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Mechanisms of antihyperuricemic effect of *Phyllanthus niruri* and its lignan constituents

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ABSTRACT

Ethnopharmacological relevance: *Phyllanthus niruri* Linn. (Euphorbiaceae) is used as folk medicine in South America to treat excess uric acid. Our initial study showed that the methanol extract of *Phyllanthus niruri* and its lignans were able to reverse the plasma uric acid of hyperuricemic animals.

Aim of the study: The study was undertaken to investigate the mechanisms of antihyperuricemic effect of *Phyllanthus niruri* and its lignan constituents.

Material and methods: The mechanisms were investigated using xanthine oxidase assay and uricosuric studies in potassium oxonate- and uric acid-induced hyperuricemic rats.

Results: *Phyllanthus niruri* methanol extract exhibited *in vitro* xanthine oxidase inhibition with an IC_{50} of 39.39 μg/mL and a moderate *in vivo* xanthine oxidase inhibitory activity. However, the lignans display poor xanthine oxidase inhibition *in vitro* and a relatively weak *in vivo* inhibitory activity at 10 mg/kg. On the other hand, intraperitoneal treatment with *Phyllanthus niruri* methanol extract showed 1.69 folds increase in urinary uric acid excretion when compared to the hyperuricemic control animals. Likewise, the lignans, phyllanthin, hypophyllanthin and phyltetralin exhibited up to 2.51 and 11.0 folds higher in urinary uric acid excretion and clearance, respectively. The co-administration of pyrazinamide with phyllanthin exhibited a significant suppression of phyllanthin's uricosuric activity resembling that of pyrazinamide with benzbromarone.

Conclusions: The present study showed that the antihyperuricemic effect of *Phyllanthus niruri* methanol extract may be mainly due to its uricosuric action and partly through xanthine oxidase inhibition, whereas the antihyperuricemic effect of the lignans was attributed to their uricosuric action.

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1. Introduction

Hyperuricemia is one of the commonly encountered biochemical abnormalities in clinical practice and has been reported to affect about 10% of adults globally (Dincer et al., 2002). Lifestyle modifications such as weight reduction, decreased alcohol consumption and dietary purine intake may help to decrease blood uric acid but many patients will still need medication to control their hyperuricemia (Wright and Pinto, 2003; Kong et al., 2004).

Despite a long history of hyperuricemia and gout, there are only a limited number of drugs currently used in clinical practice and they belong to two classes, the xanthine oxidase inhibitors, e.g. allopurinol and the uricosuric agents, e.g. probenecid and benzbromarone. Allopurinol is widely prescribed for the treatment of hyperuricemia and gout. However, it has been frequently associated with adverse effects manifested as rash, hypersensitivity, hepatotoxicity, gastrointestinal upset, hepatitis and fever. Approximately 2–10% of patients, especially the elderly developed a pruritic erythematous rash and 0.4% of patients have developed hypersensitivity syndrome, which further prevented the administration of allopurinol (Khoo and Leow, 2000; Adel, 2001; Dincer et al., 2002; Kong et al., 2002). On the other hand, probenecid is generally ineffective in patients with concomitant renal impairment whereas benzbromarone is effective in patients with renal insufficiency but possesses a risk of severe hepatotoxicity (Perez-Ruiz et al., 1998; Adel, 2001; Dincer et al., 2002). Thus, effective and well-tolerated uric acid-lowering agents are much sought after as new therapeutic options.

Natural products, mainly of plant origin have long been used in traditional medicine for the treatment of hyperuricemia and gout. Hitherto, medicinal plants have been the source for a number of clinically important drugs such as morphine, atropine and digoxin, and are excellent sources of lead compounds in the search for new drugs. In an ongoing search for new antihyperuricemic agents from local medicinal plants, the methanol extract and lignan constituents of *Phyllanthus niruri* Linn. (Euphorbiaceae) were found to lower the plasma uric acid of hyperuricemic rats (Murugaiyah and Chan, 2006). *Phyllanthus niruri*, known in Malaysia as “dukong anak” is a

