Molecules **2008**, *13*, 904-921

molecules

ISSN 1420-3049

www.mdpi.org/molecules

Full Paper

Antimicrobial and Antioxidative Activities of Bioactive Constituents from *Hydnophytum formicarum* Jack.

Supaluk Prachayasittikul [1,*], Prasit Buraparuangsang [1], Apilak Worachartcheewan [2], Chartchalerm Isarankura-Na-Ayudhya [2], Somsak Ruchirawat [3] and Virapong Prachayasittikul [2,*]

[1] Department of Chemistry, Faculty of Science, Srinakharinwirot University, Sukhumvit 23, Bangkok 10110, Thailand

[2] Department of Clinical Microbiology, Faculty of Medical Technology, Mahidol University, Bangkok 10700, Thailand

[3] Laboratory of Medicinal Chemistry, Chulabhorn Research Institute, Vipavadee Rangsit Highway, Bangkok 10210, Thailand

* Authors to whom correspondence should be addressed; S. Prachayasittikul: E-mail: supaluk@swu.ac.th; Tel. 662-664-1000 ext. 8209, Fax 662-259-2097; V. Prachayasittikul: E-mail: mtvpr@mahidol.ac.th; Tel. 662-418-0227, Fax 662-412-4110

Received: 1 April 2008; in revised form: 17 April 2008 / Accepted: 17 April 2008 / Published: 18 April 2008

Abstract: *Hydnophytum formicarum* Jack. (Rubiaceae) is a medicinal plant whose tubers possesses cardiovascular, anti-inflammatory and antiparasitic effects and have been used for the treatment of hepatitis, rheumatism and diarrhea. Herein we report the isolation of its active constituents and the testing of their antimicrobial activity against 27 strains of microorganisms using an agar dilution method and of their antioxidative activity using the DPPH and SOD assays. The results show that the crude hexane, dichloromethane, ethyl acetate and methanol extracts exert such activities. Particularly, the crude ethyl acetate extract exhibits antigrowth activity against many Gram-positive and Gram-negative bacteria with MIC 256 μg/mL. *Shewanella putrefaciens* ATCC 8671 is completely inhibited at a lower MIC (128 μg/mL). Interestingly, *Corynebacterium diphtheriae* NCTC 10356 is inhibited by all the tested extracts. Significantly, the ethyl acetate extract is also the most potent antioxidant, showing 83.31% radical scavenging activity with IC_{50} 8.40 μg/mL in the DPPH assay. The other extracts display weak to moderate antioxidative

activities, ranging from 28.60-56.80% radical scavenging. The SOD assay shows that methanol extract exhibits the highest activity (74.19% inhibition of superoxide radical). The dichloromethane and ethyl acetate extracts display comparable SOD activity. The promising bioactivities of the crude ethyl acetate extract guided the first isolation of bioactive flavonoid and phenolic compounds: isoliquiritigenin (**2**), protocatechualdehyde (**3**), butin (**4**) and butein (**5**) from this species. Their structures have been fully established by 1D and 2D NMR. In addition, stigmasterol was isolated from the crude hexane and dichloromethane extracts. The antimicrobial and cytotoxic activities of compounds **3-5** were evaluated. The tested compounds were inactive against HuCCA-1 and KB cell lines, showing ED_{50}> 10 μg/mL. Protocatechualdehyde (**3**) completely inhibits the growth of *Plesiomonas shigelloides* with MIC ≤60 μg/mL. As a result, we propose that *Hydnophytum formicarum* Jack. can serve as a new source enriched with potent antioxidative and antimicrobial agents.

Keywords: *Hydnophytum formicarum* Jack., antimicrobial, antioxidative and cytotoxic activities.

Introduction

Hydnophytum formicarum Jack. or ant plant, a medicinal plant of the Rubiaceae family [1, 2], is known in Thai as Hua roi roo [3] as well as by other local names. It is commonly found in the East and South of Thailand, South-East Asia, Papua New Guinea and Pacific Islands [2]. Its tuber possesses cardiovascular, antiinflammatory and antiparasitic effects, and is used for the relief of skin rashes and as a neurotonic [4], and as a treatment of headache [5], hepatitis, rheumatism and diarrhea [6, 7].

Hydnophytum formicarum Jack. has several branches with opposite fleshy, oval leaves and small sessile, axillary white flowers and red berries [1]. The typical size varies from 10-12 cm to 30-50 cm [8]. The tubers' cavities have one or more entrance holes communicating with the outside, through which it absorbs both water and substances to obtain essential nutrients from faecal material and debris brought in by ants. In return the plants provide food for the ants in a form of nectar or other protein-rich sources and for this reason *Hydnophytum formicarum* Jack. is well known as the ant plant.

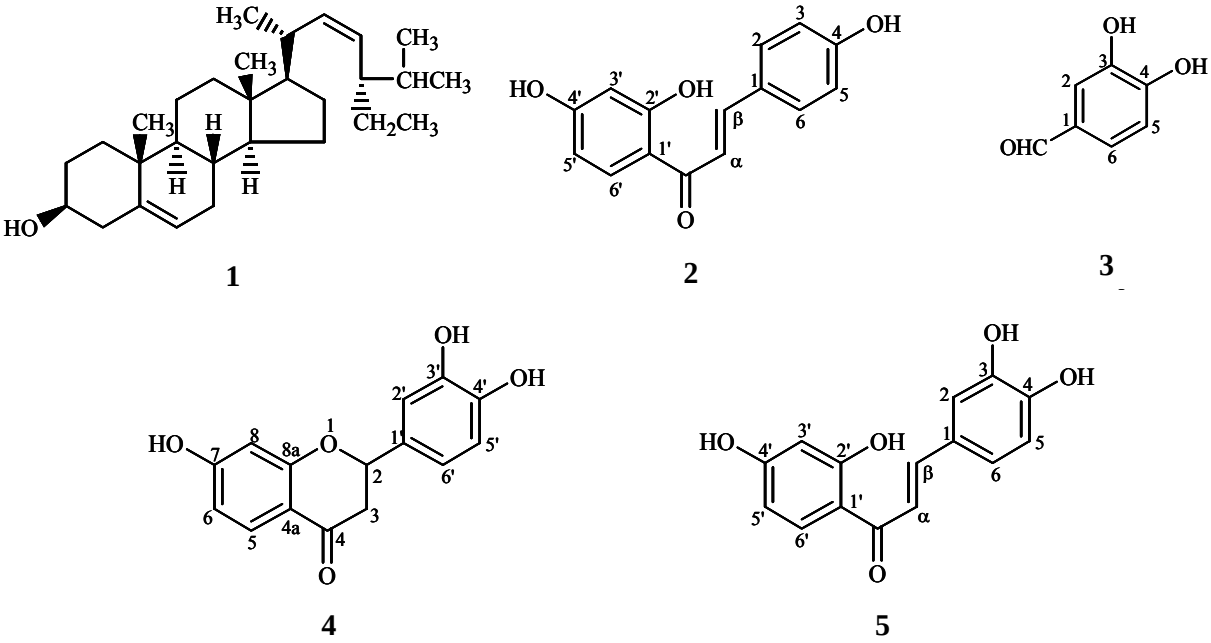
The plant has a long tradition of use in Thai traditional medicine as a mixture with other plant extracts for the treatment of diabetes. However, no chemical isolation and characterization of the components of this medicinal plant have been reported. The xanthine oxidase inhibitory activity of the *Hydnophytum formicarum* Jack. was previously reported [6]. Additionally, its antiproliferative activity against human HT-1080 fibrosarcoma cells was described [7]. The many interesting reports concerning the medicinal value of *Hydnophytum formicarum* Jack. as a folk remedy and its diverse reported biological activitives prompted us to investigate the bioactive ingredients of the tubers of *Hydnophytum formicarum* Jack to search for new sources of medicinal agents. Herein, we report the isolation, structure elucidation and biological evaluation of the antimicrobial and antioxidative activities , including cytotoxic effects, of the identifed components.

