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Fitoterapia 77 (2006) 109 – 115

FITOTERAPIA

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Antioxidant and heme oxygenase-1 (HO-1)-induced effects of selected Taiwanese plants

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Received 9 February 2005; accepted 17 November 2005
Available online 3 January 2006

Abstract

Recent studies have shown biological effects of heme oxygenase-1 (HO-1) induction and antioxidation in cardiovascular disorders. The ethanol extracts of leaves of 12 selected indigenous Taiwanese plants were investigated for their antioxidant activities, evaluated using assays of 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl, and superoxide radicals scavenging and reducing power activities as well as the induction of heme oxygenase-1 (HO-1). *Acer albopurpurascens*, *Cinnamomum kanehirai*, *Diospyros discolor*, *Excoecaria kawakamii*, *Koelreuteria henryi*, and *Syzygium formosanum* showed better DPPH-scavenging activities than the other plants. IC_{50} values ranged from 1.7 to 8.7 μg/mL. Excepting *Millettia pulchra* var. *microphylla* and *Pittosporum moluccanum*, the extracts displayed hydroxyl-scavenging activities (IC_{50} of 0.16–0.67 μg/mL). *A. albopurpurascens*, *D. discolor*, *K. henryi*, and *S. formosanum* also showed good superoxide anion radical scavenging activities and IC_{50} values ranged from 12.9 to 28.5 μg/mL. *D. discolor*, *K. henryi*, and *S. formosanum* showed potent reducing power and *M. pulchra* var. *microphylla* and *S. formosanum* exhibited potent HO-1 induced activity. These active plant extracts also contained abundant phenolic constituents. The present results provide candidates to isolate the active constituents and develop natural antioxidants.

Keywords: Indigenous Taiwanese plants; Superoxide; Reducing power; Heme oxygenase-1

1. Introduction

Oxidative stress, which is a common prelude to increased amounts of reactive oxygen species (ROS), is a causative factor in several human chronic degenerative diseases including inflammatory and neurodegenerative disorders and cardiovascular diseases [1]. ROS are highly reactive molecules. Examples include the hydroxyl radical ($OH^{\bullet}$), superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and peroxyl ($ROO^{\bullet}$).

The reactivity of ROS species generates metabolic products that attack deoxyribonucleic acid and the lipid component in cell membranes. Eukaryotic cells are equipped with endogenous scavenging systems or other substances that act to protect the cells from ROS activity.

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doi:10.1016/j.fitote.2005.11.012

Cells are impaired by an imbalance between ROS-generating and -scavenging systems. Recently, heme oxygenase-1 (HO-1) was shown to be another protective antioxidant. Its increased expression reduces oxidative damage of tissues [2].

HO is a rate-limiting catalyst in the degradation of heme to produce biliverdin, which is further converted by biliverdin reductase to yield free iron, carbon monoxide (CO) and the antioxidant bilirubin [3]. Three HO isozymes have been identified (HO-1, HO-2 and HO-3), which are encoded by separate genes [1]. HO-1 is a stress–response protein that can be induced by various oxidative-inducing agents including heme, heavy metals, UV radiation, cytokines and endotoxin [4,5]. Induction of HO-1 is an important cellular protective mechanism against oxidative injury both in vitro and in vivo [4,5]. HO-1 is also a heat shock protein that has been implicated in a cytoprotective mechanism to prevent tissues from oxidative damage [6,7].

Given these protective effects, the therapeutic potential of HO-1 has been explored. In animal models, the adenovirus-mediated gene transfer of HO-1 protects against hyperoxia-induced lung injury [8], reperfusion-induced injury of transplanted liver [9] and atherosclerosis [10].

These notable effects have prompted us to search for an alternative medicine that would function as a potent inducer of HO-1 production and activity. Plant extracts or secondary metabolites may serve as antioxidants in phytotherapeutic medicines to protect against various diseases [11]. Examples include phenolics, flavonoids, tannins, proanthocyanidins and, in particular, phenolics. Taiwan contains an abundance of plant species and so represents a suitable region for an evaluation of phenolic phytotherapeutics. Using the scavenging activities of 1,1-diphenyl-2-picrylhydrazyl (DPPH), superoxide and hydroxyl ($OH^{\bullet}$) radicals, as well as assays of reducing power and HO-1 induction, a systematic survey of selected plant material extracts from plants indigenous to Taiwan was undertaken to identify potential new sources of natural antioxidants.