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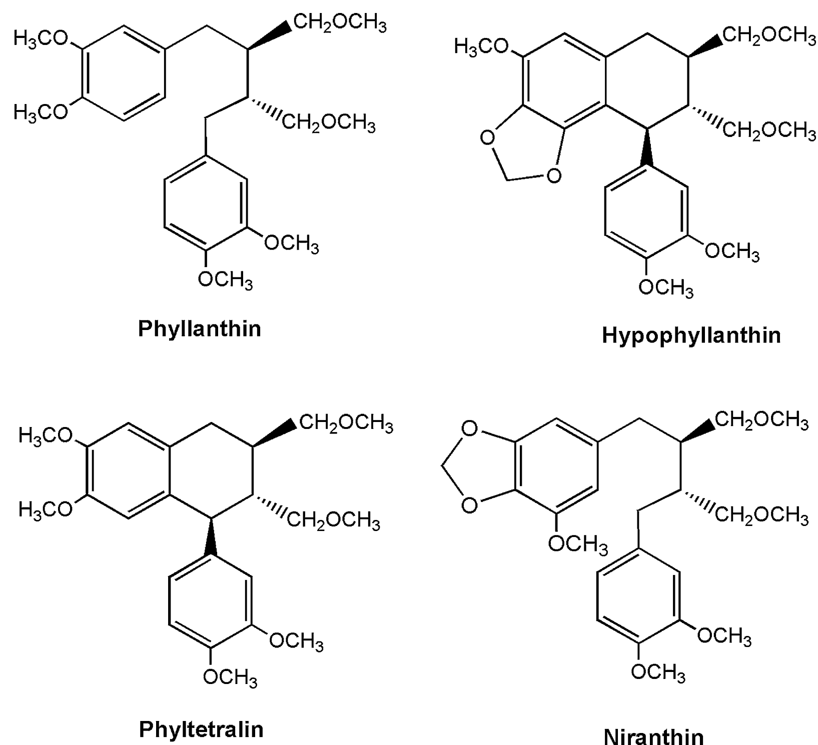

Fig. 1. Chemical structures of lignans isolated from *Phyllanthus niruri*.

small herb, found in most tropical and subtropical regions. It has been traditionally used as a remedy against fever, diarrhoea, colic and kidney problems, and as a diuretic and expectorant (Perry and Metzger, 1980). It has also been used as folk medicine to treat excess uric acid in South America (Unader et al., 1991).

The mechanism by which *Phyllanthus niruri* and its lignans exert urate lowering effect is currently unknown. The present study was undertaken to investigate the mechanisms of antihyperuricemic effect of *Phyllanthus niruri* and its lignan constituents, phyllanthin, hypophyllanthin, phyltetralin and niranthin using the xanthine oxidase assay and uricosuric studies in potassium oxonate- and uric acid-induced hyperuricemic rats.

2. Materials and methods

2.1. Chemicals and reagents

Uric acid and potassium oxonate were purchased from Merck (Darmstadt, Germany) and Aldrich Chemical Company (Milwaukee, WI, USA), respectively. Probenecid, benzbromarone, pyrazinamide, xanthine oxidase (from buttermilk), xanthine, bovine serum albumin and Bradford reagent were purchased from Sigma Chemicals (St. Louis, MO, USA). Allopurinol was kindly provided by Hovid Berhad (Ipoh, Malaysia). All the other reagents used were of analytical grade.

2.2. Extraction, fractionation and isolation of lignans

The raw materials of *Phyllanthus niruri* Linn. (Euphorbiaceae) were purchased from Nova Laboratories (Malaysia) Sdn. Bhd. A voucher specimen (No. 10843) has been deposited at the herbarium of School of Biological Sciences, Universiti Sains Malaysia. The dried powdered leaves of *Phyllanthus niruri* were soxhlet-extracted repeatedly with fresh methanol for 5 days. The pooled extracts upon solvent evaporation under partial vacuum yielded a greenish residue that was subsequently loaded onto a resin column (Diaion HP 20, Mitshubishi, Japan), and was next eluted by a stepwise percentage increase of methanol in water. Eluates from water, 30%, 50% and 80–100% of methanol in water were collected separately as fractions 1, 2, 3 and 4, respectively. Fraction 4 being bioactive was further partitioned with *n*-hexane, chloroform and *n*-butanol to yield their respective sub-fractions. Four lignans as shown in Fig. 1; phyllanthin, hypophyllanthin, phyltetralin and niranthin were isolated from the *n*-hexane sub-fraction of fraction 4 following the protocol described previously and their structure was confirmed by the nuclear magnetic resonance, mass spectrometer, ultraviolet and infra-red spectra (Murugaiyah and Chan, 2006). Due to low yield, niranthin was only used for evaluation of *in vitro* xanthine oxidase inhibition study.

2.3. Animals

Male *Sprague–Dawley* rats of 12–16 weeks old, weighing initially about 180–280 g were kept in the animal house of the School of Pharmaceutical Sciences, Universiti Sains Malaysia. The animals were maintained on a 12-h light/dark cycle, at room temperature of 25 °C and were allowed free access to standard food pellets (Gold Coin, Malaysia) and tap water. The animals were acclimatised for at least 1 week before beginning of experiments. The animals were divided into groups of six each and housed individually during the experimental period. The handling and use of animals was in accordance with the institutional guidelines. An approval was obtained from the Animal Ethics Committee, Universiti Sains Malaysia, Penang, Malaysia (Reference number: USM/PPSF/50(93) Jld.1).

