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Effects of *Phyllanthus urinaria* extract on HepG2 cell viability and oxidative phosphorylation by isolated rat liver mitochondria

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ABSTRACT

Ethnopharmacological relevance: Phyllanthus urinaria is widely used as anti-inflammatory, anti-diarrheal and hepatoprotective medicines in Asian countries such as India, China and Thailand. In Thailand, *Phyllanthus urinaria* is traditionally used as an adjuvant or alternative medicine for cancer patients, including liver cancer. However, there is limited scientific evidence supporting its use in cancer particularly hepatocellular carcinoma.

Aim of the study: To investigate the cytotoxic effect of *Phyllanthus urinaria* extract on human hepatocellular carcinoma HepG2 cells and the effect on oxidative phosphorylation by isolated rat liver mitochondria.

Materials and methods: HepG2 cells and isolated rat liver mitochondria were treated with the 50% methanolic extract of *Phyllanthus urinaria*. Cytotoxicity of the extract was assessed by trypan blue exclusion and MTT assay. Rates of oxygen consumption of isolated mitochondria were determined with a Clark oxygen electrode.

Results: It was found that the hydromethanolic extract induced cell death of HepG2 cells in a dose-dependent fashion. The IC_{50} of *Phyllanthus urinaria* extract measured by trypan blue exclusion and MTT assay were 431 ± 65 μg/ml and 445 ± 62 μg/ml, respectively. Morphological changes of the cells were also observed. With isolated rat liver mitochondria, the extract slightly stimulated mitochondrial state 4 respiration but profoundly depressed state 3 respiration and respiratory control ratio.

Conclusions: The extract impairs energy metabolism by acting as inhibitor of oxidative phosphorylation and weak mitochondrial uncoupler. These mitochondrial effects may play a role in the cytotoxic action of *Phyllanthus urinaria* extract on HepG2 cells. These results provide preliminary experimental evidence supporting the use of *Phyllanthus urinaria* against hepatocellular carcinoma and open the possibility of considering this plant an adjunctive medicine for the treatment of this deadly disease.

1. Introduction

Phyllanthus urinaria Linn. (Euphorbiaceae) is widely distributed in Southern America and many countries in Asia, such as India, China and Thailand. It is a tropical herb traditionally used for the treatment of many ailments. Several activities of *Phyllanthus urinaria*, such as anti-inflammatory, antioxidant as well as smooth muscle relaxation have been reported (Catapan et al., 2000; Kumaran and Karunakaran, 2007; Paulino et al., 1996). *Phyllanthus urinaria* is closely related in appearance to *Phyllanthus amarus* (Van Welzen and Chayamarit, 2007). Herbal products of both species have been sold in Thailand under the same local name of "Look-Tai-Bai". In Thailand, it is believed that Look-Tai-Bai can protect organs including liver from toxic substances (Chularojmontri et al., 2005; Hau et al., 2009; Kumar and Kuttan, 2005; Pramyothin et al., 2007) and increasingly used as adjuvant or alternative drug for patients with liver cancer. Like *Phyllanthus amarus*, there is evidence showing that *Phyllanthus urinaria* produces hepatoprotective effects against hepatitis B virus in clinical trial (Wang et al., 1995); and viral hepatitis is the major cause of hepatocellular carcinoma worldwide (Szabó et al., 2004). There is a report suggesting a good correlation between herbs with both antiviral and anticancer activities and the treatment of hepatocellular carcinoma (Thyagarajan et al., 2002). Thus cytotoxic effects, particularly on liver cancer cells, of the plants in *Phyllanthus* spp. are a topic of interest, both from the perspective of understanding how these plant extracts alter cell functions and from the standpoint of developing alternative agents for the treatment of liver cancer. There are increasing lines of evidence suggesting that *Phyllanthus urinaria* extract possess anticancer activity by triggering cell cycle arrest and apoptosis (Giridharan et al., 2002; Huang et al., 2004, 2006, 2003). In addition to the death receptor pathway such as Fas ligand and Bcl-2 family, apoptosis signaling transduction can also be induced through mitochondrial pathway. Moreover, mitochondrial

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dysfunction can cause a necrosis-type cell death through ATP depletion and Ca^{2+} dysregulation (Bernardi et al., 2001). Thus, the aims of this study were to investigate the cytotoxic effect of *Phyllanthus urinaria* extract on human hepatocellular carcinoma HepG2 cells and the possible involvement of mitochondrial oxidative phosphorylation in this action.