Results and Discussion

Isolation and structure elucidation

The crude hexane extract of dried *Hydnophytum formicarum* Jack. tubers was repeatedly chromatographed on a silica gel column using gradient elution with hexane-acetone mixtures to give a green gum containing a white solid. Recrystallization provided stigmasterol (**1**) of m.p. 152 – 153°C [9]. The structure was confirmed by comparison of its ^{1}H- and ^{13}C-NMR spectral data with that of an authentic sample. Similarly, the crude dichloromethane extract was isolated and purified as described to give the stigmasterol (**1**). The crude ethyl acetate extract was separated by repeated silica gel column and preparative TLC using gradient elution to obtain four compounds: isoliquiritigenin (**2**), protocatechualdehyde (**3**), butin (**4**) and butein (**5**) (Figure 1), all of which have been isolated from *Hydnophytum formicarum* Jack. for the first time. The structures of **2-5** were elucidated using UV, IR, and comparison of their ^{1}H- and ^{13}C-NMR with the literature data [10-18]. In this study, 2D NMR spectral data was also obtained.

Figure 1. Structures of compounds **1 – 5**.



Biological activities

Antimicrobial activity

The crude hexane, dichloromethane, ethyl acetate and methanol extracts, and the isolates **3-5** from *Hydnophytum formicarum* Jack. were tested for antimicrobial activity against 27 strains of microorganisms using the agar dilution method [19]. The results (Table 1) show that all the tested extracts completely inhibit the growth of the Gram-positive bacterium *C. diphtheriae* NCTC 10356 with MIC 256 μg/mL. This strain was the most sensitive, as it was the only one inhibited by the crude hexane and dichloromethane extracts. Other microorganisms: *A. xylosoxidan* ATCC 2706, *S. aureus*

ATCC 25923, *M. lutens* ATCC 10240, *S. dysenteriae*, *S. pyogenes* II, *A. hydrophila* and *B. cereus* were completely inhibited by the crude ethyl acetate extract, with MIC 256 μg/mL. The ethyl acetate extract completely inhibits the growth of *S. putrefaciens* ATCC 8671 with a low MIC (128 μg/mL). In addition, the crude methanol extract displays antigrowth activity against *S. pyogenes* II and *B. cereus*, with MICs of 256 μg/mL.

The antimicrobial activity of pure compounds **3 – 5** isolated from the crude ethyl acetate extract was similarly evaluated. It was found (Table 1) that only compound **3** displayed complete inhibiton against *P. shigelloides*, with MIC ≤ 60 μg/mL. In comparison ampicillin, a positive control, showed antigrowth activity against *P. shigelloides* at MIC 10 μg/mL.

Table 1. Antimicrobial activity of *Hydnophytum formicarum* Jack.

Compound[a,b]	**Microorganism**	**Gram**	**MIC** [c] (μg/mL)
Crude hexane extract	*C. diphtheriae* NCTC 10356	+	256
Crude dichloromethane extract	*C. diphtheriae* NCTC 10356	+	256
Crude ethyl acetate extract	*C. diphtheriae* NCTC 10356	+	256
	A. xylosoxidan ATCC 2706	-	256
	S. aureus ATCC 25923	+	256
	M. lutens ATCC 10240	+	256
	S. dysenteriae	-	256
	S. pyogenes II	+	256
	A. hydrophila	-	256
	B. cereus	+	256
	S. putrefaciens ATCC 8671	-	128
Crude methanol extract	*C. diphtheriae* NCTC 10356	+	256
	S. pyogenes II	+	256
	B. cereus	+	256
Compound **3**	*P. shigelloides*	-	≤ 60
Ampicillin	*P. shigelloides*	-	10

a: compound **4** was tested at 64 μg/mL, no growth inhibition.

b: compound 5 was tested at 115 μg/mL, no growth inhibition.

c: minimum inhibitory concentration

Antioxidant activity

The antioxidant activity of the crude extracts was tested using the DPPH (2,2-diphenyl-1-picrylhydrazyl) [20] and SOD (superoxide dismutase) [21] assays. The results (Table 2) demonstrate that all the tested crude extracts possess some antioxidative activity. In the DPPH assay, the crude ethyl acetate extract at 333.33 μg/mL exhibits highest radical scavenging activity (83.31 %) with IC_{50} of 8.40 μg/mL, while *α*-tocopherol, a positive control, shows antioxidative activity with IC_{50} 6.67 μg/mL (Figure 2). The crude dichloromethane and methanol extracts showed moderate activity (55.70

and 56.80 % radical scavanging, respectively). The nonpolar crude hexane extract is a weak antioxidant, showing 28.60% radical scavenging activity.

The SOD activity at 300 μg/mL shows that the crude methanol extract exhibits the highest (74.19 %) inhibition of superoxide anion. The native SOD, a positive control (7.6 U) exhibits 75.98 % inhibition. The crude dichloromethane and ethyl acetate extracts exert moderate SOD activity (62.24 and 67.91% inhibition, respectively) as shown in Figure 3. The hexane extract exhibits weak SOD activity.

Table 2. Antioxidative activity of *Hydnophytum formicarum* Jack.

Compound	**Radical scavenging activitya (%) (333.33 μg/mL)**	**Superoxide dismutase activityb (%) (300 μg/mL)**
Crude hexane extract	28.60	25.86
Crude dichloromethane extract	55.70	62.24
Crude ethyl acetate extract	83.31	67.91
Crude methanol extract	56.80	74.19

a: α-Tocopherol was used as a positive control.

b: Native SOD (7.6 U) from bovine erythrocytes was used as a positive control.

Figure 2. Radical scavenging activity of α-tocopherol and ethyl acetate extract.