2.4. Uricosuric activity

The animal model used in our previous antihyperuricemic study (Murugaiyah and Chan, 2006) was adopted. Briefly, the animals were chemically induced hyperuricemia by single intraperitoneal administration of potassium oxonate (200 mg/kg) and oral uric acid

(1 g/kg). Food and water were withheld overnight prior to study. Methanol extract and fractions/sub-fractions of *Phyllanthus niruri* (each at 50 mg/kg) prepared in 20% Tween 20 aqueous solution or the isolated chemical constituents phyllanthin, hypophyllanthin and phyltetralin (each at 10 mg/kg) and clinically used drugs (10 mg/kg for benzbromarone, 50 mg/kg for probenecid) prepared in a mixture of 10% ethanol in 20% Tween 20 aqueous solution were intraperitoneally administered to the rats 30 min after hyperuricemia induction. For normal and hyperuricemic controls, the animals were given the vehicle only. The animals were then placed in metabolism cages and were given 100 mL of tap water. Urine, collected in graduated tubes and water intake were measured for 5 h after treatments were given. Blood samples were taken at the end of study by cardiac puncture. The animals were anaesthetized with diethyl ether inside a chamber and placed onto its back during this procedure. Plasma was obtained after centrifugation at $3000 \times g$ for 15 min (Gallenkamp, United Kingdom). The plasma and urine samples were stored at −20 °C prior to high-performance liquid chromatography (HPLC) analysis. Plasma uric acid concentrations determinations were described previously (Murugaiyah and Chan, 2006). Urine uric acid concentrations were determined by a HPLC system comprising a Waters 510 HPLC pump (Milford, MA, USA), a Waters Spherisorb S5 C8 column (4.6 i.d. × 250 mm, Waters, USA), a Gilson 115 UV detector (Middleton, WI, USA) and a Hitachi D-2500 Chromato-integrator (Tokyo, Japan). The analytical conditions were as follows: a mobile phase of 5 mM acetate buffer with pH adjusted to 4.0 by acetic acid, a flow-rate of 1.0 mL/min and detection wavelength of 292 nm. Urinary uric acid excretion was expressed as mg of uric acid excreted per kg of animal per 5 h (mg/(kg 5 h)) whilst uric acid clearance was expressed as the volume of uric acid (L) cleared per kg animal per h (L/(kg h)). One of the lignans tested, phyllanthin was further assessed for its uricosuric activity at doses of 5 and 20 mg/kg following the same method as described above.

2.5. Pyrazinamide suppression test

The experiment was performed in four groups of animals induced hyperuricemia following the protocol described in Section 2.4. Food and water were withheld overnight prior to study. An oral dose of 300 mg/kg of pyrazinamide in 0.5% carboxymethylcellulose aqueous solution was given to all the animals 30 min after the induction of hyperuricemia. The treated groups received either phyllanthin (10 mg/kg), probenecid (50 mg/kg) or benzbromarone (10 mg/kg) intraperitoneally 30 min after pyrazinamide administration. The hyperuricemic–pyrazinamide control group received an equal volume of vehicle. All treatments were prepared in a mixture of 10% ethanol in 20% Tween 20 aqueous solution. Urinary output, collected in graduated tubes and water intake were measured for 5 h after treatments were given. Urine uric acid concentrations were determined by HPLC method as described in Section 2.4.

2.6. In vitro xanthine oxidase assay

The inhibitory effect of *Phyllanthus niruri* extract and its lignans on the xanthine oxidase activity was determined following a modified spectrophotometric method of Noro et al. (1983). The test sample was dissolved in dimethyl sulphoxide (DMSO) and subsequently diluted with phosphate buffer (pH 7.8) to a final concentration containing 1–5% of DMSO. At these concentrations, DMSO was found to possess no xanthine oxidase inhibition. The assay involved the addition of 0.1 mL of test solution to 2.9 mL of 50 mM phosphate buffer solution (pH 7.8). A 0.1 mL of freshly prepared enzyme solution containing 0.4 units per mL in 50 mM phosphate buffer at pH 7.8 was next added and the assay mixture was pre-incubated at 37 °C for 10 min. The enzymatic reaction was initiated by the addition of 2.0 mL solution of freshly prepared 0.15 mM xanthine substrate in water and the final assay mixture was incubated for 30 min. The reaction was terminated by an addition of 1.0 mL of 1 M hydrochloric acid. The absorbance of the assay mixture was measured at 292 nm. The methanol extract of *Phyllanthus niruri* was initially tested for xanthine oxidase inhibitory activity at 100 μg/mL, while its fractions/sub-fractions and lignan constituents were tested at 40 μg/mL.