2. Materials and methods

2.1. Plant material

Phyllanthus urinaria was collected in the month of June to September 2005 from Paktongchai and Muang districts in Nakhon Ratchasima province, Thailand. The plants were identified by a botanist, Dr. Paul J. Grote, School of Biology, Suranaree University of Technology (SUT) and specimen of the plants has been kept at School of Biology, SUT. The voucher specimen number is Pharm-Chu-003.

Dried whole plants (10 g) were 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 concentrated crude extract was freeze-dried in a lyophilizer and stored at −20 °C.

2.2. Total phenolic compounds

The concentration of phenolic compounds in the extract was measured according to the method described previously (Kahkonen et al., 1999) 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% Na_2CO_3 was added with 100 μl of 50% Folin-ciocalteu reagent. After 30 min of incubation, the absorbance was measured at 765 nm using a spectrophotometer. Result was expressed as milligrams per gram of gallic acid equivalents (GAE).

2.3. Cytotoxicity studies

HepG2 cells (ATCC HB 8065, USA) were cultured in RPMI-1640 medium, supplemented with 10% fetal bovine serum and 100 U/ml antibiotic-antimycotic (Gibco, USA). Cells were grown at 37 °C with 5% CO_2 atmosphere in fully humidified air. The cells were used to determine the cytotoxicity of the extract of *Phyllanthus urinaria* by trypan blue exclusion and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Mosmann, 1983). The final concentration of DMSO in the medium was adjusted not to exceed 1% (v/v) in all cultures in order to minimize the effect of DMSO.

2.3.1. Trypan blue exclusion assay

Cells (5×10^5 cells/well) were plated in triplicate in a 96-well culture plate overnight. They were treated with various concentrations (ranging from 0 to 1000 μg/ml) of the extract of *Phyllanthus urinaria* 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 number of viable (unstained) and dead (stained) cells were counted by hemacytometer under a light microscope and results were calculated and expressed as percentage of live cell compared to control.

2.3.2. MTT assay

HepG2 cells (5×10^5 cells/well) were prepared similar to that described above for trypan blue exclusion assay. They were treated with the extracts of *Phyllanthus urinaria* for 24 h. MTT solution (final concentration 0.5 mg/ml in RPMI-1640) was added to each well and incubated further for 4 h. The medium was removed and 100 μl of DMSO was added to each well to dissolve purple crystals of formazan. The absorbance was measured with a spectrophotometer at wavelength of 540 nm using a microplate reader (Bio-Rad, USA).

Table 1
Yield and total phenolic compounds of *Phyllanthus urinaria* extract.

	Yield (%)	Total phenolic compounds (mg/g GAE)
Phyllanthus urinaria	4.78	354 ± 27[a]

[a] Mean ± SEM; $n = 5$.