2. Experimental

2.1. Plant materials

All indigenous plant materials were selected and collected from the Taiwan Endemic Species Research Institute (TESRI) garden in Nantou County, central Taiwan. These plants were identified by Chih-Hui Chen at TESRI. Herbarium voucher specimens were deposited in the Graduate Institute of Pharmacognosy, Taipei Medical University, Taiwan.

2.2. Preparation of crude extracts

Dried leaves of plants were pulverized and extracted with two applications of twice the volume of 95% EtOH. The extracts were concentrated in vacuo, freeze-dried and stored at −20 °C until used (Table 1).

Table 1
Yields of 12 Taiwanese plants leaves ethanolic extracts

Plants	Family	Voucher specimen	% Yield*
A. albopurpurascens Hayata	Aceraceae	M-51	8.9
A. formosana (Burk.) Makino	Betulaceae	M-54	10.2
C. kanehirai Hayata	Lauraceae	M-43	29.6
D. discolor Willd.	Ebenaceae	M-47	13.7
E. kawakamii Hayata	Euphorbiaceae	M-41	11.2
F. formosana Hayata	Oleaceae	M-44	5.8
K. henryi Dummer	Sapindaceae	M-40	17.2
M. pulchra Kurz var. *microphylla* Dunn	Fabaceae	M-53	7.6
N. sericea (Blume) Koidz. var. *aurata* (Hayata) Hatusima	Magnoliaceae	M-46	13.2
P. moluccanum Miq	Pittosporaceae	M-45	9.8
S. warburgii O. Seem	Salicaceae	M-48	8.3
S. formosanum (Hayata) Moti	Myrtaceae	M-39	14.7

2.3. 1,1-Diphenyl-2-picrylhydrazyl radical scavenging activity

Each sample was mixed with 160 μM DPPH in MeOH. After a 20-min incubation at room temperature in the dark, the absorbance was read at 517 nm [12]. The inhibitory percentage of DPPH (% scavenging activity) was calculated as

$$[(\text{absorbance of the control} - \text{absorbance of the sample})/\text{absorbance of the control}] \times 100\%$$

2.4. Hydroxyl radical activity

The reaction mixture contained 2.8 mM deoxyribose, 20 mM potassium phosphate buffer (pH 7.4), 100 μM $FeCl_3$, 780 μM EDTA, 1 mM H_2O_2 and 100 μM ascorbic acid. After incubating at 37 °C for 1 h, equal volumes of 1% 2-thiobarbituric acid (TBA) and 10% trichloroacetic acid (TCA) were added to the reactants. The mixture was boiled for 5 min, cooled, centrifuged at 13000 rpm for 5 min and the absorbance was determined spectrophotometrically at 532 nm [13].

2.5. Superoxide radical scavenging activity

Superoxide radicals were generated by the NADH-phenazine methosulfate (PMS) system according to a described procedure [14]. The reaction mixture contained 400 μl of sample and 400 μl of 630 μM nitroblue tetrazolium (NBT). After incubation at room temperature for 10 min, the reaction was started by adding 400 μl of 156 μM NADH. The reaction mixture was incubated at ambient temperature for 5 min. The absorbance (560 nm) of each sample was compared against blank samples.

2.6. Reducing power activity

Reducing power activity was determined by the $K_3Fe(CN)_6$–$FeCl_3$ method [15]. Each test sample was mixed with an equal volume of 200 mM phosphate buffer (pH 6.6) and 1% potassium ferricyanide (K_3FeCN_6). The mixture was incubated at 37 °C for 20 min. After an equal volume of 10% trichloroacetic acid was added to the mixture, the mixture was centrifuged at 3000 rpm for 10 min. The supernatant was mixed with deionized water and 0.1% ferric chloride ($FeCl_3$) at a ratio of 1:1:2. The resulting absorbance was measured at 700 nm.

2.7. Isolation and primary culture of rat aortic smooth muscle cells

Rat aortic smooth muscle cells (RASMC) were isolated from the thoracic aorta of male Sprague–Dawley rats (200–250 g) using the explant technique [16]. Briefly, after removal of the endothelium and adventitia, the aortic explants were cultured in DMEM supplemented with 10% FBS, penicillin (100 U/mL), streptomycin (100 μg/mL) and 25 mM HEPES (pH 7.4). After 2 weeks, cells that had migrated out of the explants were removed by trypsinization and successively subcultured. The purity and identity of cells were established by immunostaining using antibody specific against smooth muscle cell α-actin. Cells from passages 5–12 were used for the experiments.

