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Chemical Constituents and Antioxidant Activity of *Hydnophytum formicarum* Jack.

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Abstract: *Hydnophytum formicarum* Jack., a medicinal plant possesses diverse bioactivities. Herein, inorganic and organic constituents including antioxidant property of its tuber extracts are reported. Analysis of the extracts by ICP-AES, twenty-two elements (Be, Al, Ca, Cr, Mn, Fe, Zn, Ba, P, Li, Sr, Rb, Hg, Tl, In, Pb, Cd, As, Cs, Na, K and Mg) were found. Among these are common essential elements e.g., Mn, Fe, Zn and Cr with important roles in life. Repeated chromatographic isolations of methanol extract afforded sodium and potassium chlorides. Bioactive β-sitosterol was found in hexane and chloroform extracts. Significantly, radical scavenging activity of the extract derived from different growing areas exhibited comparable activity with IC_{50} range 8.40-8.79 μg mL^{-1}. The findings provide data to support the use of *H. formicarum* Jack. as a traditional medicine.

Key words: *Hydnophytum formicarum* Jack., antioxidants, essential elements, ICP-AES

INTRODUCTION

Hydnophytum formicarum Jack. (Rubiaceae) has a long history of uses, in the Thai folk remedy, as a combination with other medicinal plants. Its tuber has cardiovascular, anti-inflammatory and antiparasitic effects (Prommee, 1988) as well as has been used for treatment of cancer (Itharat *et al*., 2004), hepatitis, rheumatism, diarrhea (Nguyen *et al*., 2004; Ueda *et al*., 2002) and headache (Beckstrom-Sternberg *et al*., 1994). The plant species has been reported as a potent source of natural antioxidants constituting flavonoids and phenolic compounds (Prachayasittikul *et al*., 2008) e.g., isoliquiritigenin, butin, butein, protocatechualdehyde including stigmasterol. All of which were isolated from the *H. formicarum* Jack. extracts (hexane, dichloromethane and ethyl acetate). Phenolic compounds constitute one of the most abundant groups of natural metabolites and are synthesized by plants for self-protection from biological and environmental stresses (Ahmad *et al*., 2010). Besides a variety of pharmacological active compounds, medicinal plants contain essential and trace elements (Anhwange *et al*., 2004; Shar *et al*., 2002; Mahmud *et al*., 2002) that can be available to the human body from the consumption of herbs and their extracts (Queralt *et al*., 2005). This leads to determine inorganic constituents of the *H. formicarum* Jack. extracts using an Inductively Coupled Plasma-Atomic Emission Spectrometry, ICP-AES (El-Sayed *et al*., 2011; Moshki *et al*., 2012). Considering the literature reports therefore, isolations of constituents from methanol plant extract was investigated. Our previous study showed that the ethyl acetate extract of *H. formicarum* Jack. was the most potent antioxidant. Thus, the antioxidant activity (Aisha *et al*., 2011; Geethalakshmi *et al*., 2010; Gill *et al*., 2009; Uddin *et al*., 2008) of the plant extracts obtaining from different growing areas was compared.

MATERIALS AND METHODS

General: Melting points were determined on the Electrothermal melting point apparatus (Electrothermal

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9100) and are uncorrected. ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian XL-300 MHz using deuterochloroform solution with tetramethylsilane as an internal standard. Mass spectra were determined using a Finnigan 4021 (Data System InCos 2100). Infrared spectra (IR) were obtained on a Perkin Elmer System 2000 FTIR. Inorganic constituents were analyzed by the Inductively Couple Plasma-Atomic Emission Spectrometry (ICP-AES), SPS 7000, Seiko Instruments. Column chromatography was carried out using silica gel 60 (0.063-0.200 mm) and silica gel 60 (particle size less than 0.063 mm). Thin Layer Chromatography (TLC) was carried out on silica gel 60 PF_{254} (cat. No. 774 E., Merck). 2,2-diphenyl-1-picrylhydrazyl (DPPH) and α-tocopherol were obtained from Sigma Chemical Co. (USA).

Plant material: Tubers of *H. formicarum* Jack. were collected from Makham district, Chanthaburi Province and Khlong Takrao district, Sa Kaeo Province and have been identified (BKF 135252) by The Forest Herbarium, Royal Forestry Department, Bangkok. The voucher specimens have been deposited at the Department of Chemistry, Faculty of Science, Srinakharinwirot University, Bangkok, Thailand.