For the 50% inhibitory concentration (IC_{50}) determination, the methanol extract of *Phyllanthus niruri*, its fractions and sub-fractions were examined at six concentrations in the range of 3.13–500 μg/mL. No IC_{50} determination was performed for the isolated lignans.

2.7. In vivo xanthine oxidase assay

After 5 h of administration of the methanol extract, fractions, sub-fractions and lignans of *Phyllanthus niruri*, the rats were anesthetized with diethyl ether and their liver excised and processed to obtain the cytosolic fraction following the method described by Dan et al. (1994). Briefly, the liver was perfused with 50 mL of ice-cold 0.25 mol/L sucrose in 100 mmol/L phosphate buffer of pH 7.8. Approximately 3 g of the liver was excised and homogenized in a MSE homogenizer (England) with four volumes of ice-cold 0.25 mol/L sucrose in 100 mmol/L phosphate buffer of pH 7.8. The homogenate was centrifuged in an Eppendorf centrifuge 5403 (Engelsdorf, Germany) at $1500 \times g$ for 10 min at 4 °C. The resulting supernatant was filtered and further centrifuged at $14{,}100 \times g$ for 20 min. The resulting supernatant was again centrifuged at $14{,}100 \times g$ for another 20 min at 4 °C. The supernatant after third centrifugation (cytosolic fraction) was used for *in vivo* assay of xanthine oxidase activity. The liver cytosolic fraction of 0.4 mL was pre-incubated in 1.4 mL of 50 mmol/L phosphate buffer and 0.2 mL of xanthine solution was then added. The mixtures were incubated for 30 min at 37 °C. The reaction was stopped by the addition of 2.0 mL of 0.4 M perchloric acid. The samples were centrifuged at $1000 \times g$ for 10 min. The concentration of uric acid in the supernatant was determined at 292 nm using a Perkin Elmer Lambda 45 spectrophotometer (Perkin Elmer Instruments, Norwalk, CT, USA). The enzyme activity was expressed as nmoles of uric acid produced per min by 1 mg of protein (nmol/(min mg protein)). Protein concentration was determined following the Bradford method using bovine serum albumin as the standard (Bradford, 1976).

2.8. Statistical analysis

The results were presented as the mean ± standard error of the mean (S.E.M.) of six animals. The statistical significance of difference was evaluated by the analysis of variance (ANOVA) followed by the Tukey *post hoc* test.

3. Results

3.1. Uricosuric effect of the methanol extract, fractions, sub-fractions and isolated lignans of Phyllanthus niruri

The effect of *Phyllanthus niruri* methanol extract, its fractions and sub-fractions as well as the isolated lignans on water intake, urine output, urinary excretion and clearance of uric acid of the hyperuricemic rats are shown in Table 1. In general, treatment with *Phyllanthus niruri* methanol extract, its fractions, sub-fractions of fraction 4 (*n*-hexane, chloroform and *n*-butanol) or lignans caused insignificant changes in water intake and urine output of the hyperuricemic rats. Only hyperuricemic rats treated with phyllanthin at 10 mg/kg showed a significant increase in urine output when compared to those of the hyperuricemic control rats even though their water intake was not affected. However, the changes in urine

Table 1
Effect of methanol extract, fractions, sub-fractions and lignans of *Phyllanthus niruri* on water intake, urine output, uric acid excretion and clearance of hyperuricemic rats.