2.8. Constructs of plasmid variants and luciferase activity assay

The pGL3/hHO-1 reporter plasmid, which contains a 3293-bp fragment, −3106 to +186 relative to the transcription start of the human HO-1 gene, was amplified from the human BAC clone CTA-286B10 [17] using the primers 5′-AGAGAACAGTTAGAAAAGAAAG-3′ (sense) and 5′-TACGGGCACAGGCAGGATCAGAA-3′ (anti-sense). The PCR products were inserted to the pCR2.1-TOPO cloning vector (Invitrogen, Carlsbad, CA, USA), and cut with KpnI/XbaI, such that the resulting PCR products contained the KpnI/XbaI site. This was ligated in frame into the unique KpnI/NheI site present within the pGL3 plasmid (Promega, Madison, WI, USA) to generate a pGL3/hHO-1 reporter construct containing an approximately 3.3-kbp region of human HO-1 promoter driving expression of the gene for luciferase. The identities of the sequences were confirmed using an ABI PRISM 377 DNA Analysis System (Perkin-Elmer, Taiwan Branch, Taipei, Taiwan).

For the reporter activity assay, cells were seeded in six-well plates at a density of 1×10^5 cells/well. HASMC were transiently transfected with 3 μg of plasmid DNA containing 1 μg of the Renilla luciferase construct, phRL-TK (Promega) to control transfection efficiency and 2 μg of the appropriate HO-1 promoter firefly luciferase construct. The next day, cells were transfected with pGL3/hHO-1 and phRL-TK (Promega) as an internal control plasmid using LipofetAMINE 2000™ (Invitrogen). Twelve hours after transfection, the medium was replaced with fresh medium and incubation was continued for 36 h. Transfected cells were then treated with drugs for 12 h and cell lysates were collected. Luciferase activities were recorded in a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA) using the dual luciferase assay kit (Promega) according to the manufacturer's instructions. Luciferase activities of reported plasmids were normalized to luciferase activities of the internal control plasmid.

2.9. Determination of total phenolics

The total phenolic content of each extract was determined by a modified Folin–Ciocalteu method [18]. The sample solution (250 μl) was mixed with an equal volume of 1 N Folin–Ciocalteu reagent, 500 μl of 20% sodium carbonate (Na_2CO_3) and 4 mL water. After a 25-min incubation at room temperature, the reaction mixture was centrifuged at 5000 rpm for 10 min. The supernatant was measured at 730 nm using a spectrophotometer. The amount of total phenolics was expressed as gallic acid equivalent (GAE) in milligrams per gram dry plant extract.

2.10. Statistical analysis

Data are presented as the mean±standard deviation (S.D.) of each triplicate test.

3. Results and discussion

3.1. Scavenging activity on DPPH radical

Among the 12 extracts, *Acer albopurpurascens*, *Cinnamomum kanehirai*, *Diospyros discolor*, *Excoecaria kawakamii*, *Koelreuteria henryi* and *Syzygium formosanum* showed good DPPH radical scavenging activities, with IC_{50} values ranging from 1.7 to 8.7 μg/mL (Table 2). *Alnus formosana*, *Fraxinus formosana*, *Millettia pulchra* var. *microphylla*, *Neolitsea sericea* var. *aurata*, *Pittosporum moluccanum* and *Salix warburgii* showed comparatively moderate scavenging activities. Compared with Trolox positive control, the IC_{50} value was 100.8 μM [19]. The DPPH radical scavenging assay is a common method to evaluate the antiradical activity of numerous substances [20,21]. Accordingly, the pronounced reactivities of *A. albopurpurascens*, *C. kanehirai*, *D. discolor*, *E. kawakamii*, *K. henryi*, and *S. formosanum* are consistent with their proton-donating abilities.