Extraction: The milled air dried tubers of *H. formicarum* Jack. (4 kg), collected from Chanthaburi Province, were extracted twice with hexane 8 L (7 days) at room temperature, followed by filtration. The combined filtrate was evaporated *in vacuo* to give the hexane extract (30 g). Similar extractions were conducted using chloroform and methanol to afford the corresponding chloroform (59 g) and methanol extracts (92 g).

Elemental analysis: The analysis of plant extract was performed by the ICP-AES using argon as plasma gas, carrier gas and auxiliary gas with flow rate of 6-7 L min^{-1}. The plant extracts (hexane 280, chloroform 230 and methanol 580 mg) were digested by conc H_2SO_4 (2 mL) and H_2O_2 (5 mL) at 300°C for 5 h. After cooling, distilled water was added to make a total volume of 100 mL, then filtered to obtain the solutions for the analysis. Each plant extract was injected to the analyzer in triplicates.

Isolation: The hexane extract (10 g) was isolated and purified by a silica gel (300 g) column, then eluted with increasing polarity of solvents. Fractions were collected and combined as appropriate based on TLC chromatograms; hexane: $CHCl_3$ (3:7) gave a solid (7 g) which was re-separated by silica gel (130 g) column. Elution with $CHCl_3$: MeOH (7:3) provided a viscous oil (4.8 g). Recrystallization from methanol afforded β-sitosterol (90 mg) of m.p. 138-140°C (Lit m.p. 141°C (Pouchert and Bekke, 1993). ^{1}H- and ^{13}C-NMR, MS and IR spectral data were recorded. The chloroform extract (21 g) was separated by the silica gel (400 g) column to give a solid (4 g) from $CHCl_3$: MeOH (7:3) elution. Recrystallization from ethyl acetate gave an unidentified solid (70 mg). The filtrate was evaporated to dryness and recrystallized from methanol to afford β-sitosterol (5 mg). The methanol extract (30 g) was separated by the silica gel (600 g) column, eluting by $CHCl_3$: MeOH (4:6) provided a brown solid (15 g) which was re-separated by silica gel column to give 4.1 g of solid (A), mp>250°C.

Inorganic analysis: The solid A was tested with a solution of $AgNO_3$, white precipitate of AgCl was observed. When A was tested with a solution of zinc uranyl acetate, a yellow precipitate of zinc uranyl sodium acetate was formed. Similarly, the solid A gave a yellow precipitate of potassium hexanitro-cobaltate when tested with a solution of sodium hexanitro cobaltate (Vogel, 1968).

Antioxidant assay (DPPH): When DPPH (a stable purple color radical) reacts with an antioxidant, it is reduced to form a light-yellow colored of diphenylpicrylhydrazine which can be spectrophotometrically recorded. A solution of DPPH (0.1 mM) was prepared in methanol. After an incubation of the DPPH solution and sample for 30 min, an absorbance was measured using UV-Visible spectrophotometer (UV-1610, Shimadzu) at 517 nm. The percentage of radical scavenging activity was calculated from the following equation:

$$\text{Radical scavenging activity (\%)} = \left(1 - \frac{\text{Abs.}_{\text{sample}}}{\text{Abs.}_{\text{control}}}\right) \times 100$$

where, $\text{Abs.}_{\text{control}}$ is the absorbance of the control reaction and $\text{Abs.}_{\text{sample}}$ is the absorbance of the tested compound. α-Tocopherol was used as a control (Prachayasittikul *et al*., 2010). The ethyl acetate extracts for this study were prepared from the plants collected from Chanthaburi and Sa Kaeo provinces, as described previously (Prachayasittikul *et al*., 2008).

RESULTS AND DISCUSSION

The ICP-AES results showed that the *H. formicarum* Jack. extracts (hexane, chloroform and methanol) contained twenty-two elements (Table 1). Six essential elements (Mn, Fe, Zn, Ca, Cr and P) were present in ppm levels in the hexane extract. A therapeutic element, Li was contained in the chloroform extract. On the other hand,

Table 1: Elements of *H. formicarum* Jack.