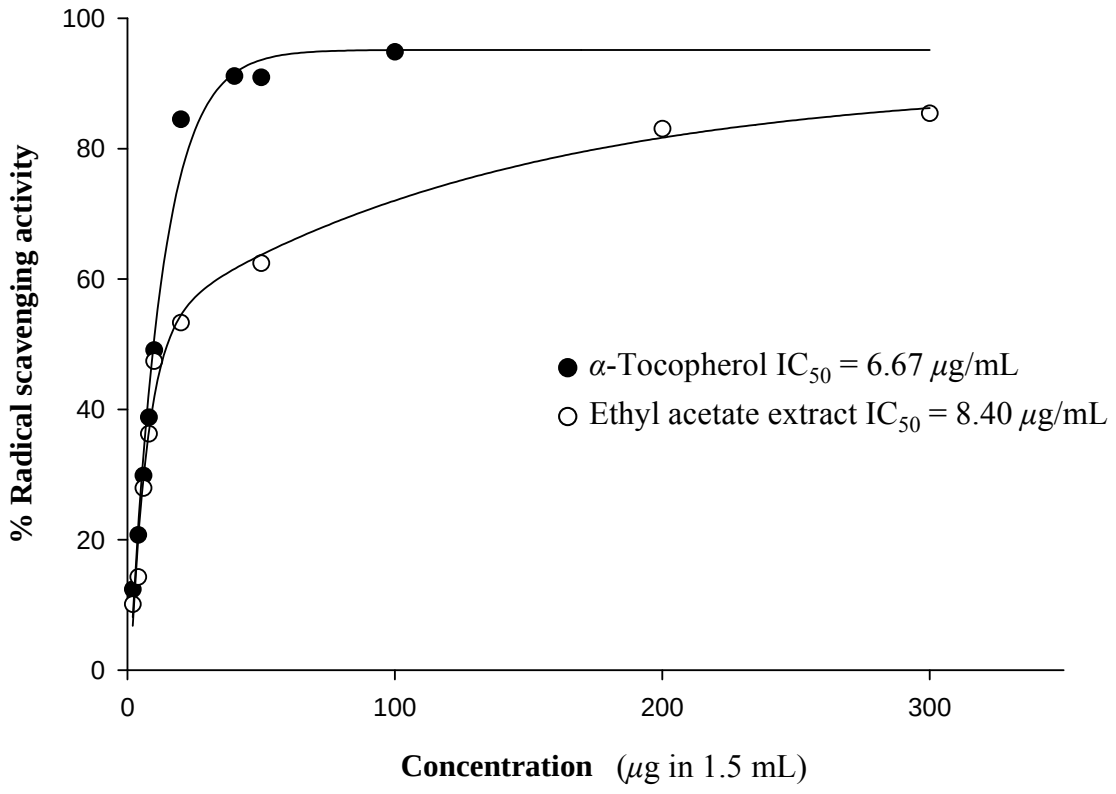
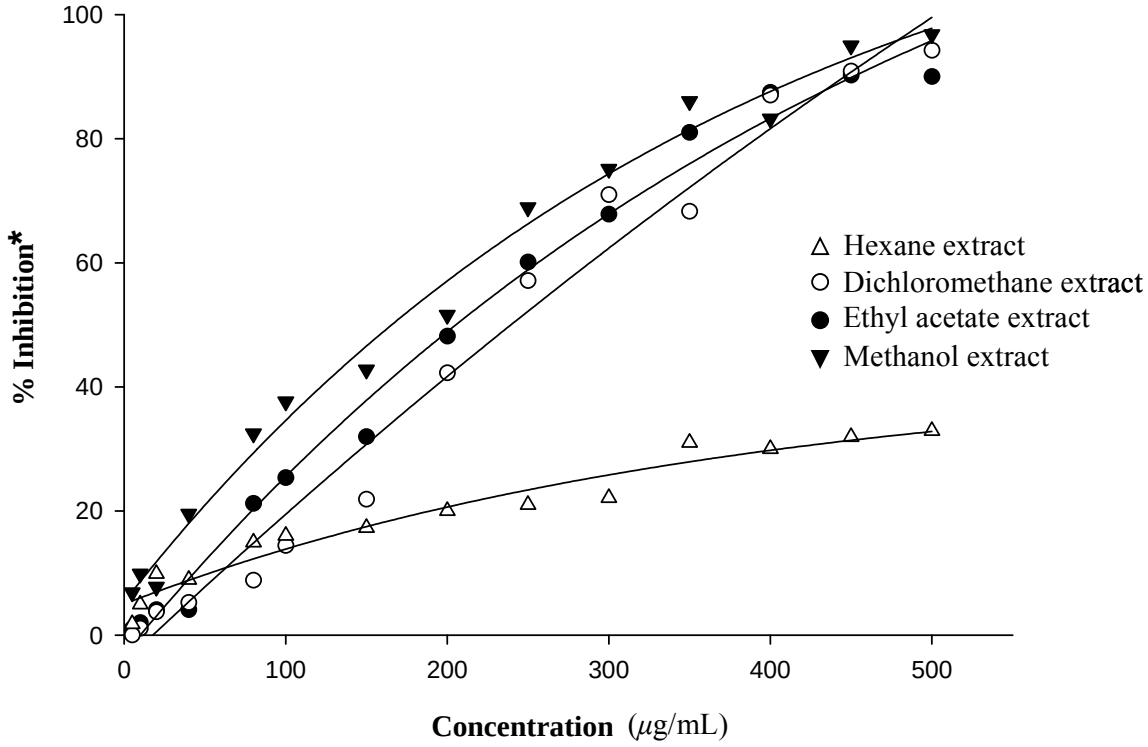


Figure 3. Superoxide dismutase activity of crude extracts.



*Note: Native SOD (7.6 U) = 75.98% inhibition

Cytotoxic activity

The isolates **3, 4** and **5** were evaluated for cytotoxic activity [22] against two cell lines: HuCCA-1 and KB, using etoposide as a positive control. It was observed that the tested compounds **3 – 5** (Table 3) were all inactive, showing ED_{50} > 10 μg/mL.

Table 3. Cytotoxic activities of compounds **3 – 5.**

Compound	**ED_{50} (μg/mL)***	
	HuCCA-1 cell line	**KB cell line**
3	>10	>10
4	>11	>11
5	>11	>11
Etoposide	4.0	0.25

* ED_{50} ">" indicates that compounds are inactive at that concentration or inhibit cell growth less than 50%.

The antimicrobial and antioxidative assay results (Tables 1 and 2) have shown that all the tested extracts exert such some biological activity. Particularly, the crude ethyl acetate extract exhibited antigrowth activity against many Gram-positive and Gram-negative bacteria with MIC 256 μg/mL,

while *S. putrefaciens* ATCC 8671 is completely inhibited at a lower MIC of 128 μg/mL. It is interesting to note that all the tested extracts can inhibit the growth of *C. diphtheriae* NCTC 10356. However, antigrowth activity against the yeasts tested, *S. cereviseae* ATCC 2601 and *C. albican*s ATCC 90028, was not observed.

The antioxidative activity (DPPH assay) of the tested extracts indicated moderate to high (55.70-83.31 %) radical scavenging activities, except for the hexane extract, is a weak antioxidant (28.60 % radical scavenging). Significantly, the ethyl acetate extract is the most potent in this respect, exhibiting 83.31% radical scavenging activity with IC_{50} 8.40 μg/mL. In the SOD assay the methanol extract exerted the highest inhibition (74.19 %) of superoxide radical. It is interesting to note that the activity increases with higher polar solvent extracts. It was reported that methanol and methanol-water extracts of *Hydnophytum formicarum* Jack. showed xanthine oxidase inhibitory (XO) activity (10.3 and 32.5%, respectively) at 50 μg/mL, while its water extract at 100 μg/mL exhibited < 0.1% XO activity [6]. In addition, methanol and methanol-H_2O (1:1) extracts of *Hydnophytum formicarum* Jack. displayed potent antiproliferative activity against human lung A549 adenocarcinoma cell, with an ED_{50} value of less than 4 μg/mL. Such activity was observed against other cell lines, such as highly metastatic human HT-1080 fibrosarcoma, human cervical HeLa and murine colon 26-L5 carcinoma cells [7]. Our findings have led to the first isolation of flavonoid and phenolic pure compounds **2-5** from the ethyl acetate extract of *Hydnophytum formicarum* Jack. so it is conceivable that the potent antioxidative activity (DPPH assay) of the ethyl acetate extract may result from these constituents **2-5**. Their structures have been fully established by 1D and 2D NMR. Additionally, stigmasterol was also isolated from the crude hexane and dichloromethane extracts.

The isolated compounds **3-5** were tested for antimicrobial and cytotoxic activities. The tested compounds were inactive against HuCCA-1 and KB cell lines, ED_{50} >10 μg/mL. Only protocatechualdehyde (**3**) exhibits antigrowth activity against *P. shigelloides*, with MIC ≤60 μg/mL. Due to the limited quantity available of the isolated compounds, compounds **4** and **5** were tested at 64 and 115 μg/mL, respectively. No antimicrobial activity was observed at these concentrations.

Isoliquiritigenin (**2**) was first found in nature in 1953 from *Dahlia variabilis* (Compositae) [23]. It was also isolated from *Glycyrrhiza glabra* (licorice) [24], *Sinofranchetia chinensis* [25] and recently from *Broussonetia papyrifera* (paper mullery) [26]. Additionally, isoliquiritigenin glycoside was isolated from woody parts of the thickening root of *Glycyrrhiza glabra* [27].

Diverse biological activities of isoliquiritigenin were reported. For example, it exhibited effects on cardiac [28] and aortic smooth muscle [29], and showed anti-platelet [30], vasorelaxant [31], antioxidant [32] and antitumor activities [33]. Particularly, isoliquiritigenin was very potent antioxidant toward LDL oxidation [24], as well as, xanthine oxidase inhibitor [25] and tyrosinase inhibitor [26]. It was first reported to inhibit proliferation and induce apoptosis of human hepatoma (Hep G2 cells) via p53-dependent pathway [34]. Inhibition of tumor necrosis factor-α (TNF-α) on the induction of ROS production and neutrophil adhesion to endothelial cell was also described [35]. Moreover, its biological activities including the decrease of prostaglandin E2 and nitric oxide production in RAW 264.7 mouse macrophages cell [36], potent antitumor activity and inhibition of prostate cancer cell [37] were documented.

