+	+
Alkaloids	-	+	-	+
Sterols	+	+	+	-
Saponins	+	-	+	+
Cardiac glycosides	+	+	+	-

Note + present, - absent

The possession of considerable amounts of TPC, AAC and alkaloids was found in the AH-EtOH extract, while massive amounts of TFC and tannins were detected in the AH-EtOAc extract. However, the AH-PET extract possessed the highest amount of saponins (Table 2).

Table 2. Phytochemical compounds in various extracts of *A. harmandii*.

Phytochemicals	Extracts of *A. harmandii*			
	AH-PET	AH-EtOAc	AH-EtOH	AH-DW
TPC (mgGAE/g extract)	1.76 ± 0.07	6.46 ± 0.43	9.00 ± 0.23	4.15 ± 0.02
TFC (mgQE/g extract)	0.16 ± 0.04	3.38 ± 0.06	0.35 ± 0.03	0.25 ± 0.01
AAC (mg/g extract)	1.68 ± 0.18	2.60 ± 0.40	8.81 ± 0.24	2.08 ± 0.13
Tannins (mgTAE/g extract)	0.17 ± 0.00	1.45 ± 0.00	0.84 ± 0.00	1.10 ± 0.00
Alkaloids (mg/g extract)	0.56 ± 0.01	1.44 ± 0.01	1.56 ± 0.02	1.42 ± 0.01
Saponins (mg/g extract)	1.33 ± 0.09	1.27 ± 0.09	0.73 ± 0.03	0.50 ± 0.06

All values are exhibited as mean ± S.E.M.

3.2 Antioxidant Efficacies

As showed in Table 3, the highest antioxidants were found in the AH-EtOAc extract in all assays except for $ABST^{\bullet+}$ radical scavenging assay. The AH-EtOAc exhibited the strongest TTA, reducing power, anti-LPO and scavenging activity against $DPPH^{\bullet}$ radicals. The TTA ability of the investigated extracts was as follows: AH-EtOAc > AH-EtOH > AH-DW > AH-PET. In the reducing power assay, the level of reduction of Fe^{3+} to Fe^{2+} was dependent on the concentration. The greatest reducing power was found in the AH-EtOAc and AH-EtOH extracts (Figure 1). From the IC_{50} values of the anti-LPO of various extracts, the greatest ability to inhibit LPO in liver homogenate was found in the AH-EtOAc extract (Figure 2). The IC_{50} values of the scavenging activity against $DPPH^{\bullet}$ radicals of the various extracts ranged from 0.83 ± 0.01 mg/ml to 2.97 ± 0.11 mg/ml. The strongest scavenging activity against $ABTS^{\bullet+}$ radicals was found in the AH-EtOH extract (IC_{50} = 1.82 ± 0.02 mg/ml). However, the ability to scavenge $ABTS^{\bullet+}$ of all the extracts was less than standard gallic acid (IC_{50} = 0.69 ± 0.00 mg/ml) (Table 3).

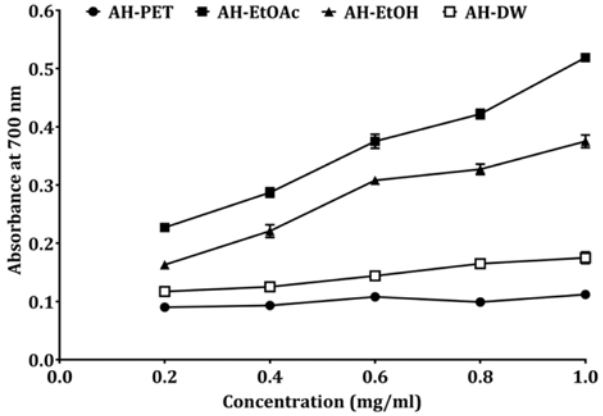

Figure 1. Reducing power of *A. harmandii* extracts. All values are exhibited as mean ± S.E.M.

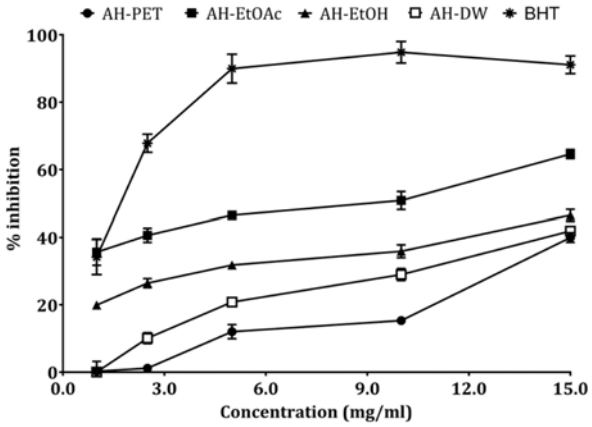

Figure 2. Anti-LPO of *A. harmandii* extracts. All values are exhibited as mean ± S.E.M.

Table 3. Antioxidant activity of various extracts from *A. harmandii* stems.

