Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm

Document heading doi:

Whole *Leea macrophylla* ethanolic extract normalizes kidney deposits and recovers renal impairments in an ethylene glycol-induced urolithiasis model of rats

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ARTICLE INFO

Article history:
Received 5 December 2011
Received in revised form 27 January 2012
Accepted 15 March 2012
Available online 20 July 2012

Keywords:
Urolithiasis
Ethylene glycol
Leea macrophylla
Urinary parameter
Rats

ABSTRACT

Objective: To investigate the antilithiatic effect of the whole *Leea macrophylla* (*L. macrophylla*) Roxb (Leeaceae) ethanol extract in ethylene glycol-induced urolithiasis model of rats. **Methods:** Forty two seven weeks old male wistar albino rats were randomly divided into two major groups namely: preventive (n=18) and therapeutic (n=24). Preventive group was further subdivided into 3 groups of 6 rats namely: preventive control (PC), preventive lithiatic control (PLC) and preventive lithiatic *L. macrophylla* (PLLM). Similarly, therapeutic group was also subdivided into 4 groups of 6 rats namely: therapeutic control (TC), therapeutic lithiatic control (TLC), therapeutic lithiatic *L. macrophylla* (TLLM) and therapeutic lithiatic cystone (TLCYS). The lithiasis was induced by 0.75% (v/v) ethylene glycol in the drinking water of all groups except the PC and TC groups. The urinary ionic parameters such as calcium, inorganic phosphate, oxalate, magnesium & creatinine and renal morphology were altered by ethylene glycol, which were partially recovered by 14 d preventive and almost fully recovered by 28 d therapeutic intervention trials with *L. macrophylla* extract (500 mg/kg BW daily). **Results:** Significant difference on recovery was observed between preventive and therapeutic interventional trials. Anti-urolithiatic effect of cystone was significantly (P<0.001) higher than extracts. *L. macrophylla* extract was found nontoxic in the acute toxicity test. **Conclusions:** The results of this study demonstrated very promising anti-urolithiatic effect of *L. macrophylla* extract with preventive and therapeutic treatments in this experimental condition.

1. Introduction

Urolithiasis (kidney stone formation) is a very common kidney disorder in all over the world which is an estimated lifetime risk of 2%-5% in Asia, 8%-15% in Europe and America and around 20% in the Middle East. It is associated with high rate of recurrence, which is around 10%-23% per year, 50% in 5-10 years and 75% in 20 years, due to an imbalance between promoters and inhibitors in the kidneys[1-3]. The majority of stones, up to 80%, found very often both in humans and in rats[4] are composed mainly of calcium oxalate[5-6]. Many remedies have been employed during ages to treat renal stones most of which were taken from plants and proved to be useful[7]. However, the rationale behind their use is not well established except for a few plants and some proprietary composite herbal drugs which were reported to be effective without any side effects[8].

Now a days, the management of urolithiasis with open renal surgery is unusual and rarely used only since the introduction of extracorporeal shock wave lithotripsy which is a standard procedure to remove kidney stones, however it may leave persistent stone fragments and cause acute renal injury, a decrease in renal function and an increase in stone recurrence[8-10]. The procedure is not widely available and very costly to the people in developing countries. In

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addition, the standard drugs used to prevent such lithiasis are not effective consistently in all patients, and many of them have adverse effects that compromise their long term use. Hence, the search for antilithiatic drugs from natural sources has gained more interest compared to earlier as shown in a recent study[11].

Leea macrophylla (L. macrophylla), primitively identified as an Indian habitat, has long been known as Hastikarnapalasa[12] in the ancient textual references[13] as well as in its use as a traditional medicine in the region. Hastikarnapalsa seems to be known in the North Eastern India from the very ancient times as evidenced by the ancient textual references and its traditional name is Hathikana or Hatkana (Elephant's ear). This traditional name of this plant might be come from the morphological structures of leaf which looks like an Elephant's ear. This plant is traditionally used by the local tribes for urinary problems, which has botanically been identified as *L. macrophylla*[14].