2.4. Isolation of rat liver mitochondria and determination of mitochondrial oxygen consumption

Male albino Wistar rats weighing 200–250 g were obtained from Institutional Animal Care, SUT, fed with standard chow diet and water ad libitum. Rat liver mitochondria were isolated by differential centrifugation as described previously (Slinde et al., 1975). Briefly, rats were sacrificed by cervical dislocation and livers were immediately removed, rinsed and minced in ice-cold medium (containing 0.25 M sucrose, 5 mM HEPES buffer (pH 7.4) and 1 mM EGTA) and homogenized with teflon pestle tissue homogenizer. All centrifugations were carried out at 4 °C. Homogenates were centrifuged at 700 × *g* for 7 min and the resulting supernatant further centrifuged at 5000 × *g* for 10 min. Pellets were resuspended in 0.25 M sucrose and centrifuged at 12,000 × *g* for 10 min. The final mitochondrial pellet was washed with 0.25 M sucrose to remove a top layer of microsome and then gently resuspended in 1 ml of 0.25 M sucrose with teflon pestle tissue homogenizer by hand. The final mitochondrial suspensions were stored in ice-bath until used, which was within 3 h after isolation. Mitochondrial protein content was determined using the Bio-Rad protein assay reagent. The rates of mitochondrial respiration were measured using a Clark oxygen electrode (Hansatech, UK). The mitochondria (2 mg protein) were incubated in 1 ml of medium consisting of 10 mM HEPES buffer (pH 7.4), 225 mM sucrose, 5 mM $MgCl_2$, 20 mM KCl, and 10 mM KH_2PO_4 at 25 °C. Glutamate plus malate (5 mM each) were used as substrates. The rates of oxygen consumption were expressed as nmol O/ml/min. The uncoupling agent CCCP (carbonyl cyanide 3-chlorophenylhydrazone), was used as a positive control of the uncoupled respiration.

2.5. Statistical analysis

Data were expressed as means ± SEM. Statistical differences between treated and control groups were determined using one way repeated measures analysis of variance (ANOVA) followed by Student–Newman–Keuls test. Differences between groups were considered significantly different when the P value < 0.05.

3. Results

3.1. The total phenolic content of Phyllanthus urinaria extract

The amount of phenolic compounds in *Phyllanthus urinaria* extract is shown in Table 1. The phenolic content was determined spectrometrically according to the Folin-Ciocalteu procedure. It was found that *Phyllanthus urinaria* extract contained 354 ± 27 mg/g GAE.

3.2. Cytotoxic effect

The cytotoxic activities of the extract of *Phyllanthus urinaria* expressed by its IC_{50} values from the two assays are comparable. The IC_{50} calculated from trypan blue exclusion and MTT assay were 431 ± 65 μg/ml and 445 ± 62 μg/ml, respectively (Fig. 1).

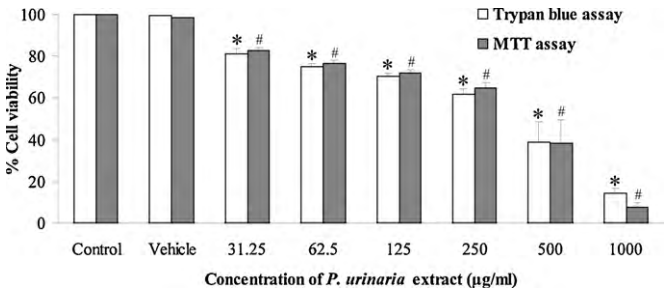

Fig. 1. Concentration-response of *Phyllanthus urinaria* extract on viability of HepG2 cells. Cytotoxicity of the extract was measured by trypan blue exclusion and MTT assay as described in Section 2. *, # $P < 0.05$ ($n = 5$) compared to control.

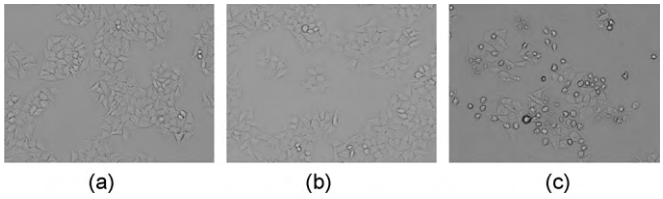

Fig. 2. Effect of *Phyllanthus urinaria* extract on HepG2 morphology. Microphotographs (100×) of HepG2 cells were taken after treating with vehicle or the extract (500 μg/ml) for 24 h as described in Section 2; (a) control, (b) 0.5% DMSO, and (c) *Phyllanthus urinaria* extract (500 μg/ml).