Our studies show that protocatechualdehyde (**3**) exhibits complete antigrowth activity against *P. shigelloides*, with MIC ≤ 60 μg/mL. Recently, the phenolic compound **3** has been reported to

significantly inhibit the growth of *Oenococcus oen*i VF [38]. Such compound was isolated from fruit of *Amomun tsao-ko* [39], root of *Salvia miltiorrhiza* [40], *Salvia officinalis* [41] and fruit of *Ganoderma applanatum* [42]. It exerted diverse biological activities e.g. antiproliferative, antioxidant, antitumor [43], reduced blood glucose level and inhibited lens opacity in streptozotocin induced diabetic cataract in rats [44] and inhibited HIV-1 replication in acute cytolytic infection of CEM cells [45].

The specific rotation of butin (**4**) was –87° and was first found in *Hydnophytum formicarum* Jack. Previously, butin was isolated from the seeds of *Butea frondosa* and was shown to possess postcoital antiimplantation and anticonceptive activities in the pregnant rats when administered orally during the implantation period [46]. Furthermore, butin was found in *Vernonia anthelmintica* Willd and in dried vine stem of *Spatholobus suberectus* Dunn [47], which showing the greatest (80.1%) anti-tyrosinase activity of human melanocytes.

Butein was isolated from *Butea frondosa* [48], *Viguiera multiflora* (Nutt.) [49], *Baeria chrysostoma* (F. and M.) [49], *Cosmos sulphureus* [50], *Dahlia variabilis* [49], *Coreopsis maritima* [51], *Coreopsis gigantea* [51], heartwood of *Dalbergia odorifera* T. [52] and from *Rhus verniciflua* Stokes [53]. This report constitutes the first isolation of butein (**5**) from *Hydnophytum formicarum* Jack. It was reported that butein served as a powerful antioxidant against lipid and LDL peroxidation by its free radical scavenging action and metal ion chelation [52, 54-56]. Moreover, butein exhibited other bioactivities such as anti-inflammatory [53, 57-58], strong aromatase inhibitor with IC_{50} less than 5 μM (3.75 μM) [59], inhibited TNF-α [58], inhibited cyclooxygenase 2 by lipopolysaccharide, inhibited nitric oxide production and inducible nitric oxide synthase (iNOS) [57], inhibited cAMP-specific phosphodiesterase [60] and angiotensin converting enzyme [61] as well as antinephritic effects [62], induced apoptosis in HL-60 cells [63] and B16 melanoma cells [64].

It is well known that stigmasterol is a phytosterol isolated from soy beans and other plants. Stigmasterol showed antioxidative activity, determined by the thiocyanate method [65], and lipid antioxidant properties [66]. In addition, stigmasterol significantly suppressed HMG-CoA reductase activity leading to approximately 11% reduction in plasma cholesterol levels in Wistar and WKY rats feeding 0.5 % stigmasterol [67].

According to the reported biological activities of isoliquiritigenin (**2**), protocatechualdehyde (**3**), butin (**4**), butein (**5**) and stigmasterol (**1**) our results provide remarkable support for the use of *Hydnophytum formicarum* Jack. as traditional medicine, e.g. for treatment of diabetes, rheumatism and diarrhea. Furthermore, *Hydnophytum formicarum* Jack. can be considered as a new natural source of the bioactive flavonoid and phenolic compounds **2-5**.

Conclusions

In conclusion, we have provided the experimental data to support the use of *Hydnophytum formicarum* Jack. as a herbal medicine. Significantly, the crude ethyl acetate extract exhibited potent antioxidative activity (DPPH assay), with IC_{50} 8.40 μg/mL, as well as antimicrobial activity. In addition, the other tested crude hexane, dichloromethane and methanol extracts show antimicrobial and antioxidative activities. The compounds **2**-5 isolated from the ethyl acetate extract are effective antioxidants and antimicrobials. Although these compounds **2-5** were previously isolated from other

plants, they have been first found in *Hydnophytum formicarum* Jack. by our group. Their structures have been fully established by 1D and 2D NMR. The compounds **3-5** were tested for antimicrobial and cytotoxic activities. The tested compounds were inactive against HuCCA-1 and KB cell lines, showing ED_{50} >10 μg/mL. Protocatechualdehyde (**3**) completely inhibits the growth of *P. shigelloides*, with MIC ≤60 μg/mL. As a result, the *Hydnophytum formicarum* Jack. could serve as a new natural source enriched with potent antioxidative and antimicrobial agents. Our isolates **2-5** are potential lead compounds to be further structurally modified leading to new and effective therapeutics. Other biological activities of *Hydnophytum formicarum* Jack. are being investigated.

Experimental

General

Melting points were determined on an Electrothermal melting point apparatus (Electrothermal 9100) and are reported without correction. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AM 400 instrument with a 400/100 MHz operating frequency using deuterochloroform or deuteromethanol solutions with tetramethylsilane as internal standard. Mass spectra were determined using a Finnigan MAT INCOS 50 mass spectrometer. Infrared spectra (IR) were obtained on Perkin Elmer System 2000 FTIR. Ultraviolet (UV) spectra were measured with Milton Roy Spectronic 3000 Array. Optical rotation was measured in methanol solution with sodium D line (590 nm) on JASCO DIP370 digital polarimeter. 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) and preparative Thin Layer Chromatography were carried out on silica gel 60 PF_{254} (cat. No. 7747 E., Merck).

Plant material

Tubers of *Hydnophytum formicarum* Jack. were purchased from the "Chao krom pur" traditional drug store, Bangkok. It has been identified (BKF 135252) by The Forest Herbarium, Royal Forestry Department, Bangkok. A voucher specimen has been deposited at Department of Chemistry, Faculty of Science, Srinakharinwirot University, Bangkok, Thailand.

Cell cultures

HuCCA-1 cells were established from chlolangiocarcinomas experimentally induced in hamsters. The cell lines were characterized and have been maintained in CRI laboratory ever since 1994 in Ham's F12 culture medium (GIBCO Laboratories, Grand Island NY) supplemented with 10% fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT), 100 U/mL penicillin and 100 μg/mL streptomycin. The KB cell lines, originally derived from epidermoid carcinoma of the floor of the oral cavity and commonly used as a reference laboratory standard for cytotoxicity assay, has been maintained in CRI laboratory in DMEM (Dulbecco's modified Eagle medium).

Extraction and Isolation

Extraction

The air-dried tubers of *Hydnophytum formicarum* Jack. (4 kg) were ground and extracted with hexane (10 L × 7 days × 3), followed by filtration. The filtrates were combined and evaporated to dryness under reduced pressure to obtain crude hexane extract. Similary, the extraction was carried out using dichloromethane, ethyl acetate and methanol to give the corresponding dichloromethane, ethyl acetate and methanol extracts.

Isolation

The crude hexane extract (15 g) was purified by column chromatography on silica gel. Elution was conducted initially with hexane gradually enriched with acetone. All fractions were collected, monitored by TLC and combined as appropriate. The solvents were evaporated to dryness *in vacuo* to afford 14 fractions (**H1 – H14)**. Fractions **H9** and **H10**, eluted by 3-5% acetone-hexane, were combined and evaporated to give green gum containing white crystals (2.7 g). This mixture was further separated by column chromatography over silica gel using gradient elution (hexane-acetone) to give a white solid. Recrystallization of the solid from hexane-ethyl acetate gave stigmasterol (**1**, 496 mg), m.p. 152-153^{o}C.

The crude dichloromethane extract (20 g) was subjected to silica gel column. Initial elution with hexane then gradually increasing with acetone and finally enriched with methanol gave fractions which were collected and combined according to TLC chromatogram. The solvents were evaporated to dryness to afford 18 fractions (**C1 – C18**). Fractions **C9** and **C10** (2.31 g) was repeatedly isolated by silica gel column. Elution with increasing polarity (hexane-acetone) afforded white amorphous compound which was recrystallized from hexane-ethyl acetate to give stigmasterol (**1**, 77 mg), m.p. 152-153^{o}C.