It is herb or herbaceous shrub, 90 cm or more in height, with switchy branches and perennial tuberous roots, distributed to the relatively hotter parts of India, from the Eastwards of Ganges, Bihar, Bengal, Assam, the Terai and its contiguous plains and also in the western India from Konkan westwards. In Bangladesh, it is very rarely distributed in some parts of the Chittagong Hill Tracts. The tuberous roots are astringent and alexipharmic; traditionally used to kill guinea worm, and pounded is applied to obstinate sores to promote cicatrisation. It is also applied externally to allay pain and to stop the effusion of blood[15]. The leaves of this plant are usually used by local tribes as first line therapy to get rid of arthritic pain and urinary disturbances but no research work has been conducted for scientific documentation of such uses. The present study investigated the anti-urolithialic effect of the ethanol extract of whole *L. macrophylla* in an ethylene glycol-induced urolithiasis model of rats.

2. Materials and methods

2.1. Plant material

Fresh *L. macrophylla* whole plant (including root) was collected from Hillside of the University of Chittagong, Bangladesh and was taxonomically identified by Dr. Mostafa Kamal Pasha, Professor, Department of Botany, University of Chittagong, Bangladesh. A voucher specimen (Accession no. ACCU-2011/07) that contains the identification characteristics of the plant has been preserved for future reference.

2.2. Preparation of whole plant extract

The fresh whole plants were chopped into small pieces, air dried at room temperature (23±5) ℃ for about 10 d, ground into powder (751 g) by using Willy mill mini (mesh size, 40-80). The powder was extracted with 6 L pure ethanol (99%, Sigma-Aldrich, Germany) for 15 d at room temperature with occasional stirring. Then filtered ethanol was concentrated under reduced pressure by using a rotatory vacuum evaporator (RE200 Sterling, UK) at a temperature below 50 ℃. The concentrated extract (79.92 g, blackish-green semisolid, yield 5.5% w/w) was preserved at 4 ℃ for further use.

2.3. Experimental animals

Six to seven week-old male Wistar albino rats weighing 150-200 g were obtained from the animal house of Bangladesh Council of Scientific and Industrial Research (BCSIR), Chittagong, Bangladesh. The animals were housed individually in stainless steel wire meshed plastic cages in a temperature (23±2) ℃ and humidity (55%-60%) controlled room with a 12 h light-dark cycle. The animals were supplied with standard rat pellet diet and drinking water *ad libitum* during the entire period of the study. Animals were maintained and experiment was carried out according to the rules and regulations of the Institutional Animal Ethics Committee (AEIIUC-Pharm/2011-03).

2.4. Acute toxicity test

Acute toxicity test of *L. macrophylla* was conducted by established method[16]. Briefly, the *L. macrophylla* ethanolic extract was injected intraperitoneally to mice at various dose levels such as 0.75, 1.5, 2.5 and 3.5 g/kg BW. Five mice in each dose group were closely observed for 24 h for any mortality and next ten days for any delayed toxic effect. Hence, the effective therapeutic dose was taken 500 mg/kg BW as one tenth of the approximate median lethal dose (LD_{50} >3.5 g/kg)[17].

2.5. Anti-urolithiatic activity of whole plant extracts

In order to examine the preventive as well as therapeutic effects of the ethanolic extract of whole *L. macrophylla*, animals were divided into two major groups namely: Preventive group and Therapeutic group. Then the preventive group was further randomly divided into 3 groups as preventive control (PC), preventive lithiatic control (PLC) and preventive lithiatic *L. macrophylla* (PLLM) with 6 animals in each group. On the other hand, the therapeutic group was randomly divided into 4 groups as therapeutic control (TC), therapeutic lithiatic control (TLC), Therapeutic *L. macrophylla* (TLM), and therapeutic cystone (TLCYS) with 6 animals in each group. The induction of urolithiasis and intervention trials has been performed according to the following:

2.6. Preventive group (1-14 d)

i. Preventive control (PC) group: Received only drinking water *ad libitum*

ii. Preventive lithiatic control (PLC) group: Received 0.75% ethylene glycol in drinking water *ad libitum* for 14 d.

iii. Preventive lithiatic *L. macrophylla* (PLLM) group: Received 0.75% ethylene glycol in drinking water *ad libitum* for 14 d along with *L. macrophylla* extract at a dose of 500 mg/kg BW per day.