Groups	Dose (mg/kg)	n	Water intake (mL)	Urine output (mL)	Uric acid excretion (mg/(kg 5 h))	Plasma uric acid (mg/dL)	Uric acid clearance (mL/(kg h))
			Mean ± S.E.M.	Mean ± S.E.M.	Mean ± S.E.M.	Mean ± S.E.M.	Mean ± S.E.M.
Methanol extract, fractions and sub-fractions of *Phyllanthus niruri*							
Normouricemic control	–	6	12.7 ± 1.9	1.88 ± 0.69	1.26 ± 0.12	0.47 ± 0.06	0.06 ± 0.01
Hyperuricemic control	–	6	11.7 ± 0.8	1.86 ± 0.25	3.83 ± 0.52	3.32 ± 0.72[d]	0.03 ± 0.01
Methanol extracts	50	6	12.5 ± 0.7	1.41 ± 0.43	6.46 ± 0.69[d]	1.65 ± 0.28	0.08 ± 0.01
Fraction 1	50	6	12.3 ± 1.3	2.32 ± 0.56	4.84 ± 0.74	1.98 ± 0.57	0.07 ± 0.02
Fraction 2	50	6	13.0 ± 1.3	2.18 ± 0.91	4.46 ± 0.65	3.74 ± 0.86[e]	0.05 ± 0.02
Fraction 3	50	6	13.5 ± 1.4	1.70 ± 0.47	3.79 ± 0.20	2.76 ± 0.78	0.06 ± 0.03
Fraction 4	50	6	12.0 ± 0.4	2.50 ± 0.69	7.54 ± 0.76[e,g]	0.64 ± 0.16[g]	0.30 ± 0.06[g]
n-Hexane sub-fraction of fraction 4	50	6	17.0 ± 2.4	2.45 ± 0.55	10.16 ± 1.74[f,h]	0.66 ± 0.20[g]	0.43 ± 0.11[e,h]
Chloroform sub-fraction of fraction 4	50	6	13.2 ± 3.5	1.87 ± 0.54	6.56 ± 1.91	2.25 ± 0.77	0.09 ± 0.04
n-Butanol sub-fraction of fraction 4	50	6	16.5 ± 2.5	1.97 ± 0.78	5.60 ± 1.62	2.49 ± 0.78	0.20 ± 0.15
Lignans of *Phyllanthus niruri* and standard drugs							
Normouricemic control	–	6	12.3 ± 0.8	1.26 ± 0.51	1.85 ± 0.35	0.38 ± 0.07	0.13 ± 0.04
Hyperuricemic control	–	6	13.7 ± 1.3	1.97 ± 0.33	4.11 ± 0.65	3.04 ± 0.69[f]	0.03 ± 0.01
Benzbromarone	10	6	14.5 ± 2.2	2.70 ± 0.48	11.51 ± 1.18[f,h]	0.65 ± 0.16[i]	0.47 ± 0.11[d,h]
Probenecid	50	6	16.2 ± 1.4	3.13 ± 0.43	11.00 ± 0.99[f,h]	1.38 ± 0.39[h]	0.24 ± 0.08
Phyllanthin	5	6	12.8 ± 3.3	3.32 ± 0.80	9.53 ± 0.86[e,g]	1.02 ± 0.28[i]	0.27 ± 0.07
Phyllanthin	10	6	13.5 ± 1.8	5.27 ± 0.79[e,h]	10.31 ± 1.67[f,h]	0.71 ± 0.07[i]	0.33 ± 0.09[g]
Phyllanthin	20	6	13.2 ± 2.6	3.35 ± 0.51	15.48 ± 1.77[f,i]	0.53 ± 0.07[i]	0.62 ± 0.09[f,i]
Hypophyllanthin	10	6	12.7 ± 1.9	3.70 ± 0.55	9.34 ± 1.79[e,g]	1.08 ± 0.15[h]	0.19 ± 0.05
Phyltetralin	10	6	15.7 ± 1.5	2.75 ± 0.70	8.38 ± 0.93[e]	1.98 ± 0.48[d]	0.12 ± 0.04

Values are expressed as mean ± S.E.M. for six animals.
[d] $P < 0.05$, [e] $P < 0.01$, [f] $P < 0.001$ significantly different compared to normal control.
[g] $P < 0.05$, [h] $P < 0.01$, [i] $P < 0.001$ significantly different compared to hyperuricemic control.

output observed with phyllanthin treated animals were not dose-dependent.

Hyperuricemic induction by a single administration of potassium oxonate intraperitoneally (200 mg/kg) and uric acid orally (1 gm/kg) caused an insignificant increase in uric acid excretion than those of the normal control rats. In contrast, hyperuricemic rats treated with the *Phyllanthus niruri* methanol extract had a 5.13 and 1.69 times, respectively higher in urinary uric acid excretion than those of normal and hyperuricemic control animals. Among its fractions, fraction 4 exhibited a 5.98 and 1.97 times higher excretion of uric acid than those of normal and hyperuricemic controls, respectively. Subsequent partitioning of fraction 4 yielded three sub-fractions with the *n*-hexane component being the most potent and exhibited a significant 8.06 and 2.65 times higher urinary excretion of uric acid than those of normal and hyperuricemic controls, respectively. Treatment with benzbromarone (10 mg/kg), probenecid (50 mg/kg) and the lignans, phyllanthin, hypophyllanthin and phyltetralin at 10 mg/kg each also produced 6.22, 5.95, 5.57, 5.05 and 4.53 times, respectively higher urinary excretion of uric acid than those of normal rats and 2.80, 2.68, 2.51, 2.27 and 2.04 times, respectively higher when compared to that of hyperuricemic control animals (Table 1). Further studies on phyllanthin showed a significant dose-dependent increase of 2.32 and 3.77 times in urinary excretion of uric acid at 5 mg/kg and 20 mg/kg, respectively when compared to that of hyperuricemic controls.