The crude ethyl acetate extract (59 g obtained from 10 kg of *Hydnophytum formicarum* Jack. which was extracted as described) was isolated by silica gel column. Elution with hexane, then gradually increasing polarity with acetone and finally enriched with methanol were performed. All fractions were collected and combined as usual using TLC monitoring. The solvents were evaporated to dryness to give 11 fractions (**E1 – E11**). Three selected main fractions (**E1 – E3**) were isolated and purified.

Fraction **E1** (13.51 g, yellowish green) was obtained by elution with 20% acetone-hexane. The mixture was subjected to silica gel column eluting with 2% methanol-dichloromethane, and then further purified by preparative TLC using 5% methanol-dichloromethane as a mobile phase to afford a yellow solid. Recrystallization from dichloromethane-methanol furnished *1-(2,4-dihydroxyphenyl)-3-(4-hydroxyphenyl)-2-propen-1-one* (2′,4,4′-trihydroxychalcone, isoliquiritigenin, **2**, 15 mg); yellow solid; m.p. 182-183˚C; (lit. 202°C [10], 193.5-195°C [11]); UV λ_{max} (MeOH) nm (log ε): 369 (7.42) (in methanol [13] 258 sh, 298 sh, 367); FTIR υ_{max} (KBr, cm^{-1}): 3514, 3288, 1634, 1589, 1514, 1371, 1229, 1175 (lit. [11]: 3380, 1626, 1607, 1585, 1543, 1370, 1343, 1320, 1287); ^{1}H-NMR (CD_3OD): δ 7.75 (1H, *d*, *J* = 15.4 Hz, H-*β*), 7.74 (1H, *d*, *J* = 8.8 Hz, H-6′), 7.47 (2H, *d*, *J* = 8.6 Hz, H-2,6), 7.36 (1H, *d*, *J* = 15.4 Hz, H-*α*), 6.80 (2H, *d*, *J* = 8.6 Hz, H-3,5), 6.36 (1H, *dd*, *J* = 8.8, 2.4 Hz, H-5′), 6.31

(1H, *d*, *J* = 2.4 Hz, H-3′); ^{13}C-NMR (CD_3OD): δ 191.92 (CO), 165.71 (C-4′), 164.36 (C-2′), 159.50 (C-4), 144.40 (CH-*β*), 131.72 (CH-6′), 130.48 (CH-2,6), 126.58 (C-1), 117.20 (CH-*α*), 115.90 (CH-3,5), 113.61 (C-1′), 108.18 (CH-5′), 103.08 (CH-3′); EIMS *m/z* (% relative intensity):256 (M^+, 65), 255 (60), 239 (15), 163 (15), 150 (27), 137 (29), 28 (100) (lit. [12]: 256 (M^+, 100), 255 (55), 239 (14), 192 (26), 163 (37), 150 (20), 137 (90), 120 (50)).

Fraction **E2** (6.75 g) obtained from elution with 30% acetone-hexane, was further purified by silica gel column chromatography. Elution with increasing amounts of methanol in dichloromethane, then purification by preparative TLC using 3% methanol-dichloromethane as a developing solvent afforded a semi-solid. Recrystallization from methanol-ethyl acetate gave 3,4-dihydroxy-benzaldehyde (protocatechualdehyde, **3**, 15 mg). Brown crystals, m.p. 153-154°C (lit. 154 °C [10], 155-156°C [14], 151-153°C [15]); UV λ_{max} (MeOH) nm (log ε): 232 (6.81), 278 (6.89), 312 (6.94) (in ethanol [16]: 209 (4.17), 234 (4.15), 281 (4.01), 315 (3.97), 360 (3.05)); FTIR υ_{max} (KBr, cm^{-1}): 3326, 2875, 2825, 1647, 1595, 1537, 1443, 1168, 1119 (lit. [14]: 3220, 1650, 1595, 1440, 1375, 1296, 1165; [15]: 3240, 1680, 1600); ^{1}H-NMR (CD_3OD): δ 9.63 (1H, s, CHO), 7.25 (1H, *br s*, H-2), 7.23 (1H, *br d*, H-6), 6.87 (1H, *d*, *J* = 8.0 Hz, H-5); ^{13}C-NMR (CD_3OD): δ 191.93 (CHO), 151.74 (C-4), 145.18 (C-3), 129.29 (C-1), 125.83 (CH-6), 114.90 (CH-5), 113.97 (CH-2); EIMS *m/z* (% relative intensity) : 138(M^+,70), 137(100), 109(18).

Fraction **E3** (2.26 g of brown solid obtained from 40% acetone-hexane elution) was rechromatographed on a silica gel column. Elution with 6% methanol-dichloromethane afforded a brown gum, which was further purified by preparative TLC on silica gel developing five times with 6% methanol-dichloromethane to provide two bands. The lower band (R_f = 0.40) was recrystallized from acetone-methanol to give *2-(3,4-dihydroxyphenyl)-2,3-dihydro-7-hydroxy-4H-1-benzopyran-4-one* (3,4′,7-trihydroxyflavanone, butin, **4**, 27.9 mg). The higher band (R_f = 0.44) was recrystallized from dichloromethane to yield *1-(2,4-dihydroxyphenyl)-3-(3,4-dihydroxyphenyl)-2-propen-1-one* (2′,3,4,4′-tetrahydroxychalcone, butein, **5**, 18.7 mg).

Butin (**4**). Yellow solid, m.p. 204-205°C (lit. 205°C [10], 224-225°C [17]); UV λ_{max} (MeOH) nm (log ε): 233 (6.76), 278 (6.84), 311 (6.89) (lit. [17] 225 (4.21), 327 (3.99) in methanol); FTIR υ_{max} (KBr, cm^{-1}): 3491, 3371, 3146, 1666, 1607, 1585, 1327, 1287, 1171, 1115; $[\alpha]_D^{29}$ -87° (c 0.03, MeOH)(-); lit. [17],–18.7° ± 0.8°; c 0.5, MeOH [18]); ^{1}H-NMR (CD_3OD): δ 7.69 (1H, d, *J* = 8.7 Hz, H-5), 6.87 (1H, *d*, *J* = 1.8 Hz, H-2′), 6.77 (1H, *d*, *J* =8.2 Hz, H-5′), 6.74 (1H, *dd*, *J* = 8.2, 1.8 Hz, H-6′), 6.44 (1H, *dd*, *J* = 8.7, 2.2 Hz, H-6), 6.32 (1H, *d*, *J* = 2.2 Hz, H-8), 5.22 (1H, *dd*, *J* = 13.2, 2.8 Hz, H-2), 2.94 (1H, *dd*, *J* = 13.2, 17.0 Hz, H-3a), 2.65 (1H, *dd*, *J* = 17.0, 2.8 Hz, H-3b); ^{13}C-NMR (CD_3OD): δ 192.07 (CO), 164.86 (C-7), 163.77 (C-8a), 144.96 (C-3′), 144.59 (C-4′), 130.31 (C-1′), 128.74 (CH-5), 118.15 (CH-6′), 114.98 (CH-5′), 113.64 (C-4a), 113.21 (CH-2′), 110.65 (CH-6), 102.77 (CH-8), 79.45 (CH-2), 43.62 (CH_2-3); EIMS *m/z* (% relative intensity): 272 (M^+, 100), 271 (55), 255 (24), 163 (20), 150 (87), 137 (52), 136 (24) (lit. [17]: 272 (M^+, 65)).