Sample	TTA (mgGAE/g extract)	IC_{50} (mg/ml)			EC_{50} of reducing power (mg/ml)
		DPPH	ABTS	Anti-LPO	
AH-PET	0.56 ± 0.20	ND	ND	20.24 ± 1.15	18.08 ± 3.77
AH-EtOAc	10.03 ± 0.46	0.83 ± 0.01	2.98 ± 0.01	7.95 ± 0.56	0.97 ± 0.01
AH-EtOH	8.93 ± 0.39	2.05 ± 0.02	1.82 ± 0.02	17.21 ± 1.93	1.44 ± 0.05
AH-DW	6.23 ± 0.45	2.97 ± 0.11	3.25 ± 0.01	18.39 ± 1.43	5.23 ± 0.48
Gallic acid	-	0.67 ± 0.01	0.69 ± 0.00	-	0.07 ± 0.00
BHT	-	-	-	1.48 ± 0.03	-

All values are exhibited as mean ± S.E.M. ND is not detectable.

4. DISCUSSION

Currently, although modern pharmaceutical drugs are available and greatly effective in treating various diseases and promoting health, many people throughout the world still prefer to use traditional medicines because of their low cost and because of the belief that they have less adverse effects. Bioactivity of plant materials in prevention or promoting health depends on the phytochemicals produced by plants, so we investigated the varieties of phytochemicals in four extracts from the stems of *A. harmandii* using qualitative and quantitative methods.

Since each phytochemicals can be dissolved in different solvents, in this study the solvents in different polarities were used to extract *A. harmandii*. Detecting phytochemical compounds in the extracts of *A. harmandii* revealed that the AH-EtOH extract possessed a greater variety of secondary metabolites than other extracts (Table 1). The possession of considerable amounts of TPC, AAC and alkaloids was found in the AH-EtOH extract, while massive amounts of TFC and tannins were detected in the AH-EtOAc extract (Table 2). These results imply that each phytochemical can be dissolved in different solvents. The ethanol could extract more of the phytochemicals than other three solvents.

Even though phytochemicals naturally occur in small quantities [19], they have a broad spectrum in curing or preventing many diseases.

A wide variety of phytochemicals, especially TPC, TFC and AAC, isolated from plants, have been reported to have anti-cancer, anti-tumor, anti-diabetes, anti-inflammation, anti-virus and anti-bacteria [20-21]. A potential role of tannins, alkaloids and saponins as antioxidants was previously reported [22]. Therefore, the extracts which had high amounts of these bioactive compounds which were obtained in this study could be a powerful source for preventing degenerative disorder or promoting health.

We investigated antioxidant activity in four extracts of *A. harmandii* using various assays. Total antioxidant, $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ scavenging activities and reducing power are simple, rapid, sensitive and reproducible assays for investigating antioxidant properties in plant extracts, beverages and foods [23]. From our results, the highest antioxidants were found in the AH-EtOAc extract in all assays, except $ABTS^{\bullet+}$ scavenging activity which were found in the AH-EtOH extract. The antioxidant properties of the *A. harmandii* extracts found in this study may be due to the presence of phytochemicals within the extracts. A correlation between phytochemical constituents and efficacy of extracts to inhibit free radicals was found. The extracts which exhibited potent antioxidant efficacies had high amounts of TPC, TFC, AAC, alkaloids and tannins. Anti-LPO efficacy of the investigated extracts also depended on the amount of TPC, TFC and AAC. It is clear that a significant efficacy of extracts investigated in this study mainly come from phytochemicals like TPC, TFC and AAC. Previous studies showed significant correlations between TPC or TFC and antioxidant activity [24]. There are hydroxyl groups in the structures of phenolics and flavonoids [25]. Hydroxyl groups attribute to the human defense system by donating a hydrogen molecule to ROS including superoxide anion radicals, hydroxyl radicals, and lipid peroxy radicals [26].

Formation of lipid hydroperoxide from LPO can damage to every molecule especially DNA, causing DNA strand breaking, mutation and carcinogenesis [27]. The anti-LPO efficacies of AAC, TPC and TFC have been reported in a variety of plants [28]. These compounds may inhibit LPO via different mechanisms. Therefore, the underlining mechanisms of antioxidant activity of *A. harmandii* extracts are reducing, neutralizing, quenching, and decomposing peroxide which eliminate free radicals by acting as antioxidants [29]. The anti-LPO properties of *A. harmandii* can prevent various pathological events such as inflammation, atherosclerosis and liver injury induced by LPO [30]. From the phytochemical and antioxidant results obtained from this study, we suggest that the AH-EtOAc and AH-EtOH extracts act as potent antioxidants and probably have the ability to prevent oxidative-related diseases.

5. CONCLUSIONS

It could be concluded that ethyl acetate and ethanolic extracts from the stems of *A. harmandii* possessed high amounts of TPC, TFC and AAC. They exhibited the antioxidants and free radical scavenging activities. These results indicate that *A. harmandii* can be a powerful source of natural antioxidant for preventing oxidative-related degenerative diseases and promoting health. Further study is needed to isolate bioactive compounds from *A. harmandii* and determine the antioxidant mechanisms in an *in vivo* model.

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