Comparison of the Antioxidant and Cytotoxic Activities of *Phyllanthus virgatus* and *Phyllanthus amarus* Extracts

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Key Words

Phyllanthus virgatus • *Phyllanthus amarus* • Antioxidant • HepG2 • Cytotoxicity • Oxygen consumption

Abstract

Objective: To determine the antioxidant activity and cytotoxicity of *Phyllanthus virgatus* crude extract compared to *Phyllanthus amarus*. **Methods:** Phenolic contents of the hydromethanolic extracts were measured using Folin-Ciocalteu reagent. Antioxidant activity was evaluated by the 2,2-diphenyl-1-picrylhydrazyl hydrate free radical scavenging and antilipid peroxidation assays. Cytotoxicity on human hepatoma HepG2 cells was assessed by trypan blue and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide viability assays. A stereomicroscope was used to observe and photograph the morphology of the cells. Oxygen consumption of the HepG2 cells was measured using a Clark oxygen electrode. **Results:** The extract of *P. virgatus*, which contained more phenolic compounds than *P. amarus* extract, had higher cytotoxicity and showed higher free radical scavenging activity and more inhibition of peroxidation in a linoleic acid system. *P. virgatus* extract conspicuously increased the oxygen consumption of HepG2 cells, while *P. amarus* extract had little stimulatory effect. **Conclusion:** The hydromethanolic

extract of *P. virgatus* had stronger antioxidant and cytotoxic action than *P. amarus* extract. The stimulation of HepG2 cell respiration by *P. virgatus* extract suggests the extract alters mitochondrial function; this action could play a role in the cytotoxicity of this plant.

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Introduction

Oxidative stress induced by oxygen radicals, causing damage to lipids, proteins and nucleic acid, has been shown to be involved in the initiation and progression of various diseases such as inflammation, liver injury, renal failure, cardiovascular diseases and cancer [1]. Several studies have revealed the potential of medicinal plants for serving as antioxidants against various diseases induced by oxidative radicals [2–4]. It is widely known that almost all chemotherapeutic agents currently used produce serious toxicities to several organs. Naturally occurring phenolic compounds of plants have been shown to have antioxidant properties [5–7] and to exhibit cytotoxic activity on a variety of cancer cell types, such as Jurkat and human hepatoma HepG2 cells, without toxicity to non-cancer cells [8]. Moreover, there is increasing evidence

showing the chemopreventive potential of many plant compounds such as green and black tea polyphenols, lycopene, soy isoflavones, curcumin, phenethyl isothiocyanate, indole-3-carbinol and perillyl alcohol [9–11].

The genus *Phyllanthus* consists of several species in the family Euphorbiaceae. *Phyllanthus virgatus* and another two species, *P. amarus* and *P. urinaria*, are closely related in appearance and phytochemical structure [12]. In Thailand, they have the same local name (Look Tai Bai). All of them traditionally have been used for the treatment of many ailments such as gonorrhea, jaundice, diabetes and liver diseases [13]. The anticancer activity of *Phyllanthus* species has also been documented [14–19]. For example, *P. amarus* inhibits the growth of human adenocarcinoma cell line Caco-2 [14], hepatoma induced by *N*-nitrosodiethylamine in rats [15] and sarcoma induced by 20-methylcholanthrene in mice [16]. Similarly, *P. urinaria* has also been shown to possess cytotoxic activity against several cancer cell lines including human promyelocytic leukemia [17], Lewis lung carcinoma cells [18] and HepG2 cells [19]. However, the pharmacological studies on *P. virgatus* mostly involved its antimicrobial [20, 21] and antihyperglycemic effects [22]. Cytotoxicity of *P. virgatus* to cancer cells, particularly HepG2 cells, has never been reported. In this survey, we studied and compared the antioxidant and cytotoxic activities of the hydromethanolic extracts of *P. virgatus* and *P. amarus*. In addition, the effects of both extracts on HepG2 cell oxygen consumption were also investigated.