Butein (**5**). Orange-yellowish needles, m.p. 211-212°C (lit. [10]: 214°C); UV λ_{max} (MeOH) nm (log ε): 262 (6.88), 380 (7.04) (lit. [13]: 239sh, 266, 319sh, 379 in methanol); FTIR υ_{max} (KBr, cm^{-1}): 3546, 3471, 3295, 1638, 1591, 1557, 1513, 1351, 1234, 1123; ^{1}H-NMR (CD_3OD): δ 7.81 (1H, *d*, *J* = 8.9 Hz, H-6′), 7.70 (1H, *d*, *J* = 15.3 Hz, H-*β*), 7.40 (1H, *d*, *J* = 15.3 Hz, H-*α*), 7.15 (1H, *d*,*J* = 1.8 Hz, H-2), 7.05 (1H, *dd*, *J* = 8.2, 1.8 Hz, H-6), 6.82 (1H, *d*, *J* = 8.2 Hz, H-5), 6.40 (1H, *dd*, *J* = 8.9, 2.4 Hz, H-5′),

6.33 (1H, *d*, *J* = 2.4 Hz, H-3′); ^{13}C-NMR (CD_3OD): δ 191.87 (CO), 165.49 (C-2′), 164.38 (C-4′), 147.87 (C-3), 144.83 (C-4), 144.61 (CH-*β*), 131.56 (CH-6′), 126.78 (C-1), 122.19 (CH-6), 116.86 (CH-*α*), 115.10 (CH-5), 114.28 (CH-2), 113.21 (C-1′), 107.86 (CH-5′), 102.66 (CH-3′); EIMS *m/z* (% relative intensity): 272 (M^+, 60), 255 (9), 163 (30), 150 (30), 137 (100), 123 (12), 110 (18).

Biological evaluation: Antimicrobial assay [19]

Antimicrobial activity of the crude extracts and isolates **3–5** was investigated using the agar dilution method [19]. Briefly, the tested compounds dissolved in either CH_2Cl_2 or MeOH were individually mixed with Müller Hinton (MH) broth to obtain a final volume of 2 mL. Two-fold dilution was prepared and the solution was then transferred to the MH agar solution to yield the final concentrations ranging from 256-4 μg/mL. Twenty seven strains of microorganisms (listed below), cultured in MH broth at 37°C for 24 h, were diluted with 0.9 % normal saline solution to adjust the cell density of 10^8 CFU/mL.

Gram-negative bacteria
Escherichia coli ATCC 25922
Klebsiella pneumoniae ATCC 700603
Serratia marcescens ATCC 8100
Salmonella typhimurium ATCC 13311
Salmonella choleraesuis ATCC 10708
Shewanella putrefaciens ATCC 8671
Achromobacter xylosoxidans ATCC 2706
Pseudomonas aeruginosa ATCC 15442
Pseudomonas stutzeri ATCC 17587
Gram-positive bacteria
Staphylococcus aureus ATCC 29213
Staphylococcus aureus ATCC 25923
Enterococcus faecalis ATCC 29212
Enterococcus faecalis ATCC 33186
Micrococcus lutens ATCC 10240
Bacillus subtilis ATCC 6633
Corynebacterium diphtheriae NCTC 10356
Yeasts
Saccharomyces cereviseae ATCC 2601
Candida albicans ATCC 90028

Clinical specimens
Gram-negative bacteria
Shigella dysenteriae
Salmonella enteritidis type C
Morganella morganii
Aeromonas hydrophila
Citrobacter freundii
Plesiomonas shigelloides
Gram-positive bacteria
Streptococcus pyogenes II
Bacillus cereus
Listeria monocytogenes

The organisms were inoculated onto each plate using a multipoint inoculator and further incubated at 37°C for 18-48 h. Compounds which possessed high efficacy to inhibit bacterial cell growth were analyzed.

Antioxidative assay [20, 21]

Two methods were used, DPPH and SOD assays. The antioxidative activity of the crude extracts was elucidated by DPPH radical scavenging assay [20]. When DPPH (a stable purple color) reacts with an antioxidant compound, it is reduced to yield a light-yellow color of diphenylpicrylhydrazine. Changes of the color can be spectrophotometrically measured. In this study, experiment was initiated by preparing 0.2 mM solution of DPPH in methanol. One mL of this solution was added sample solution (1 mg/mL dissolved in methanol, 0.5 mL). After 30 min, absorbance was measured at 517 nm and the percentage of radical scavenging activity was calculated from the following equation:

$$\% \text{ Radical scavenging} = (1\text{-Abs.sample/Abs.cont}) \times 100$$

where Abs.cont is the absorbance of the control reaction and Abs.sample is the absorbance in the presence of sample.

The SOD activity was assayed by measuring inhibition of the photoreduction of nitro blue tetrazolium (NBT) [21]. The indirect assay is comprised of several reactions: the photochemically excited riboflavin was first reduced by methionine into a semiquinone, which donated an electron to oxygen to form the superoxide source. The superoxide readily converted NBT into a purple formazan product. In this regard, the SOD activity was inversely related to the amount of formazan formed.

Cytotoxic assay [22]

Cytotoxic activity was determined by a slightly modified quantitative cytotoxicity assay from other investigators [22]. Briefly, the confluent cell monolayers were trypsinized and diluted with appropriate culture medium to a final concentration of 3×10^5cells/mL. Portions (100 μL) containing approximately 3×10^4 cells were distributed into 96-well flat-bottomed tissue culture plates and incubated overnight at 37°C in a humidified 5% CO_2 incubator. Solutions (100 μL) containing different concentrations of tested compounds **3 – 5** (0.001 – 10 μg/mL) or taxol (0.012 – 1.2 μg/mL) were added to each well and the plates were incubated as above for an additional 48 h. After the incubation, each well was washed (x 3) with phosphate-buffered saline (PBS, pH 7.2) and then stained with crystal violet. After the excess dye was removed, the stained cells were lysed with 100 mM HCl (100 μL) in absolute methanol and the optical density was determined by a microtitre plate reader (Titertek, Multiskan MCC/340) set to read at a wavelength of 540 nm. All tests were carried out in quadruplicate and the mean value was calculated. The activity was expressed as ED_{50} (the effective dose that inhibits 50% of cell growth).

Acknowledgements

We thank the Faculty of Science, Srinakharinwirot University, the Faculty of Medical Technology, Mahidol University, and Chulabhorn Research Institute for facilities and supports. This project was partially supported by the annual budget grant of Mahidol University (B.E. 2551-2555).

References

1. Morse, C. *Ecology & Evolutionary Biology Conservatory* (Online 2000, 30 March). http://florawww.eeb.uconn.edu/acc_num/198500652.html.
2. Huxley, C. R.; Jebb, M. H. P. The tuberous epiphytes of the rubiaceae 1: A new subtribe-the hydnophytinae. *Blumea* **1991**, *36*, 1-20.
3. Smitinund, T. *Thai plant names (botanical names-vernacular names)*; Funny Publishing: Bangkok, **1980**; pp. 183-184.
4. Prommee, P. *Thai traditional medicine*; Mahachulalongkon Publishing: Bangkok, **1988**; p. 51.
5. Beckstrom-Sternberg, S. M.; Duke J. A.; Wain K. K. *The Ethnobotany Database*; (Online 1994, July). http://probe.nal.usda.gov:8300/cgi-bin/webace?db=ethnobotdb& class =Taxon&object= Hydnophytum+formicarium.
6. Nguyen, M. T.; Awale, S.; Tezuka, Y.; Tran, Q. L.; Watanabe, H.; Kadota, S. Xanthine oxidase inhibitory activity of Vietnamese medicinal plants. *Biol. Pharm. Bull.* **2004**, *27*, 1414-1421.
7. Ueda, J. Y.; Tezuka, Y.; Banskota, A. H.; Tran, Q. L.; Tran, Q. K.; Hariyama, Y.; Saiki, I.; Kadota, S. Antiproliferative activity of Vietnamese medicinal plants. *Biol. Pharm. Bull.* **2002**, *25*, 753-760.
8. Huxley, C. Symbiosis between ants and epiphytes. *Biol. Rev.* **1980**, *55*, 321-340.
9. Pouchert, C. J.; Bekke, J. *The Aldrich library of ^{13}C and ^{1}H FTNMR spectra*; Aldrich Chemical Company: Wisconsin, **1993**; p. 569.
10. Devon, T. K.; Scott, A. I. *Handbook of naturally occurring compounds: acetogenins, shikimates and carbohydrates*; Academic Press: New York, **1975**; Vol. 1, pp. 10-167.
11. Saitoh, T.; Noguchi, H.; Shibata, S. A new isoflavone and the corresponding isoflavanone of licorice root. *Chem. Pharm. Bull.* **1978**, *26*, 144-147.
12. Yenesew, A.; Midiwo, J. O.; Waterman, P. G. Rotenoids, isoflavones and chalcones from the stem bark of *Mellettia usaramensis* subspecies *usaramensis*. *Phytochemistry* **1998**, *47*, 295-300.
13. Mabry, T. J.; Markham, K. R.; Thomas, M. B. *The systematic identification of flavonoids*; Springer-Verlag: New York, **1970**; pp. 165-166.
14. Kuroyanagi, M.; Fukuoka, M.; Yoshihira, K; Natori, S. Chemical and toxicology studies on bracken fern, *Pteridium aquilinum* var. *latiusculum*. III. Further characterization of pterosins and pterosides, sesquiterpenes and the glucosides having 1-indanone skeleton, from the rhizomes. *Chem. Pharm. Bull.* **1979**, *27*, 592-601.
15. Hata, K.; Baba, K; Kozawa, M. Chemical studies on the heartwood of *Cassia garrettiana* CRAIB. II. nonanthraquinonic constituents. *Chem. Pharm. Bull.* **1979**, *27*, 984-989.
16. Santavy′, F.; Walterova′, D.; Hruban, L. Ultraviolet spectra, methoxy and methylenedioxy groups of some aromatic compounds and alkaloids. IV; The effect of hydroxyl, methoxyl and methylenedioxy groups on the ultraviolet spectra of aromatic compounds conjugated with an oxo-group or with and α, β-double bond in the side chain. *Coll.t Czech. Chem. Commun.* **1970**, *37*, 1825-1850.
17. Van der Westhuizen, J. H.; Ferreira, D.; Roux, D. G. Photochemical deoxygenation of an α-ketol: The dihydroflavonol-flavanone conversion. *J. Chem. Soc. Perkin Trans I.* **1980**, 1003-1006.