3.3. Effect of Phyllanthus urinaria extract on HepG2 cell morphology

Morphological features of cell treated with the extract of *Phyllanthus urinaria* (500 μg/ml) were observed under microscope with 100× magnification. The normal morphology of HepG2 maintained in RPMI-1640 medium is illustrated in Fig. 2a. The epithelial-like feature forming a monolayer on the surface of the culture flask was apparent. In the presence of the extract, the morphological changes (Fig. 2c) were observed such as rounding up, loss of contact with neighbouring cells and detachment from plate. The 0.5% DMSO did not affect cell proliferation and survival of HepG2 cells compared to control (Fig. 2b).

3.4. Effect of Phyllanthus urinaria extract on mitochondrial function

Fig. 3 shows that 500 μg/ml of the extract increased oxygen consumption by isolated rat liver mitochondria respiring in the presence of glutamate and malate as substrates while DMSO had no effect. It was also apparent that this uncoupling activity of the extract was much weaker than CCCP, a powerful mitochondrial uncoupler. Fig. 4 demonstrates the inhibition of mitochondrial oxidative phosphorylation by the extract. Tracing 4a showed the control respiratory response of isolated mitochondria to ADP. Addition of ADP greatly stimulated mitochondrial respiration (state 3 respiration) as ADP was phosphorylated to form ATP. After ADP was exhausted, the respiration returned to slow level (state 4 respiration) with a clear cut-off (transition from state 3 to state 4 respiration). The extract at 500 μg/ml caused a small increase in respiration but strongly depressed state 3 respiration (tracing 4c). In addition there was no clear cut-off and state 4 respiration after ADP was enhanced. This increase in state 4 respiration was, at least partly, due to the uncoupling action of the extract (Fig. 3). Fig. 5 shows that the extract inhibited state 3 respiration and reduced respiratory control ratio (the ratio of state 3/state 4 respiration) in a dose-related manner.

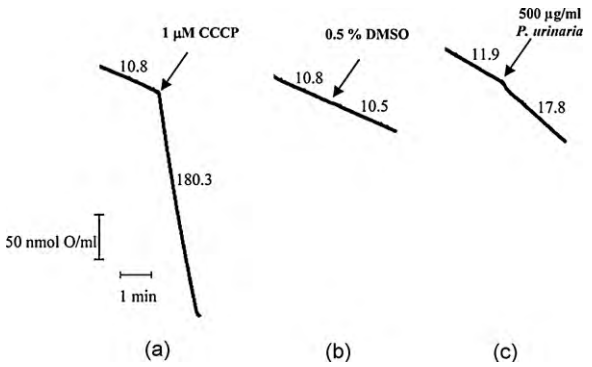

Fig. 3. Uncoupling effect of *Phyllanthus urinaria* extract on isolated rat liver mitochondria. Incubation of the mitochondria and measurement of respiration were described in Section 2. Mitochondria were treated with *Phyllanthus urinaria* at the concentration of 500 μg/ml. CCCP (1 μM) was used as positive control (tracing a) to show the effect of the uncoupler on mitochondrial respiration. Mitochondria treated with 0.5% DMSO as negative control (tracing b) showed no effect (no significant change in rate of oxygen consumption). Arrows indicate the time at which each compound was added. Tracings are representative of four experiments with different mitochondrial preparations.

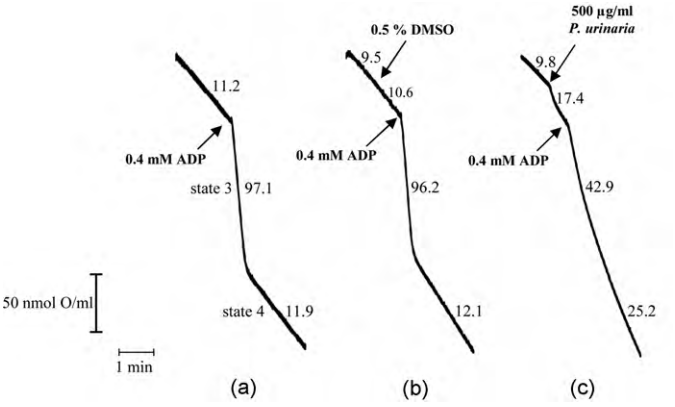

Fig. 4. Inhibition of oxidative phosphorylation by *Phyllanthus urinaria* extract. Rat liver mitochondria were incubated in medium and oxygen consumption was measured as described in Section 2. Tracing a is a control; tracings b and c show the effects of 0.5% DMSO (negative control) and *Phyllanthus urinaria* extract (500 μg/ml), respectively. The extract, vehicle and ADP were added as indicated. Tracings are representative of four experiments with different mitochondrial preparations.