Materials and Methods

Plant Materials and Extraction

Plants were collected from June to September 2005 in the Pak Tong Chai and Mueang districts of Nakhon Ratchasima province, Thailand, and were identified by a botanist, Dr. Paul J. Grote, School of Biology, Suranaree University of Technology. Specimens of the plants are archived at the same School of Biology. Whole plants were thoroughly washed with distilled water to remove dirt and contaminants, and then oven-dried at 40°C. Each dried plant (10 g) was extracted twice with 100 ml of 50% methanol in distilled water. The pooled extracts were filtered and concentrated at 40°C using a rotary evaporator under low pressure. The residue was freeze-dried in a lyophilizer and stored at –20°C until used.

Measurement of Total Phenolic Compounds

The concentration of phenolic compounds in an extract was measured according to the method described previously [23], using gallic acid as a standard. The reaction mixture consisted of 250 µl of the extract (1 mg/ml) or standard (0.025–0.5 mg/ml gallic acid), and 2.5 ml of 2% Na₂CO₃ was added with 100 µl of 50% Folin-Ciocalteu reagent. After 30 min of incubation, the absor-

bance was measured at 750 nm using a spectrophotometer. Results were expressed as milligrams per gram of gallic acid equivalents.

Determination of Antioxidant Activity: DPPH Free Radical Scavenging Activity

To measure antioxidant activity, the 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) radical scavenging assay was carried out according to the procedure previously described [8]. Briefly, each sample (500 µl; final concentration range: 0–300 µg/ml) was added to 4.0 ml of 50 µM DPPH in methanolic solution, and the final volume was adjusted to 5.0 ml with distilled water. After vortexing, the mixture was incubated for 30 min in the dark at room temperature. The decrease in absorbance at 517 nm was then measured using a spectrophotometer. Antioxidant activity was expressed as half maximal inhibitory concentration (IC₅₀), which was defined as the concentrations of the extracts required to inhibit the formation of DPPH radicals by 50%.

Determination of Antioxidant Activity: Inhibition of Linoleic Acid Oxidation

The antilipid peroxidation activity of the extracts was determined using the ferric thiocyanate method, as previously described [24]. Each sample (500 µl; final concentration range: 0–2,000 µg/ml) was mixed with 2.5 ml of linoleic acid emulsion (0.2 M, pH 7.0) and 2.0 ml of phosphate buffer (0.2 M, pH 7.0). The linoleic acid emulsion was prepared by mixing 0.2804 g of linoleic acid with 0.2804 g of Tween 20 and 50 ml of phosphate buffer. The reaction mixture was incubated in the dark at 60°C for 8 h. An aliquot (0.1 ml) of the mixture was diluted with 4.5 ml of 75% ethanol, followed by the addition of 0.2 ml of 30% ammonium thiocyanate and 0.2 ml of 20 mM ferrous chloride in 3.5% HCl. Absorbance of the solution was measured at 500 nm using a spectrophotometer. The percentage of inhibition of lipid peroxidation in linoleic acid emulsion was calculated as follows:

$$\text{inhibition of lipid peroxidation (\%)} = (1 - A_1/A_0) \times 100\%,$$

where A₀ is the absorbance of the control, while A₁ is the absorbance in the presence of the extracts.

Cytotoxicity Studies

HepG2 cells (ATCC HB-8065; Manassas, Va., USA) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 100 U/ml antibiotic-antimycotic (Gibco, Langley, Okla., USA). The cells were grown at 37°C in a fully humidified atmosphere of air with 5% CO₂. The cells were used to determine the cytotoxicity of the extracts by trypan blue exclusion and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays [8].