2.7. Therapeutic group (1-28 d)

i. Therapeutic control (TC) group: Received only drinking water *ad libitum*.

ii. Therapeutic lithiatic control (TLC) group: Received 0.75% ethylene glycol in drinking water *ad libitum* for 28 d.

iii. Therapeutic lithiatic *L. macrophylla* (TLLM) group: Received 0.75% ethylene glycol in drinking water *ad libitum* from 1st to 15th day and *L. macrophylla* extract at a dose of 500 mg/kg BW per day from 15th to 28th day.

iv. Therapeutic lithiatic cystone (TLCYS) group; Received standard urolithiatic drug cystone (500 mg/kg BW) from 15th day till 28th day[18,19].

Either plant extract or urolithiatic drug was given once daily by oral route using gastric tube to their respective group of animals.

2.8. Sampling

On day 14 and 28, all animals from preventive and therapeutic groups respectively were kept in metabolic cages and 24 h urine samples were collected and analyzed the concentrations of each of them by using previously published methods for calcium[4], inorganic phosphate[20], oxalate[21], magnesium[4] and creatinine[22]. At the end of the each experimental period, all animals were sacrificed by decapitation and kidneys from each rat were sampled for subsequent histopathological study.

2.9. Analytical methods

Calcium and magnesium were estimated with Atomic Absorption Spectroscopy (Perkin Elmer AAnalyst 200) when inorganic phosphate was determined by spectroscopic analysis (Spectrophotometer, Shimadzu UV Vis 1200). The concentration of urinary creatinine was analyzed by an autoanalyzer (Cobas Integra 800, auto analyzer). The urinary oxalate concentration was measured in unprocessed urine by decarboxylase method as described by Petter Urdal[23].

2.10. Histopathological study

To confirm the incidence of lithiasis and its healing, the kidneys were subjected to histopathological study. The kidneys were washed, weighed and fixed rapidly with 10% neutralized formalin (pH 7.4), and soaked in paraffin; cut at 5 μm intervals and the slices were stained with hematoxylin and eosin. Tissue slices were photographed using optical microscopy and observed the pathological changes as described previously[24].

2.11. Statistical analysis

All data are presented as mean ± error of mean (SEM) of 6 animals and were analyzed by one-way Analysis of Variance (ANOVA) (SPSS for windows, version 18.0). The values were considered significantly different at $P < 0.05$.

3. Results

No acute toxicity was found to the dose up to 3.5 g/kg BW of *L. macrophylla* ethanolic extract. The concentrations of urinary calcium, inorganic phosphate, oxalate, magnesium and creatinine for preventive groups at 14th d are shown in Table 1. The levels of calcium, phosphate and oxalate were significantly ($P<0.001$) increased and the levels of magnesium and creatinine were significantly decreased in 24 h urine of ethylene glycol feeding PLC group compared to the PC group in the urine sample of same duration. In contrast, the levels of calcium, phosphate and oxalate were significantly decreased and magnesium level was significantly increased in 24 h urine in the in parallel ethylene glycol and *L. macrophylla* extract feeding PLLM group compared to the ethylene glycol feeding PLC group when no significant difference was observed for creatinine level between these groups.

The concentrations of urinary calcium, phosphate, oxalate, magnesium and creatinine of therapeutic groups are presented in Table 2. As similar to the preventive groups, the feeding ethylene glycol significantly increased the concentrations of above-mentioned urinary parameters in the TLC group compared to the TC group. On the other hand, feeding of *L. macrophylla* extract significantly decreased the levels of calcium, phosphorous and oxalate and increased the levels of magnesium and creatinine in TLLM group compared to the ethylene glycol treated TLC group. Elevated actions were observed for the anti-lithiatic drug, cystone, feeding TLCYS group compared to TLC and TLLM group.

The data of kidney weights of therapeutic groups at the end of 28 d experimental period is presented in Table 3. The mean weight of kidney of the ethylene glycol fed TLC group was significantly increased compared to the TC group when this induction of TLC group was similarly recovered by the feeding of *L. macrophylla* extract and antilithiatic drug, cystone, in TLLM and TLCYS groups, respectively.

The slides of the histopathological study of kidney tissues

Table 1

Preventive antiurolithiatic effects of *L. macrophylla* extract (500 mg/kg BW/day) on various urinary parameters at the end of 14 d treatment period (mg in 24 h urine sample).