Treatment with *Phyllanthus niruri* methanol extract produced an insignificant increase in uric acid clearance when compared to that of normal and hyperuricemic controls, respectively. However, treatment with its fraction 4 produced a significant increase in uric acid clearance of 5.0 and 10.0 times that of normal and hyperuricemic controls, respectively whilst the *n*-hexane sub-fraction derived from fraction 4 treated rats had a 7.17 and 14.33 times higher uric acid clearance than those of control animals, respectively. Similar to the results obtained for urinary excretion of uric acid, administration of benzbromarone, probenecid and lignans caused a higher uric acid clearance, but a significant increase of 15.67 and 11.0 times compared to that of hyperuricemic controls was only observed in animals treated with benzbromarone (10 mg/kg) and phyllanthin (10 mg/kg), respectively. Further phyllanthin administration also produced a dose-dependent increase in uric acid clearance of 9.0 and 20.67 times at 5 mg/kg and 20 mg/kg, respectively when compared to that of hyperuricemic controls.

3.2. Pyrazinamide suppression test

Table 2 shows the effect of benzbromarone, probenecid and phyllanthin on the urinary uric acid excretion of hyperuricemic rats with co-administration of pyrazinamide. Hyperuricemic rats given an oral dose of 300 mg/kg of pyrazinamide showed a significant decrease in urinary excretion of uric acid compared to the hyperuricemic animals without pyrazinamide (Table 1). Pyrazinamide administered hyperuricemic rats treated with probenecid showed a significant increase of 6.96 times in urinary uric acid excretion whereas those treated with benzbromarone and phyllanthin

Table 2
Effect of clinically used uricosuric drugs and phyllanthin on the urinary excretion of uric acid of hyperuricemic rats with co-administration of pyrazinamide.

Group	Dose		n	Uric acid excretion (mg/(kg 5 h))
	(mg/kg)	(μmol/kg)		Mean ± S.E.M.
Hyperuricemic + pyrazinamide control	–	–	6	1.37 ± 0.35
Benzbromarone	10	23.58	6	4.81 ± 1.74
Probenecid	50	175.19	6	9.53 ± 2.47[a]
Phyllanthin	10	23.92	6	5.83 ± 0.92

Values are expressed as mean ± S.E.M. for six animals.
[a] $P < 0.01$ significantly different compared to hyperuricemic + pyrazinamide control.

Table 3
In vitro xanthine oxidase inhibitory activity and IC_{50} values of methanol extract, fractions, sub-fractions and lignans of *Phyllanthus niruri*.

Sample (n = 3)	Initial XO inhibition		IC_{50} (μg/mL)
	Concentration (μg/mL)	Percentage inhibition (Mean ± S.E.M.)	
Allopurinol	0.05	84.20 ± 5.67	0.022 ± 0.001
Methanol extract of *Phyllanthus niruri*	100	67.66 ± 1.81	39.39 ± 6.56
Fractions of methanol extract of *Phyllanthus niruri*			
Fraction 1	40	4.02 ± 1.80	427.73 ± 56.54
Fraction 2	40	36.47 ± 1.38	86.94 ± 8.18
Fraction 3	40	59.14 ± 7.37	28.60 ± 0.49
Fraction 4	40	65.01 ± 6.01	22.72 ± 1.62
Sub-fractions of fraction 4 and the isolated lignans			
n-Hexane	40	53.87 ± 4.37	42.87 ± 3.51
Chloroform	40	56.42 ± 9.29	26.70 ± 2.03
n-Butanol	40	69.93 ± 7.44	12.30 ± 0.99
Phyllanthin	40	17.78 ± 1.36	–
Hypophyllanthin	40	20.83 ± 7.74	–
Phyltetralin	40	15.42 ± 1.36	–
Niranthin	40	6.87 ± 0.71	–

showed an insignificant increase of 3.51 and 4.26 times, respectively in urinary uric acid excretion. Thus, in pyrazinamide administered hyperuricemic animals, the uricosuric effect of benzbromarone or phyllanthin was suppressed while the uricosuric effect of probenecid was unaffected.

3.3. *In vitro xanthine oxidase inhibitory activity*

Allopurinol, a known inhibitor of xanthine oxidase, was adopted as positive control in the studies and has an IC_{50} of 0.022 μg/mL (0.162 μM). The inhibitory activity of the methanol extract, fractions, sub-fractions and lignans of *Phyllanthus niruri* against xanthine oxidase and their respective IC_{50} values are shown in Table 3. The inhibitory activity of the methanol extract was initially assessed at 100 μg/mL and its IC_{50} was 39.39 μg/mL. Therefore, the xanthine oxidase inhibitory activity of *Phyllanthus niruri* fractions, sub-fractions and lignans was initially determined at 40 μg/mL. The results demonstrated that upon fractionation the xanthine oxidase inhibitory activity of methanol extract of *Phyllanthus niruri* was concentrated in its fractions 3 and 4 with 59.14% and 65.01% inhibition, respectively. Further partitions of fraction 4 resulted in concentration of xanthine oxidase inhibitory activity in the *n*-butanol sub-fraction with 69.93% inhibition. However, they were less potent when compared to allopurinol. In contrast, the isolated lignans showed no appreciable xanthine oxidase inhibitory activity *in vitro*. The lignans could not be tested at a higher concentration of more than 40 μg/mL due to their poor solubility and tendency to precipitate in the buffered assay mixture. Moreover, the IC_{50} value of the methanol extract was 39.39 μg/mL, thus indicating that further studies of the lignans beyond 40 μg/mL was not beneficial.

