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In vitro xanthine oxidase inhibitory activity of *Piper betle* and *Phyllanthus niruri*

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Abstract
Xanthine oxidase inhibitors are commonly employed for the treatment of gout in birds but their side effects limit their use, making the search for an alternative very essential. Based on the literature reports, *Piper betle* and *Phyllanthus niruri* herbs were selected for the present study to find out their xanthine oxidase inhibitory potential. Alcoholic extracts of both herbs were analysed for qualitative phytochemical contents. Alkaloids, flavonoids, phenol, saponin and terpenoids were detected in both herbs. The xanthine oxidase inhibitory activity was assessed in different concentrations of both herbs *viz*., 10, 25, 50 and 100 µg/ml in double beam UV/Visible Spectrophotometer in comparison with Allopurinol. The results revealed that both herbs possess xanthine oxidase inhibitory activity at 100 µg/ml concentration and among herbs *P. betle* showed superior xanthine oxidase inhibitory activity (81.50±0.57 per cent). Hence it was concluded that the presence of phytochemicals in both herbs might contribute to their xanthine oxidase inhibitory action and they may be studied alone or in combination through *in vivo* experiments to authenticate the present findings.

Keywords: Xanthine oxidase; allopurinol; *Piper betle; Phyllanthus niruri*

Introduction
Gout is commonly observed in poultry as they are uricotelic and lack uricase enzyme. Gout condition leads to severe renal disorder associated with hyperuricemia, resulting in the precipitation of mono sodium urate crystals on the visceral surface(s) and rarely in articular surfaces of the joints of birds. Xanthine oxidase is an enzyme that catalyzes the final steps in purine catabolism, ultimately generating uric acid. Therefore, xanthine oxidase inhibitors are employed as an effective mediator for the suppression of uric acid generation in the treatment of gout (Borges *et al.*, 2002) [4]. Allopurinol is one of the synthetic xanthine oxidase inhibitor which has been widely used in the therapeutic and clinical management of gout. However the drawback of using allopurinol is that it generates superoxide radicals (Berry and Hare, 2004) [3] and also causes allergic reactions (Kong *et al.*, 2000). Since nature contains rich source of medicinal plants and some of them have been reported to inhibit xanthine oxidase enzyme (Azmi *et al.*, 2012) [12], these medicinal properties of plants can be used as alternate sources of gout medication and a substitute for synthetic xanthine oxidase inhibitors. Based on the literatures *P. betle* (Dwidedi and Tripathi, 2014) [5] and *P. niruri* (Lee *et al*., 2016) [8] were chosen for the present study. These plants are commonly available in Indian conditions and can be easily recognized by the farmers. Hence a preliminary study was undertaken to explore the *in vitro* xanthine oxidase inhibitory potentials of *P. betle* and *P. niruri* leaves using spectrophotometer.

Materials and methods
P. betle and *P. niruri* herbs were collected locally and authenticated by the Botanical Survey of India (No. BSI/SRI/5/23/2017/Tech/1921) Coimbatore, Tamil Nadu. The *P. betle* and *P. niruri* leaves were dried and processed for alcoholic extract preparation.

Qualitative phytochemical screening
The qualitative phytochemical analysis of alcoholic extracts of *Piper betle* L. and *Phyllanthus niruri L.* was done by using the method of Trease and Evans (1983) [11] and Kokate *et al.* (1990) [6] at the laboratory of Ethno Veterinary Herbal Research Centre for Poultry, Veterinary Clinical Complex Campus, VCRI, Namakkal, Tamil Nadu. The tests for the presence of alkaloids, amino acids and proteins, carbohydrates, flavonoids, glycosides, phenol, phylobatannin, saponin, tannin, terpenoids, vitamin C and volatile oils were conducted for both herbs.

Xanthine oxidase inhibitory assay

In vitro Xanthine oxidase enzyme inhibitory activity assessment was carried out in double beam UV/Visible Spectrophotometer (Model-2210, Systronics) as per the method described by Apaya and Hernandez (2011) [1] for the alcoholic extracts of *P. betle* and *P. niruri* leaves in comparison with Allopurinol. Allopurinol, xanthine, xanthine oxidase from bovine milk source were purchased from Sigma Aldrich company. The allopurinol solution was prepared by dissolving 5.0 mg of allopurinol in 5.0 ml of 0.15 M phosphate buffer (pH 7.5). Xanthine oxidase enzyme solution was prepared by diluting 30 µl of a 5.0 unit's enzyme solution to a final volume of 3.0 ml. The substrate solution was prepared by addition of 5 drops of 1.0 M NaOH to 22.7 mg of xanthine to aid its dissolution and deionized water to a final volume of 250 ml. The herbal extracts were dissolved in 1% dimethyl sulfoxide (DMSO) to a concentration of 1 mg/ml.

The assay mixture consists of 1 ml extract of each plant (10/25/50/100 µg/ml), 2.9 ml of phosphate buffer (pH 7.5) and 0.1 ml of xanthine oxidase enzyme solution (pH 7.5). After pre incubation at 25°C for 10 minutes, the reaction was initiated by addition of 1 ml of 0.6 mM substrate solution of xanthine. The assay mixture was again incubated at 25°C for 30 minutes. The reaction was then stopped by the addition of 1ml of 1N hydrochloric acid and the absorbance was measured at 290 nm in double beam visible spectrophotometer. The assay for each concentration was carried out in triplicate. One unit of xanthine oxidase activity is defined as the amount of enzyme required to produce 1 mmol of uric acid per min at 25°C. Xanthine oxidase activity was expressed as percentage inhibition of xanthine oxidase in the above assay system and the percentage of Inhibition was calculated as follows.

$$\text{Percentage of inhibition} = \frac{(A-B) - (C-D)}{(A-B)} \times 100$$

Where, A is the activity of the enzyme without the compound, B is the control of A without the compound and enzyme; C and D are the activities of the compound with and without the enzyme respectively. Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. Results are expressed as mean ± SEM.