Trypan Blue Exclusion Assay

Cells (5 × 10⁵ cells/well) were plated in triplicate in a 96-well culture plate overnight. They were treated with various concentrations (ranging from 0 to 1,000 µg/ml) of the extracts for 24 h. Cells were harvested after digestion with 0.25% trypsin-EDTA solution at 37°C for 5 min. The cell suspension was mixed with an equal volume of 0.4% (w/v) trypan blue. The numbers of viable (unstained) and dead (stained) cells were counted by hemacytometer under a light microscope and the results calculated and expressed as the percentage of live cells compared to control.

Table 1. Comparison of total phenolic contents and antioxidant activity of *Phyllanthus* extracts

	Total phenolic contents, mg/g GAE	Scavenging of DPPH, IC ₅₀ , µg/ml	Inhibition of linoleic acid oxidation, %	
			250 µg/ml	2,000 µg/ml
<i>P. virgatus</i>	43.6 ± 1.0***	30.4 ± 5.1*	62.9 ± 3.1**	84.0 ± 3.2***
<i>P. amarus</i>	34.2 ± 1.8	39.0 ± 4.3	57.5 ± 1.1	65.5 ± 3.1

Values are means ± SD. GAE = Gallic acid equivalents. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; $n = 5$.

MTT Assay

HepG2 cells (5×10^5 cells/well) were prepared as described above for the trypan blue exclusion assay and treated with each extract for 24 h. MTT solution (0.5 mg/ml) was added to each well and incubated for a further 4 h. The medium was removed and 100 µl of DMSO (99.9%) added to each well to dissolve purple crystals of formazan. Absorbance was measured with a spectrophotometer at a wavelength of 540 nm using a microplate reader (Biorad, USA). Data are expressed as percent cell viability compared to control.

Measurement of HepG2 Cell Oxygen Consumption

Rates of oxygen consumption by HepG2 cells were determined with a Clark oxygen electrode (Hansatech Oxygraph, King's Lynn, UK). HepG2 cells (2×10^7 cells/ml) were incubated in an oxygraph chamber. Glucose (2 mg/ml) was used as a substrate. After a stable rate of oxygen consumption, carbonyl cyanide 3-chlorophenylhydrazone (CCCP), which is a well-known and potent mitochondrial uncoupler (as a positive control), or the extracts were added to the chamber. Respiratory rates were expressed as nanomoles of oxygen per milliliter per minute.

Statistical Analysis

Data are expressed as means ± SD, and the unpaired t test was used to compare phenolic content and antioxidant activity between *P. virgatus* and *P. amarus* groups. However, the data from cytotoxicity and oxygen consumption studies were not normally distributed. The data were expressed as medians with interquartile ranges. The Mann-Whitney U test was used to compare cytotoxicity (IC₅₀) between *P. virgatus* and *P. amarus* groups. Statistical differences between treated and control groups in the oxygen consumption study were determined using the Friedman repeated measures analysis of variance by ranks followed by the Student-Newman-Keuls method for multiple comparisons. Differences between groups were considered significant when $p < 0.05$ ($n = 5$ in each group for all experiments).

Results

Total Phenolic Content and Antioxidant Activity

The amounts of phenolic compounds in extracts as well as antioxidant activity are shown in table 1. *P. virgatus* extract contained more phenolic compounds than

P. amarus extract. The former also showed significantly greater antioxidant capacity both in DPPH scavenging ($p < 0.05$) and inhibition of linoleic acid oxidation ($p < 0.01$). Significant differences in the inhibition of linoleic acid oxidation were found at concentrations of ≥ 250 µg/ml of the extracts, and the maximal inhibition rates of the *P. virgatus* and *P. amarus* extracts were about 84.0 and 65.5% ($p < 0.001$), respectively.

Cytotoxic Effect

The cytotoxic activity of the extracts of *P. virgatus* and *P. amarus*, expressed as IC₅₀ values, from the two assays was similar (fig. 1). The results showed that *P. virgatus* extract was more toxic to HepG2 cells than *P. amarus* extract. The IC₅₀ of *P. amarus* and *P. virgatus* extracts calculated from trypan blue exclusion were 500 (424–604) and 380 (305–401) µg/ml, respectively (fig. 1a), whereas those calculated from MTT assays were 372 (336–593) and 258 (208–296) µg/ml, respectively (fig. 1b).