Sample	Element (ppm)[a]								
Hexane extract	Be	Al	Ca	Cr	Mn	Fe	Zn	Ba	P
	0.15	1.47	0.74	0.23	0.14	0.20	0.05	0.09	7.57
Chloroform extract	Li	Sr	Rb	Hg	Tl	In	Pb	Cd	As	Cs
	0.34	0.03	8.31	1.18	9.67	9.42	2.59	0.08	4.99	1910.00
Methanol extract	Na		K		Mg		Mn		Fe	
	35.60		40.60		0.49		0.02		0.19	

[a]Approximate values

heavy toxic metals e.g., Hg, Pb and Cd were found in the chloroform extract together with the highest content of Cs (1910 ppm). The methanol extract constituted K and Na as major essential metal ions including ions of Mg, Mn and Fe.

It is notable that the elements found in this medicinal plant have vital roles in life e.g., ions of K, Ca, Mg, Fe and Zn are essential to all organisms with the possible exception of blue green algae (in case of K^+) (Singh *et al*., 2010; Tounekti *et al*., 2010; Ranade-Malvi, 2011; Rodriguez-Navarro and Rubio, 2006). Recently, K^+ has been reported to have direct synergistic effect with two ionic micronutrients namely, Fe and Mn (Ranade-Malvi, 2011). Arsenic plays a role in metabolism of methyl compounds. Deficiency of the arsenic will impair growth reproduction and heart function (Singh *et al*., 2010). Other elements, for example Fe, Cu, Mn and Co are important components of many antioxidant processes (Slavica *et al*., 2005). Toxicities of heavy toxic metals like Pb, Hg and Cd depend on the allowed daily intake amount (Singh *et al*., 2010).

The methanol extract of the plant species was isolated and purified by repeated silica gel column and recrystallization to provide a solid A (4.1 g) with m.p.>250°C, highly water soluble but insoluble in organic solvents. Its IR spectra showed no absorption bands of any functional groups. This observation suggested that the solid A was likely to be inorganic compounds. Thus, the inorganic analysis was conducted (Vogel, 1968). The results suggest that the solid A possibly contains sodium and potassium chlorides. This is in accorded with the ICP-AES analysis that the methanol extract contained Na (35.60 ppm) and K (40.60 ppm).

Additionally, the hexane and chloroform plant extracts were isolated and purified by silica gel column to give β-sitosterol in 90 and 5 mg, respectively. Its structure was confirmed by comparing the spectral data (data not shown) with that of an authentic sample (Pouchert and Bekke, 1993). Previously, the methanol extract of *H. formicarum* Jack. was reported to contain a mixture of stigmasterol and β-sitosterol, hydroxybenzoic acid ester, resorcine and 7,3′,5′-trihydroxyflavanone. Some of these exhibited antiproliferative activity (Hasmah *et al*., 2008). β-sitosterol is the bioactive compound with antiinflammatory and antipyretic effects (Gupta *et al*., 1980) as well as antihypercholesterolemic activity (Ikeda *et al*., 1981).

Table 2: Radical scavenging activity (DPPH) of *H. formicarum* Jack.

Ethyl acetate extract	DPPH activity, IC_{50} (μg mL^{-1})
Chanthaburi	8.79
Sa Kaeo	8.49
Drug store	8.40[a]

[a]From (Prachayasittikul *et al*., 2008)

Our previous study showed that the ethyl acetate extract displayed the highest radical scavenging activity (Prachayasittikul *et al*., 2008). To determine whether the plant species collected from different growing areas will exhibit the same or different antioxidant activity. Thus, the radical scavenging activity (DPPH) of the plant ethyl acetate extracts; collected from Chanthaburi and Sa Kaeo were investigated to compare with the one obtained from the drug store (Prachayasittikul *et al*., 2008). Significantly, the extracts (Table 2) of *H. formicarum* Jack. from three different sources displayed comparable antioxidant activity with IC_{50} range 8.40-8.79 μg mL^{-1}. This is crucial for its efficacy as traditional medicine.

It was reported that antioxidant compounds exerted their activity through radical scavenging capacity and metal binding catalyst (Prachayasittikul *et al*., 2008; Ahmad *et al*., 2010; Kaur *et al*., 2008). From the analysis results of hexane, chloroform and methanol extracts (Table 1), it could be assumed that those containing inorganic constituents may originate as metal ligands (electron donor groups) coordinated compounds. Similarly, the ethyl acetate extract of phenolic compounds with the highest antioxidative activity (Prachayasittikul *et al*., 2008). could possibly contain some inorganic ions.

CONCLUSION

The study describes the presence of essential metal ions and bioactive sterol of *H. formicarum* Jack.. The significant antioxidant potency of the *H. formicarum* Jack. collected from different areas is observed. This provides data to support the use of *H. formicarum* Jack. as the Thai traditional medicine.

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