Table 2
Phenolic contents and IC_{50} values against DPPH, hydroxyl, and superoxide radicals of Taiwanese plant leaves ethanolic extract

Plants	IC_{50} value			Total phenolics (mg of GAE /g)
	DPPH radical (μg/mL)	OH radical (μg)	Superoxide radical (μg/mL)	
A. albopurpurascens	1.7	0.32	28.5	28.9
A. formosana	11.3	0.36	181.7	15.6
C. kanehirai	6.4	0.16	68.3	32.1
D. discolor	4.4	0.24	16.6	36.8
E. kawakamii	8.7	0.32	86.7	23.2
F. formosana	13.2	0.58	114.4	27.0
K. henryi	3.6	0.36	19.8	36.7
M. pulchra var. *microphylla*	10.6	2.14	265.6	29.4
N. sericea var. *aurata*	12.9	0.24	70.4	20.8
P. moluccanum	14.9	1.24	223.3	19.7
S. warburgii	12.7	0.67	39.0	24.7
S. formosanum	5.8	0.42	12.9	36.1

3.2. The scavenging activity on hydroxyl radical

Hydroxyl radical is one of the most reactive radicals which abstract hydrogen atoms from biologic molecules to damage the living cells. Among the 12 extracts, all but *M. pulchra* var. *microphylla* and *P. moluccanum* showed good activities (IC_{50} 0.16–0.67 μg/mL). The IC_{50} value of the glutathione positive control was 0.10 μg/mL. The observations are consistent with the suggestion that the active plant extracts may stimulate deoxyribose degradation via reaction with hydroxyl radical or via the chelation of iron, thus exhibiting the pro-oxidant effects ascribed to actual or proposed antioxidants [22].

3.3. Scavenging activity on superoxide radical

The superoxide anion radical ($O_2^{\bullet -}$) has been linked to oxidant-mediated DNA damage and certain forms of cancer [23]. Although the roles of the different enzymatic and metal catalysts in specific pathological processes are still being clarified, increased $O_2^{\bullet -}$ generation is implicated in many human diseases [24]. *A. albopurpurascens*, *D. discolor*, *K. henryi*, and *S. formosanum* extracts displayed good scavenging activities, with IC_{50} values ranging from 12.9 to 28.5 μg/mL. Scavenging activities for 20, 40, and 60 U of the superoxide dismutase positive control

Table 3
Reducing power activity of Taiwanese plant leaves ethanolic extract

Plants	Extract (μg)	Reducing power activity ρA 700 nm±S.D.
A. albopurpurascens	25	0.381±0.0262
	50	0.803±0.0033
	75	1.116±0.0011
A. formosana	25	0.410±0.0182
	50	0.767±0.0183
	75	1.076±0.0044
C. kanehirai	25	0.331±0.0195
	50	0.590±0.0232
	75	0.731±0.0143
D. discolor	25	0.665±0.0371
	50	1.303±0.0472
	75	1.821±0.2171
E. kawakamii	25	0.362±0.0113
	50	0.660±0.0615
	75	0.945±0.0422
F. formosana	25	0.208±0.0021
	50	0.417±0.0162
	75	0.628±0.0331
K. henryi	25	0.827±0.0132
	50	1.590±0.0253
	75	1.952±0.0136
M. pulchra var. *microphylla*	25	0.188±0.0061
	50	0.390±0.0162
	75	0.511±0.0283
N. sericea var. *aurata*	25	0.289±0.0101
	50	0.607±0.0042
	75	0.866±0.0153
P. moluccanum	25	0.197±0.0231
	50	0.397±0.0172
	75	0.515±0.0711
S. warburgii	25	0.189±0.0283
	50	0.379±0.0184
	75	0.546±0.0072
S. formosanum	25	0.532±0.0594
	50	1.091±0.0262
	75	1.381±0.0061

were 19.8%, 43.0%, and 61.3%, respectively. The observations indicate that the four plants may be contributing factors in the development of inflammation and age-related diseases [25].

3.4. Reducing power

In the reducing power assay, the reduction and consequent formation of the ferrous product is monitored via the coincident formation of Perl's Prussian blue at 700 nm. An increasing absorbance is indicative of potent antioxidant activity. The reducing powers of the 12 selected extracts displayed a dose-dependent behavior. *D. discolor*, *K. henryi*, and *S. formosanum* initially showed potent activities, with activity being evident for the *A. albopurpurascens* and *A. formosana* extracts at a higher concentration (Table 3). The differing quantitative reduction activities of the might be a consequence of their differing mechanisms of antioxidation, which can include prevention of chain initiation, chelation of metal ions, reduction of lipid peroxyl radicals, and the scavenging active oxygen species and hydroxyl radicals [26].