Effects of Phyllanthus Extracts on Morphological Changes of HepG2 Cells

Morphological features of cells treated with the extracts at 500 µg/ml were studied after 24 h of incubation (fig. 2). Normal cell morphology characterized by epithelial-like features forming a monolayer on the surface of the culture flask was seen in controls and cells treated with 1% DMSO. In the presence of the plant extracts, morphological changes were observed, such as rounding up, loss of contact with cells and detachment from the plate. *P. virgatus* extract caused more extensive morphological changes in HepG2 cells than *P. amarus*.

Effects of Phyllanthus Extracts on Oxygen Consumption of HepG2 Cells

The rate of oxygen consumption by HepG2 cells was measured in the presence of the extracts (fig. 3). It was found that *P. amarus* extract caused a small and statisti-

Fig. 1. Cytotoxic effects of *Phyllanthus* extracts. Cell viability was measured by two different methods. Values are expressed as medians with interquartile ranges in parentheses. The IC_{50} of *P. amarus* and *P. virgatus* measured by the trypan blue method (**a**)

were 500 (424–604) and 380 (305–401) $\mu\text{g/ml}$, respectively ($p < 0.05$; $n = 5$). The IC_{50} of *P. amarus* and *P. virgatus* measured by MTT viability assays (**b**) were 372 (336–593) and 258 (208–296) $\mu\text{g/ml}$, respectively ($p < 0.05$; $n = 5$).

Fig. 2. Effects of *Phyllanthus* extracts on HepG2 morphology. Morphological features of cells treated with the extracts (500 $\mu\text{g/ml}$ each) were observed under a microscope after 24 h of incubation. **a** Control. **b** 0.5% DMSO. **c** *P. amarus*. **d** *P. virgatus*.

Fig. 3. Effects of *Phyllanthus* extracts on oxygen consumption of HepG2 cells. Tracings **a** and **b** show the effects of vehicle (0.5% DMSO) and CCCP (1 μM) as negative and positive controls, respectively. Tracings **c** and **d** show the effects of *P. amarus* and *P. virgatus* (500 $\mu\text{g/ml}$ each), respectively. Arrows: time at which the vehicle, CCCP and the extracts were added. Numbers in parentheses: rates of oxygen consumption in nanomoles of oxygen per milliliter per minute. The tracings are representative of 5 experiments ($n = 5$). The respiration rates of HepG2 cells in the presence of *P. virgatus* extract [16.9 (13.3–20.7) $\text{nmol O}_2/\text{ml/min}$] and CCCP [9.8 (8.1–14.2) $\text{nmol O}_2/\text{ml/min}$], but not of *P. amarus* extract [7.5 (5.7–11.0) $\text{nmol O}_2/\text{ml/min}$], were significantly higher ($p < 0.05$) than that of the negative control [5.5 (4.2–8.2) $\text{nmol O}_2/\text{ml/min}$]. Data are presented as medians with interquartile ranges.

cally insignificant respiratory stimulation ($p > 0.05$), while *P. virgatus* extract clearly enhanced the respiration of HepG2 cells. This effect of *P. virgatus* extract was comparable to that produced by CCCP.

Discussion

The findings of a higher total phenolic content of *P. virgatus* extract from five separate plant collections and the stronger antioxidant activity of *P. virgatus* extract are comparable to those presented by Kumaran and Karunakaran [13], who have previously reported that the total phenolic content and antioxidant activity of five *Phyllanthus* species from India can be placed in the following order: *P. debilis* > *P. urinaria* > *P. virgatus* > *P. maderaspatensis* > *P. amarus*. *P. virgatus* extract also showed a stronger cytotoxic effect than *P. amarus*.