3.4. *In vivo xanthine oxidase inhibitory activity*

The effect of the methanol extract of *Phyllanthus niruri* and its fractions, sub-fractions and lignans on rat liver xanthine oxidase is shown in Table 4. Hyperuricemia induction did not cause any appreciable changes in liver xanthine oxidase activity. The methanol

Table 4
Effect of methanol extract, fractions, sub-fractions and isolated lignans and allopurinol on rat liver xanthine oxidase of hyperuricemic rats.

Groups	Dose (mg/kg)	n	Xanthine oxidase activity (nmol/(min mg protein))	Percentage inhibition
Normouricemic control	–	6	1.34 ± 0.11	–
Hyperuricemic control	–	6	1.36 ± 0.14	–
Methanol extracts	50	6	$0.45 \pm 0.04^{d,g}$	76.84 ± 2.01
Fraction 1	50	6	1.18 ± 0.31	39.09 ± 15.92
Fraction 2	50	6	0.64 ± 0.29	66.96 ± 14.77
Fraction 3	50	6	$0.31 \pm 0.09^{e,h}$	84.10 ± 4.55
Fraction 4	50	6	$0.24 \pm 0.04^{f,i}$	87.49 ± 2.15
n-Hexane sub-fraction of fraction 4	50	6	0.81 ± 0.20	58.30 ± 10.53
Chloroform sub-fraction of fraction 4	50	6	0.98 ± 0.14	49.23 ± 7.40
n-Butanol sub-fraction of fraction 4	50	6	$0.35 \pm 0.12^{e,h}$	81.98 ± 5.97

Groups	Dose (mg/kg)		n	Xanthine oxidase activity (nmol/(min mg protein))	Percentage inhibition
	(mg/kg)	(μmol/kg)			
Normouricemic control	–	–	6	1.22 ± 0.25	–
Hyperuricemic control	–	–	6	1.31 ± 0.41	–
Allopurinol	10	73.47	6	$0.24 \pm 0.07^{d,g}$	82.06 ± 5.37
Phyllanthin	10	23.92	6	0.43 ± 0.07	66.97 ± 5.45
Hypophyllanthin	10	23.26	6	0.81 ± 0.13	37.83 ± 9.88
Phyltetralin	10	24.04	6	0.65 ± 0.17	50.72 ± 13.15

Values are expressed as mean ± S.E.M. for six animals.
$^{d}P < 0.05$, $^{e}P < 0.01$, $^{f}P < 0.001$ significantly different compared to normal control.
$^{g}P < 0.05$, $^{h}P < 0.01$, $^{i}P < 0.001$ significantly different compared to hyperuricemic control.

extract at 50 mg/kg dose exhibited a significant 76.84% inhibition of xanthine oxidase activity whereas, among its fractions at similar dose, the xanthine oxidase inhibitory activity was found to be concentrated in fractions 3 and 4 with a significant inhibition of 84.10% and 87.49%, respectively. Further partition of fraction 4 yielded the *n*-hexane, chloroform and *n*-butanol sub-fractions which caused an inhibition of 58.30%, 49.23% and 81.98% in liver xanthine oxidase activity, respectively at 50 mg/kg. None of the lignans isolated from the *n*-hexane sub-fraction demonstrated significant changes in liver xanthine oxidase activity at 10 mg/kg dose. In contrast, a single dose of allopurinol at 10 mg/kg caused a significant 82.06% reduction of liver xanthine oxidase activity.

4. Discussion

Hyperuricemia is commonly encountered in clinical practice but currently the therapeutic agents for lowering blood uric acid are very few in numbers (Dincer et al., 2002). A potential source of novel antihyperuricemic agents may be derived from the natural products. In our previous study, the methanol extract from the leaves of *Phyllanthus niruri* (Euphorbiaceae) showed uric acid lowering activity in hyperuricemic rats. Further antihyperuricemic-guided fractionation and purification of the methanol extract afforded lignans; phyllanthin, hypophyllanthin, phyltetralin and niranthin as the bioactive constituents that significantly reversed the plasma uric acid level of hyperuricemic animals to its normal level comparable to that of clinically used drugs (Murugaiyah and Chan, 2006). The present studies investigated their mechanism of uric acid lowering effect.