Evaluation of IC_{50} concentrations

In the present study different concentrations of allopurinol (10, 25, 50 and 100 µg/ml) as well as *P. betle* and *P. niruri* extracts were evaluated for xanthine oxidase inhibitory activity. The IC_{50} value of Allopurinol and both herbs were obtained through the dose-response logarithmic function curve by plot of different concentration against percentage of inhibition (Werns *et al*., 1991) [13].

Results and discussion

The results of qualitative phytochemical analysis of *P. betle and P. niruri* leaf extracts are given in table 1. The results revealed the presence of alkaloids, carbohydrates, flavonoids, phenol, saponin, terpenoids and volatile oil in *P. betle* and alkaloids, carbohydrates, flavonoids, phenol, saponin, tannins and terpenoids in *P. niruri*.

Table 1: Qualitative Phytochemical analysis results of *P. betle and P. niruri* extracts.

Phytochemical	*P. betle*	*P. niruri*
Alkaloids	Present	Present
Amino acids and Proteins	Absent	Absent
Carbohydrates	Present	Present
Flavonoids	Present	Present
Glycosides	Absent	Absent
Phenol	Present	Present
Phylobatannin	Absent	Absent
Saponin	Present	Present
Tannin	Absent	Present
Terpenoids	Present	Present
Vitamin C	Absent	Absent
Volatile oil	Present	Absent

The results of xanthine oxidase inhibition by the standard drug Allopurinol and *P. betle* and *P. niruri* extracts at different concentrations are given in table 2.

Table 2: *In vitro* Xanthine oxidase Inhibitory activity of Allopurinol, *P. betle* and *P. niruri*

Drug/Herb	Concentration	Percentage of Inhibition	IC_{50} value (µg/mL)
Allopurinol	10 µg/ml	64.73^{e}± 0.90	6.16^{a}±0.10
	25 µg/ml	73.67^{g} ± 0.71	
	50 µg/ml	84.83^{i}± 1.26	
	100 µg/ml	93.10^{j}± 0.33	
Piper betle	10 µg/ml	39.58^{b}±0.61	16.50^{b}±0.34
	25 µg/ml	59.40^{d}±1.16	
	50 µg/ml	71.44^{fg}±0.52	
	100 µg/ml	81.50^{h}±0.57	
Phyllanthus niruri	10 µg/ml	28.70^{a}±0.98	38.30^{c}±0.54
	25 µg/ml	42.11^{c}±0.56	
	50 µg/ml	57.88^{d}±0.84	
	100 µg/ml	70.15^{f}±0.46	

Rows bearing common superscript did not vary significantly at (P<0.05) level.

A dose dependent xanthine oxidase inhibitory activity was observed for the drug as well as herbs. In the present study the standard drug Allopurinol showed greater percentage of inhibition of xanthine oxidase enzyme than the respective concentrations of *P. betle* and *P. niruri* herbs in all the tested concentrations. Similarly *P. betle* produced superior xanthine oxidase inhibition than the *P. niruri* in the all the respective test concentrations. Allopurinol at the concentration of 25 µg/ml could produce an equivalent effect to that of *P. betle* 50 µg/ml and *P. niruri* 100 µg/ml concentrations, which reflects that the standard drug has better inhibition of xanthine oxidase enzyme than both the herbs.

Also, the calculated IC_{50} values for Allopurinol, *P. betle* and *P. niruri* herbs were 6.16 ± 0.10, 16.50 ±0.34 and 38.30 ±0.54 µg/ml respectively. It showed that Allopurinol IC_{50} value was lower than both herbs and among herbs, *P. betle* IC_{50} value was lower than *P. niruri*. These results revealed that the standard drug Allopurinol is the most potential drug of all the three bio active molecules taken for the study, with respect to xanthine oxidase inhibition. Among the herbs *P. betle* is more potential. However due to the side effects of Allopurinol, the *P. betle* which showed the significant xanthine oxidase inhibitory activity (81.50 ± 0.57) at 100 µg/ml concentration could be used as a suitable alternative either alone or in combination with *P. niruri* for future *in vivo* studies. The results are in agreement with previous studies carried out for assessing xanthine oxidase inhibition by herbs.

The presence of alkaloids, flavonoids and phenolic compounds commonly present in both herbs might have resulted in their xanthine oxidase inhibitory activity (Umamaheswari *et al.*, 2009) [12]. Nile and Khobragade (2011) [10] stated that the presence of significant amount of polyphenols and flavonoids in the *Tephrosia purpurea* Linn root extracts might be responsible for its xanthine oxidase inhibitory effect. Further, the enzyme kinetic studies carried out by Umamaheswari *et al.* (2011) indicated that all the flavonoids are competitive inhibitors of xanthine oxidase enzyme like allopurinol whereas polyphenolic compounds are non-competitive inhibitors of xanthine oxidase enzyme. Alkaloids, phenolic compounds and flavonoids are believed to inhibit uric acid generation and exert uricosuric and anti-inflammatory effects, through their xanthine oxidase inhibitory activity (Ling and Bochu, 2014) [9]. The outcome of this study indicated that *P. betle* and *P. niruri* herbs could be used as potential antigout agents instead of Allopurinol either alone or in combination. Further investigations on the above compounds and *in vivo* studies are necessary to develop potential herbal remedies for clinical use in the prevention and treatment of gout in poultry.

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