Experiments on the effect of *Phyllanthus* extracts on HepG2 cell respiration showed that at a concentration of 500 µg/ml, *P. virgatus* extract clearly stimulated oxygen consumption, whereas *P. amarus* extract produced but a small and insignificant increase in respiration. Since oxygen consumption by cells is mostly due to mitochondrial oxidative phosphorylation, changes in cellular respiration rate as a result of experimental treatment may indicate alterations in mitochondrial activity. This finding therefore suggests that *P. virgatus* extract affects mitochondrial function. The greater cytotoxic activity of *P. virgatus* extract compared to that of *P. amarus* may be related to a higher phenolic content since it has been reported that phenolic compounds could be cytotoxic [25].

The ability of the extracts to stimulate HepG2 cell oxygen consumption may be involved in cell death. Apoptosis signaling transduction can be induced through the death receptor pathway, such as the Fas ligand and Bcl-2 family, as well as through a mitochondrial pathway [26]. Mitochondrial dysfunction can cause a necrosis-type cell death via ATP depletion and Ca^{2+} dysregulation [27]. Further experiments are needed to determine whether dysregulation of mitochondrial respiration and function is caused by phenolic compounds and is thereby involved in the cytotoxicity of *Phyllanthus* extracts; these might include, for example, measurement of oxygen consumption and oxidative phosphorylation by isolated mitochondria treated with known phenolic compounds of these extracts.

Conclusion

The results of the present study demonstrate that the hydromethanolic extract of *P. virgatus* exerted greater antioxidant and cytotoxic effects on human hepatoma HepG2 cells than *P. amarus* extract. It also produced stronger stimulation of HepG2 cell respiration, which indicates the possibility of mitochondrial involvement in the cytotoxic action.

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References

- 1 Aruoma OI, Kaur H, Halliwell B: Oxygen free radicals and human diseases. *J R Soc Health* 1991;111:172–177.
- 2 Rice-Evans CA, Miller NJ, Paganga G: Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic Biol Med* 1996;21:933–956.
- 3 Sabu MC, Kuttan R: Anti-diabetic activity of medicinal plants and its relationship with their antioxidant property. *J Ethnopharmacol* 2002;81:155–160.
- 4 Vuillaume M: Reduced oxygen species, mutation, induction and cancer initiation. *Mutat Res* 1987;186:43–72.
- 5 Sithisarn P, Supabphol R, Gritsanapan W: Comparison of free radical scavenging activity of Siamese neem tree (*Azadirachta indica* A Juss var *siamensis* Valetton) leaf extracts prepared by different methods of extraction. *Med Princ Pract* 2006;15:219–222.
- 6 Rao VS, Paiva LA, Souza MF, Campos AR, Ribeiro RA, Brito GA, Teixeira MJ, Silveira ER: A correlation between the antioxidant activity and total phenolic content has been observed. *Planta Med* 2003;69:851–853.
- 7 Sithisarn P, Jarikasm S: Antioxidant activity of *Acanthopanax trifoliatum*. *Med Princ Pract* 2009;18:393–398.
- 8 Rao YK, Geethangili M, Fang SH, Tzeng YM: Antioxidant and cytotoxic activities of naturally occurring phenolic and related compounds: a comparative study. *Food Chem Toxicol* 2007;45:1770–1776.
- 9 Gann PH, Ma J, Giovannucci E, Willett W, Sacks FM, Hennekens CH, Stampfer MJ: Lower prostate cancer risk in men with elevated plasma lycopene levels: results of a prospective analysis. *Cancer Res* 1999;59:1225–1230.
- 10 Miller EC, Giovannucci E, Erdman JW Jr, Bahnson R, Schwartz SJ, Clinton SK: Tomato products, lycopene, and prostate cancer risk. *Urol Clin North Am* 2002;29:83–93.
- 11 Kelloff GJ, Crowell JA, Steele VE, Lubet RA, Malone WA, Boone CW, Kopelovich L, Hawk ET, Lieberman R, Lawrence JA, Ali I, Viner JL, Sigman CC: Progress in cancer chemoprevention: development of diet-derived chemopreventive agents. *J Nutr* 2000;130:467S–471S.