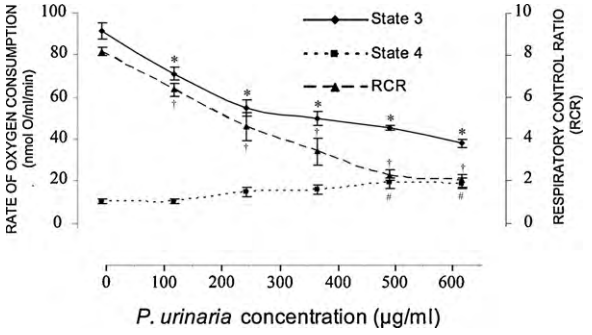

Fig. 5. Dose–response curves of *Phyllanthus urinaria* extract on state 3 and state 4 respiration and respiration control ratio of isolated rat liver mitochondria. Each point represents mean ± SEM. *, # and † $P < 0.05$ compared to control; $n = 4$.

4. Discussion

Natural medicines have played a great role in the treatment of various disorders in human. Cancer has become an important issue in health science as it is a major cause of death in both developed and developing countries. Human liver tumors, particularly hep-

atocellular carcinoma are among the most common malignancies worldwide (Roberts and Gores, 2005). It was reported that aqueous extract of *Phyllanthus urinaria* induced apoptosis of Lewis lung carcinoma cells but did not cause any cell loss in normal cells such as human umbilical vein endothelial cells (HUVEC) and human embryonic liver cells (WRL 68), suggesting that the apoptotic effect of *Phyllanthus urinaria* might be specific to tumor cells (Huang et al., 2003). Other *in vivo* study has also supported the safety of *Phyllanthus urinaria* extract on normal liver cells. Histopathological analysis of liver sections reveals that 80% methanolic extract of *Phyllanthus urinaria* ranging from 20 to 200 mg/kg body weight did not show any toxicological effects on mouse liver (Hau et al., 2009). Several mechanisms underlying apoptosis have been proposed including down-regulation of Bcl-2 expression (Huang et al., 2006), induction of Fas receptor/ligand expression (Huang et al., 2004), and activation of c-myc and caspases (Giridharan et al., 2002). It has been shown that low dose of the extracts (70% methanol) of *Phyllanthus* species including *Phyllanthus urinaria* inhibited HepG2 cell proliferation by induction of TNF-α-mediated apotosis (Sureban et al., 2006). In this study, the cytotoxicity of *Phyllanthus urinaria* extract on HepG2 hepatocellular carcinoma *in vitro* was examined by MTT and trypan blue assay. The calculated IC_{50} were 431 ± 65 μg/ml and 445 ± 62 μg/ml, respectively. The lowest concentration of the extract that caused cytoxicity to HepG2 was 31 μg/ml. In contrast, the least concentration of the extract that caused cytoxicity to HL-60 cells reported previously was 1 mg/ml (Huang et al., 2004). However, the incubation period in this experiment was longer than the previous study (24 and 4 h, respectively). The toxicity of the extract was confirmed by morphological feature of the cells. In fact, the morphological changes were observed after 4 h of incubation with the plant extract (data not shown).