18. Roux, D. G.; Paulus, E. Condensed tannins. 10. Isolation of (-)-butin and butein from wattle heartwoods. *Biochem. J.* **1961**, *80*, 62-63.
19. Baron, E. J.; Peterson, L. R.; Finegold, S. M. Methods for testing antimicrobial effectiveness; In *Bailey and Scott's diagnostic microbiology*; Mosby-Year Book: St. Louis, MI, **1994**; pp. 168-193.
20. Yen, G. C.; Hsieh, G. C. Antioxidant effects on dopamine and related compounds. *Biosci. Biotechnol. Biochem.* **1997**, *61*, 1646-1649.
21. Piacham T.; Isarankura-Na-Ayudhya C.; Nantasenamat C.; Yainoy S.; Ye L.; Prachayasittikul V. Metalloantibiotic Mn(II)-bacitracin complex mimicking manganese superoxide dismutase. *Biochem. Biophys. Res. Comm.* **2006**, *341*, 925-930.
22. Tengchaisri, T.; Chawengkirttikul, R.; Rachaphaew, N.; Reutrakul, V.; Sangsuwan, R.; Sirisinha, S. Antitumor activity of triptolide against cholangiocarcinoma growth *in vitro* and in hamsters. *Cancer Lett.* **1998**, *133*, 169-175.
23. Bate-Smith, E. C.; Swain, T. The isolation of 2,4,4'-trihydroxychalcone from yellow varieties of *Dahlia variabilis*. *J. Chem. Soc.* **1953**, 2185-2187.
24. Vaya, J.; Belinky, P. A.; Aviram, M. Antioxidant constituents from licorice roots: isolation, structure elucidation and antioxidative capacity toward LDL oxidation. *Free Radic. Biol. Med.* **1997**, *23*, 302-313.
25. Kong, L. D.; Zhang, Y.; Pan, X.; Tan, R. X.; Cheng, C. H. Inhibition of xanthine oxidase by liquiritigenin and isoliquiritigenin isolated from *Sinofranchetia chinensis*. *Cell. Mol. Life Sci.* **2000**, *57*, 500-505.
26. Zheng, Z.-P.; Cheng, K.-W.; Chao, J.; Wu, J.; Wang, M. Tyrosinase inhibitors from paper mulberry (*Broussonetia papyrifera*). *Food Chem.* **2008**, *106*, 529-535.
27. Hayashi, H.; Hiraoka, N.; Ikeshiro, Y.; Yamamoto, H. Organ specific localization of flavonoids in *Glycyrrhiza glabra* L. *Plant Sci.* **1996**, *116*, 233-238.
28. Wegener, J. W.; Nawrath, H. Cardiac effects of isoliquiritigenin. *Eur. J. Pharmacol.* **1997**, *326*, 37-44.
29. Wegener, J. W.; Nawrath, H. Differential effects of isoliquiritigenin and YC-1 in rat aortic smooth muscle. *Eur. J. Pharmacol.* **1997**, *323*, 89-91.
30. Tawata, M.; Aida, K.; Noguchi, T.; Ozaki, Y.; Kume, S.; Sasaki, H.; Chin, M.; Onaya, T. Anti-platelet action of isoliquiritigenin, an aldose reductase inhibitor in licorice. *Eur. J. Pharmacol.* **1992**, *212*, 87-92.
31. Yu, S. M.; Kuo, S. C. Vasorelaxant effect of isoliquiritigenin, a novel soluble guanylate cyclase activator, in rat aorta. *Br. J. Pharmacol.* **1995**, *114*, 1587-1594.
32. Haraguchi, H.; Ishikawa, H.; Mizutani, K.; Tamura, Y.; Kinoshita, T. Antioxidative and superoxide scavenging activities of retrochalcones in *Glycyrrhiza inflata*. *Bioorg. Med. Chem.* **1998**, *6*, 339-347.
33. Kobayashi, S.; Miyamoto, T.; Kimura, I.; Kimura, M. Inhibitory effect of isoliquiritigenin, a compound in licorice root, on angiogenesis *in vivo* and tube formation *in vitro*. *Biol. Pharm. Bull.* **1995**, *18*, 1382-1386.
34. Hsu, Y.-L.; Kuo, P.-L.; Lin, C.-C. Isoliquiritigenin induces apoptosis and cell cycle arrest through p53-dependent pathway in Hep G2 cells. *Life Sci.* **2005**, *77*, 279-292.