Urinary excretion of uric acid has been well studied in the rats because rats shows a net reabsorption of uric acid in the renal and respond well to agents known to have effect on uric acid excretion in humans (Sugino and Shimada, 1995; Yamada et al., 1999a). In the present study, hyperuricemia induction caused an increase in the urinary excretion of uric acid and the result is in agreement with the previous reports (Johnson et al., 1969; Yonetani and Iwaki, 1983). Administration of fraction 4 of methanol extract and the *n*-hexane sub-fraction of fraction 4 increased the excretion and clearance of uric acid of hyperuricemic rats. Likewise, the bioactive lignans, phyllanthin, hypophyllanthin and phyltetralin increased the urinary uric acid excretion. Phyllanthin increased the urinary uric acid excretion and clearance in a dose-dependent manner and at 10 mg/kg dose exhibited similar potency with that of benzbromarone (10 mg/kg) and probenecid (50 mg/kg). In contrast, a much higher dose of more than 100 mg/kg of scopoletin, an uricosuric agent from *Erycibe obtusifolia* increase the urinary uric acid level of the hyperuricemic rats to the same extent as probenecid (100 mg/kg) (Ding et al., 2005).

Pyrazinamide suppression test is used to elucidate the mechanism of action of uricosuric agents. The active metabolite of pyrazinamide, pyrazinoate has been reported to inhibit the secretion of uric acid at second segment of the proximal convulated tubulues (Sugino and Shimada, 1995; Yamada et al., 1999b). Meanwhile, the uricosuric drugs such as probenecid and benzbromarone inhibit uric acid uptake in the lumen. Following the four compartment model, benzbromarone inhibits post-secretory reabsorption, while probenecid mainly inhibits post-secretory and partly inhibits pre-secretory reabsorption (Dan et al., 1990; Yamada et al., 1999b).

In the present study the administration of pyrazinamide to the hyperuricemic rats caused a marked decrease in the urinary uric acid excretion similar to the earlier report (Dan et al., 1990). The co-administration of pyrazinamide with benzbromarone or phyllanthin significantly suppressed the uricosuric activity of the latter agents whereas when administered with probenecid the uricosuric activity of probenecid was reduced to a lesser extent. When probenecid was co-administered with pyrazinamide, its uricosuric effect was not much affected, as it is partly a pre-secretory inhibitor and will inhibit the uric acid reabsorption at first segment of the proximal tubules. In contrast, benzbromarone mainly inhibit post-reabsorption of uric acid and its effect is related to the tubular uric acid concentration that remains after secretion at second segment. Since pyrazinamide inhibit the secretion, the uricoscuric effect of benzbromarone is suppressed in the presence of pyrazinamide. Phyllanthin exhibited similar uricosuric activity to that of benzbromarone, probably by inhibition of reabsorption at the post-secretory reabsorption site of the proximal convulated tubule.

The uricosuric nature of *Phyllanthus niruri* lignans may be of great interest in management of hyperuricemia and gout, considering that in clinical practice about 90% of the gout patients are underexcretors of uric acid (Wright and Pinto, 2003). Unlike benzbromarone, *Phyllanthus niruri* and its lignans, phyllanthin and hypophyllanthin were also reported to have hepatoprotective properties (Syamasundar et al., 1985; Harish and Shivanandappa, 2006). An additional advantage with the use of *Phyllanthus niruri* over the other uricosuric agents is its antiurolithic properties (Freitas et al., 2002; Barros et al., 2006) which may minimize the risk of uric acid deposition in the collecting tubule commonly observed with the use of other uricosuric drugs.

Another possible mechanism by which an agent can reduce blood uric acid is via xanthine oxidase enzyme inhibition that will eventually reduce the production of uric acid. The methanol extracts of *Phyllanthus niruri* showed moderate *in vitro* and *in vivo* xanthine oxidase inhibitory activity but the isolated lignans showed a relatively poor inhibition. *Phyllanthus niruri* was also reported to contain a series of flavanoids, polyphenols and tannins (Calixto et al., 1998). Studies have indicated that these compounds showed good inhibitory activity towards xanthine oxidase (Ying et al., 1998; Hatano et al., 1990). Therefore, it seems very likely that these compounds, probably found at a lower concentration could be responsible for the observed xanthine oxidase inhibitory activity of the *Phyllanthus niruri* methanol extracts.

5. Conclusions

Based on the findings of the present study, it was evident that the progressive increase in urinary uric acid excretion shown by *Phyllanthus niruri* fractions, sub-fractions and lignans paralleled their antihyperuricemic effect, in contrast to those findings from the xanthine oxidase inhibitory studies. Therefore, it seems very likely that the antihyperuricemic effect of *Phyllanthus niruri* methanol extract may be attributable mainly to its uricosuric action and partly through xanthine oxidase inhibition, while the antihyperuricemic effect of the lignans was attributable to their uricosuric action.

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