- 12 Chantaranothai P: *Phyllanthus*; in van Welzen PC, Chayamarit K (eds): Euphorbiaceae. Flora of Thailand. Bangkok, Royal Forest Department, 2005, vol 6, pp 473–507.
- 13 Kumaran A, Karunakaran RJ: In vitro antioxidant activities of methanol extracts of five *Phyllanthus* species from India. LWT 2007;40:344–352.
- 14 Lawson-Evi P, Eklun-Gadegbeku K, Agbonon A, Aklikokou K, Moukha S, Creppy EE, Gbassor M: Toxicological assessment on extracts of *Phyllanthus amarus* Schum and Thonn. Sci Res Essay 2008;3:410–415.
- 15 Rajeshkumar NV, Kuttan R: *Phyllanthus amarus* extract administration increases the life span of rats with hepatocellular carcinoma. J Ethnopharmacol 2000;73:215–219.
- 16 Rajeshkumar NV, Joy KL, Kuttan G, Ramsewak RS, Nair MG, Kuttan R: Antitumour and anticarcinogenic activity of *Phyllanthus amarus* extract. J Ethnopharmacol 2002;81:17–22.
- 17 Huang ST, Yang RC, Chena MY, Pang JHS: *Phyllanthus urinaria* induces the Fas receptor/ligand expression and ceramide-mediated apoptosis in HL-60 cells. Life Sci 2004;75:339–351.
- 18 Huang ST, Yang RC, Yang LJ, Lee PN, Pang JHS: *Phyllanthus urinaria* triggers the apoptosis and Bcl-2 down-regulation in Lewis lung carcinoma cells. Life Sci 2003;72:1705–1716.
- 19 Sureban SM, Subramaniam D, Paramasivam R, Ramanujam RP, Dieckgraefe BK, Houchen CW, Anant S: Therapeutic effects of *Phyllanthus* species: induction of TNF- α -mediated apoptosis in HepG2 hepatocellular carcinoma cells. Am J Pharmacol Toxicol 2006;1:65–71.
- 20 Maruyama M, Yamauchi S, Akiyama K, Sugahara T, Kishida T, Koba Y: Antibacterial activity of a virgatusin-related compound. Biosci Biotechnol Biochem 2007;71:677–680.
- 21 Akiyama K, Yamauchi S, Nakato T, Maruyama M, Sugahara T, Kishida T: Antifungal activity of tetra-substituted tetrahydrofuran lignan, (–)-virgatusin, and its structure-activity relationship. Biosci Biotechnol Biochem 2007;71:1028–1035.
- 22 Shabeer J, Srivastava RS, Singh SK: Antidiabetic and antioxidant effect of various fractions of *Phyllanthus simplex* in alloxan diabetic rats. J Ethnopharmacol 2009;124:34–38.
- 23 Kähkönen MP, Hopia AI, Vuorela HJ, Rauha JP, Pihlaja K, Kujala TS, Heinonen M: Antioxidant activity of plant extracts containing phenolic compounds. J Agric Food Chem 1999;47:3954–3962.
- 24 Zhan Y, Dong CH, Yao YJ: Antioxidant activities of aqueous extract from cultivated fruit-bodies of *Cordyceps militaris* (L) link in vitro. J Integr Plant Biol 2006;48:1365–1370.
- 25 Metodiewa D, Jaiswal AK, Cenas N, Dickancaité E, Segura-Aguilar J: Quercetin may act as a cytotoxic prooxidant after its metabolic activation to semiquinone and quinoidal product. Free Radic Biol Med 1999;26:107–116.
- 26 Nagata S: Apoptosis by death factor. Cell 1997;88:355–365.
- 27 Bernardi P, Petronilli V, di Lisa F, Forte M: A mitochondrial perspective on cell death. Trends Biochem Sci 2001;26:112–117.