35. Kuma, S.; Sharma, A.; Madan, B.; Singhal, V.; Ghosh, B. Isoliquiritigenin inhibits IkB kinase activity and ROS generation to block TNF-α induced expression of cell adhesion molecules on human endothelial cells. *Biochem. Pharmacol.* **2007**, *73*, 1602-1612.
36. Takahashi, T.; Takasuka, N.; Iigo, M.; Baba, M.; Nishino, H.; Tsuda, H.; Okuyama, T. Isoliquiritigenin, a flavonoid from licorice, reduces prostaglandin E2 and nitric oxide, causes apoptosis, and suppresses aberrant crypt foci development. *Cancer Sci.* **2004**, *95*, 448-453.
37. Jung, J. I.; Chung, E.; Seon, M. R.; Shin, H.-K.; Kim, E. J.; Lim, S. S.; Chung, W.-Y.; Park, K.-K.; Park J. H. Y. Isoliquiritigenin (ISL) inhihits ErbB3 signaling in prostate cancer cells. *BioFactors* **2006**, *28*, 159-168.
38. Figueiredo, A. R.; Campos, F.; de Freitas, V.; Hogg, T.; Couto, J. A. Effect of phenolic aldehydes and flavonoids on growth and inactivation of *Oenococcus oeni* and *Lactobacillus hilgardii. Food Microbiol.* **2008**, *25*, 105-112.
39. Martin, T. S.; Kikuzaki, H.; Hisamoto, M.; Nakatani, N. Constituents of *Amomum tsao-ko* and their radical scavenging and antioxidant activities. *J. Am. Oil Chem. Soc.* **2000**, *77*, 667-673.
40. Lee, T. Y.; Chang, H. H.; Wang, G. J.; Chiu, J. H.; Yang, Y. Y.; Lin, H. C. Water-soluble extract of *Salvia miltiorrhiza* ameliorates carbon tetrachloride-mediated hepatic apoptosis in rats. *J. Pharm. Pharmacol.* **2006**, *58*, 659-665.
41. Lu, Y.; Yeap, F. L. Salvianolic acid L, a potent phenolic antioxidant from *Salvia officinalis. Tetrahedron Lett.* **2001**, *42*, 8223-8225.
42. Lee, S.; Shim, S. H.; Kim, J. S.; Shin, K. H.; Kang, S. S. Aldose reductase inhibitors from the fruiting bodies of *Ganoderma applanatum. Biol. Pharm. Bull.* **2005**, *28*, 1103-1105.
43. Shibata, M.-A.; Hirose, M.; Yamada, M.; Tatematsu, M.; Uwagawa, S.; Ito, N. Epithelial cell proliferation in rat forestomach and glandular stomach mucosa induced by catechol and analogous dihydroxybenzenes. *Carcinogenesis* **1990**, *11*, 997-1000.
44. Kim, Y. S.; Kim, N. H.; Lee, S. W.; Lee, Y. M.; Jang, D. S.; Kim, J. S. Effect of protocatechualdehyde on receptor for advanced glycation end products and TGF-β1 expression in human lens epithelial cells cultured under diabetic conditions an on lens opacity in streptozotocin-diabetic rats. *Eur. J. Pharmacol.* **2007**, *569*, 171-179.
45. Lai, P. K.; Oh-Hara, T.; Tamura, Y.; Kawazoe, Y.; Konno, K.; Sakagami, H.; Tanaka, A.; Nonoyama, M. Polymeric phenylpropenoids are the active components in the pine cone extract that inhibit the replication of type-1 human immunodeficiency virus *in vitro*. *J. Gen. Appl. Microbiol.* **1992**, *38*, 303-312.
46. Bhargava, S. K. Estrogenic and postcoital anticonceptive activity in rats of butin isolated from *Butea monosperma* seed. *J. Ethnopharmacol.* **1986**, *18*, 95-101.
47. Lee, M.-H.; Lin, Y.-P.; Hsu, F.-L.; Zhan, G.-R.; Yen, K.-Y. Bioactive constituents of *Spatholobus suberectus* in regulating tyrosinase-related proteins and mRNA in HEMn cells. *Phytochemistry* **2006**, *67*, 1262-1270.
48. Perkin F. R. S., A. G.; Hummel, J. J. CXLVII.-The colouring principle of the flowers of the *Butea Frondosa*. *J. Chem. Soc. Trans.* **1904**, *85*, 1459-1472.
49. Shimokoriyama, M.; Geissman, T. A. Anthochlor pigments. XIV. The pigments of *Viguiera multiflora* (Nutt.) and *Baeria chrysostoma* (F. and M.). *J. Org. Chem.* **1960**, *25*, 1956-1959.

50. Geissman, T. A. Anthochlor pigments. III. The pigments of *Cosmos sulphureus*. *J. Chem. Soc.* **1942**, *64*, 1704-1707.
51. Geissman, T. A; Harborne, J. B.; Seikel, M. K. Anthochlor pigments. XI. The Constituents of *Coreopsis maritima*. Reinvestigation of *Coreopsis gigantea*. *J. Am. Chem. Soc.* **1956**, *78*, 825-829.
52. Cheng, Z.-J.; Kuo, S.-C.; Chan, S.-C.; Ko, F.-N.; Teng, C.-M. Antioxidant properties of butein isolated from *Dalbergia odorifera*. *Biochim. Biophys. Acta.* **1998**, *1392*, 291-299.
53. Chan, S. C.; Chang, Y. S.; Wang, J. P.; Chen, S. C.; Kuo, S. C. Three new flavonoids and antiallergic, anti-inflammatory constituents from the heartwood of *Dalbergia odorifera*. *Planta Med.* **1998**, *64*, 153-158.
54. Sogawa, S.; Nihro, Y.; Ueda, H.; Miki, T.; Matsumoto, H.; Satoh, T. Protective effects of hydroxychalcones on free radical-induced cell damage. *Biol. Pharm. Bull.* **1994**, *17*, 251-256.
55. Lee, J. C.; Lim, K. T.; Jang, Y. S. Identification of *Rhus verniciflua* stokes compounds that exhibit free radical scavenging and anti-apoptotic properties. *Biochim. Biophys. Acta.* **2002**, *1570*, 181-191.
56. Chen, W.-J.; Song, J.-R.; Guo, P.; Wen, Z.-Y. Butein, a more effective antioxidant than α-tocopherol. *J. Mol. Struct. THEOCHEM* **2006**, 763, 161-164.
57. Lee, S. H.; Seo, G. S.; Sohn, D. H. Inhibition of lipopolysaccharide-induced expression of inducible nitric oxide synthase by butein in RAW264.7 cells. *Biochem. Biophys. Res. Comm.* **2004**, *323*, 125-132.
58. Lee, S. H.; Seo, G. S.; Jin, X. Y.; Ko, G.; Sohn, D. H. Butein blocks tumor necrosis factor α-induced interleukin 8 and matrix metalloproteinase 7 production by inhibiting p38 kinase and osteopontin mediated signaling events in HT-29 cells. *Life Sci.* **2007**, *81*, 1535-1543.
59. Wang, Y.; Chan, F. L.; Chen, S.; Leung, L. K. The plant polyphenol butein inhibits testosterone-induced proliferation in breast cancer cells expressing aromatase. *Life Sci.* **2005**, *77*, 39-51.
60. Yu, S. M.; Cheng, Z. J.; Kuo, S. C. Endothelium-dependent relaxation of rat aorta by butein, a novel cyclic AMP-specific phosphodiesterase inhibitor. *Eur. J. Pharmacol.* **1995**, *280*, 69-77.
61. Kang, D. G.; Kim, Y. C.; Sohn, E. J.; Lee, Y. M.; Lee, A. S.; Yin, M. H.; Lee, H. S. Hypotensive effect of butein via the inhibition of angiotensin converting enzyme. *Biol. Pharm. Bull.* **2003**, *26*, 1345-1347.
62. Hayashi, K.; Nagamatsu, T.; Honda, S.; Sukuki, Y. Butein (3,4,2′,4′-tetrahydroxychalcone) ameliorates experimental anti-glomerular basement membrane antibody-associated glomerulonephritis. *Eur J. Pharmacol.* **1996**, *316*, 297-306.
63. Kim, N. Y.; Pae, H. O.; Oh, G. S.; Kang, T. H.; Kim, Y. C.; Rhew, H. Y.; Chung, H. T. Butein, a plant polyphenol, induces apoptosis concomitant with increased caspase-3 activity, decreased Bcl-2 expression and increased Bax expression in HL-60 cells. *Pharmacol. Toxicol.* **2001**, *88*, 261-266.
64. Iwashita, K.; Kobori, M.; Yamaki, K.; Tsushida, T. Flavonoids inhibit cell growth and induce apoptosis in B16 melanoma 4A5 cells. *Biosci. Biotechnol. Biochem.* **2000**, *64*, 1813-1820.
65. Hung, C.-Y.; Yen, G.-C. Extraction and identification of antioxidative components of Hsian-tsao *(Mesona procumbens* Hemsl.). *Lebensm.-Wiss. u.-Technol.* **2001**, *34*, 306-311.

66. Ramadan, M. F.; Zayed, R.; El-Shamy, H. Screening of bioactive lipids and radical scavenging potential of some solanaceae plants. *Food Chem.* **2007**, *103*, 885-890.
67. Batta, A. K.; Xu, G.; Honda, A.; Miyazaki, T.; Salen, G. Stigmasterol reduces plasma cholesterol levels and inhibits hepatic synthesis and intestinal absorption in the rat. *Metabolism* **2006**, *55*, 292-299.

Sample Availability: Contact the authors.