3.5. Induced of HO-1 promoter-driven luciferase expression

The luciferase reporter plasmid, flanked by ~3.3 kbp upstream of the 5′ untranslated region of the human HO-1 gene, was constructed. This construct was transiently transfected into RASMC for 48 h followed by exposure to increasing concentrations (1–20 μg/mL) of plant extracts. Quercetin was used as the positive control in this experiment [27]. It induced more than 2.5-fold of the HO-1 production at 10 μg/mL concentration. Luciferase activity of *M. pulchra* var. *microphylla* doubled in response to the increased concentrations of the extract (Fig. 1). Extracts from *S. formosanum* (10 μg/mL) and those from *K. henryi*, *F. formosana*, *P. moluccanum*, and *N. sericea* var. *aurata* at 1 mg/mL resulted in a 1.5–2 fold increase in the promoter activity of HO-1 (Fig. 1). These observations are consistent with the potentially potent HO-1 induction activity and protection of cells and tissues from oxidative injuries of the *M.*

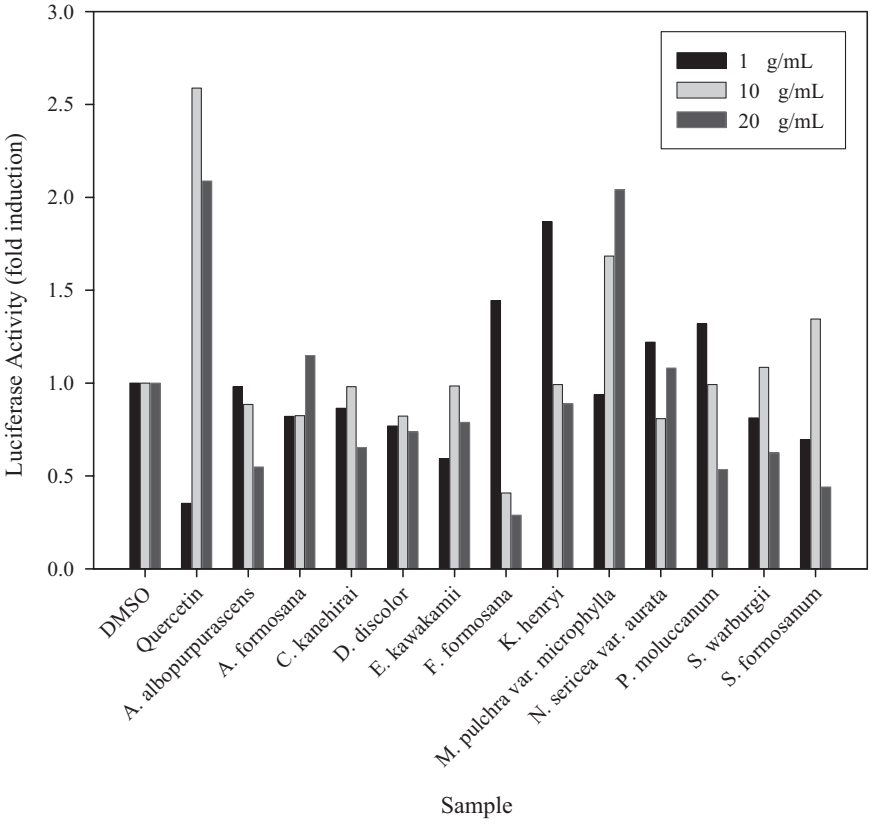

Fig. 1. HO-1 induced effect of Taiwanese plant leaves ethanol extract on rat aortic smooth muscle cells.

pulchra var. *microphylla* extract. The lack of dose–response effect observed with the induction of HO-1 among some of the Taiwanese plant extracts. The hypothetical mechanism may be stimulate the transcription factor, such as NF-κB, leading to inhibit the HO-1 production at high concentration [28]. Confirmation of this potential and mechanism require further study.

3.6. Total phenolic content

Total phenolics may play a role in antioxidant activity [29]. Among the plant extracts analyzed presently, those from *C. kanehirai*, *D. discolor*, *K. henryi*, and *S. formosanum* contained abundant phenolic constituents (more than 30 mg of GAE per g). *A. albopurpurascens*, *E. kawakamii*, *F. formosana*, *M. pulchra* var. *microphylla*, *N. sericea* var. *aurata* and *S. warburgii* contained less but still substantial quantities of phenolics, ranging from 20.8 to 29.4 mg GAE/g dry material.

Acknowledgements

This study was sponsored by the Mackay Memorial Hospital (93MMH-TMU-10). We also express thanks for the financial support for this study from the Juridical Person of Yen's Foundation, Taiwan.

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