Crude extracts of fruits, herbs and vegetables rich in phenolics are increasingly of interest in food industry as sources of natural antioxidants (Kahkonen et al., 1999). Phenolic compounds from plants possess several biological properties such as antioxidant, anti-apoptosis, anti-aging, anti-carcinogen, anti-inflammation, anti-atherosclerosis and cardiovascular protection (Han et al., 2007). Total phenolic content of *Phyllanthus urinaria* extract used in the present study was 354 ± 27 mg/g GAE which is comparable with that reported previously (325 ± 18 mg/g GAE) (Kumaran and Karunakaran, 2007). From the same study, the methanolic extracts of five different species of *Phyllanthus* have been shown to consist of phenolic compounds such as lignans, tannins, flavonoids and other unknown structures. A correlation between the antioxidant activity and total phenolic content has been observed. However, phenolic compounds, especially flavonoids, possess both antioxidant and prooxidant properties depending on concentration used. The latter which is exhibited at higher concentration of phenolic compounds has been shown to stimulate mitochondrial respiration (Dorta et al., 2005). In our attempt to search for the possible involvement of the mitochondria in the cytotoxic activity of *Phyllanthus urinaria* extract, its effect on oxygen consumption of isolated rat liver mitochondria was investigated. The results showed that the extract profoundly inhibited state 3 respiration and decreased respiratory control ratio. The extract also increased state 4 respiration both before and after ADP. Similar results were obtained when succinate was substrate (data not shown). The increased state 4 respiration as well as the decreased respiratory control ratio indicated uncoupling activity of the extract while depression of state 3 respiration indicated inhibition of oxidative phosphorylation. The very weak uncoupling activity of the extract makes it unlikely that this is the major action responsible for HepG2 cell death. Instead, the extract strongly inhibits oxidative phosphorylation which can conceivably decrease intracellular ATP level. Since cell death can be caused by mitochondrial dysfunction through ATP depletion and Ca^{2+} dysregulation (Bernardi et al., 2001), the mitochondrial effects described here, particularly the inhibition of oxidative phosphorylation, are likely to be involved in the cytotoxicity of *Phyllanthus urinaria* extract on HepG2 cells. On the other hand, the results on mitochondrial functions may suggest a toxicological potential of *Phyllanthus urinaria* in routine use as traditional medicine.

The action of *Phyllanthus urinaria* extract on mitochondrial function reported here has also been found with other medicinal plants. It has been shown that a prenylated flavanoid isolated from the roots of *Dalea elegans* has antioxidant activity and it stimulates mitochondrial state 4, but inhibits state 3 respiration (Elingold et al., 2008). Moreover, it was reported that C-methylated flavonoids, namely myrigalones, from *Myrica gale* Linn. effectively uncoupled oxidative phosphorylation in mitochondria (Mathiesen et al., 1996). These flavonoids also inhibit ATP synthesis. Additionally, several flavonoids found in plants, such as quercetin, galangin, taxifolin and catechin have been shown to affect mitochondrial energetic processes (Dorta et al., 2005). Several secondary metabolites presented in *Phyllanthus urinaria* have been identified, including lignans, alkaloids, tannins, carboxylic acids, methyl and ethyl gallates as well as flavonoids (Chang et al., 2003; Li et al., 2008; Wei et al., 2005). It is possible that chemical(s) belonging to flavonoid group might be responsible for the activity of *Phyllanthus urinaria* on cell viability and mitochondrial function. However, the identification of the active ingredients of this plant needs further investigation.

5. Conclusion

In this study, the effects of *Phyllanthus urinaria* extract on human hepatoma HepG2 cells and isolated rat liver mitochondria have been investigated. It was found that the extract induced cell death and morphological changes of HepG2 cells. With isolated rat liver mitochondria, the extract slightly stimulated state 4 respiration. More importantly, it conspicuously depressed state 3 respiration and respiratory control ratio. Thus, the extract impairs hepatic energy metabolism by acting as uncoupler and inhibitor of mitochondrial oxidative phosphorylation. In view of the importance of mitochondria in cell functions, it is proposed that these mitochondrial effects may contribute to the cytotoxic action of *Phyllanthus urinaria* extract on HepG2 cells. Our findings provide preliminary experimental evidence supporting therapeutic potential of *Phyllanthus urinaria* in human liver cancer and open the possibility of considering this plant an adjunctive medicine for the treatment of this deadly disease.

Acknowledgements

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