Groups	Calcium	Phosphate	Oxalate	Magnesium	Creatinine
PC	0.63±0.02	7.74±0.09	0.38±0.01	0.25±0.01	7.17±0.11
PLC	1.25±0.02^{a*}	13.01±0.19^{a*}	9.76±0.34^{a*}	0.15±0.01^{a*}	5.39±0.08^{a*}
PLLM	0.92±0.02^{b*}	9.80±0.25^{b*}	2.77±0.16^{b*}	0.21±0.01^{b*}	5.63±0.16^{bNS}

Data are shown as mean ± SEM of six animals in each group. PC: Preventive control; PLC: Preventive lithiatic control; PLLM: Preventive lithiatic *L. macrophylla*; NS: Not significant. Values with superscript letters are significantly different as follows: ^{a}as compared to group PC, ^{b}as compared to group PLC (*P<0.001, SPSS for windows, version 12.0).

Table 2

Therapeutic antiurolithiatic effects of *L. macrophylla* extract (500 mg/kg BW/day) on various urinary parameters at the end of 28 d experimental period (mg in 24 h urine sample).

Groups	Calcium	Phosphate	Oxalate	Magnesium	Creatinine
TC	0.68±0.02	8.14±0.28	0.37±0.02	0.25±0.01	7.24±0.28
TLC	1.78±0.08^{a***}	14.36±0.51^{a***}	12.68±0.58^{a***}	0.18±0.01^{a***}	5.61±0.20a***
TLLM	0.97±0.05^{b***}	9.32±0.26^{b***}	3.88±0.25^{b***}	0.26±0.01^{b**}	6.34±0.18^{b*}
TLCYS	0.53±0.11^{b***}	7.03±0.14^{b***}	2.26±0.02^{b***}	0.23±0.02^{b**}	5.46±0.12^{b***}

Data are shown as mean ± SEM of six animals in each group. TC: Therapeutic control; TLC: Therapeutic Lithiatic control; TLLM: Therapeutic lithiatic *L. macrophylla*; TLCYS: Therapeutic lithiatic cystone. Values with different superscript letters are significantly different as follows: ^{a}as compared to group TC, ^{b}as compared to group TLC (*P<0.05, **P<0.01, ***P<0.001, SPSS for windows, version 12.0).

are shown in Figure 1. The slides showed the healing of ethylene glycol induced urolithiasis in kidney with the treatment of *L. macrophylla* extract.

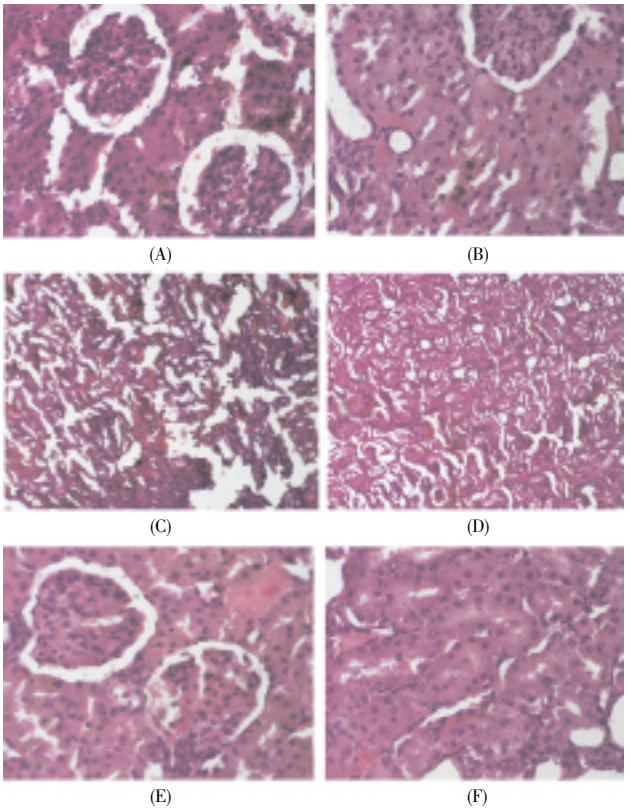

Figure 1. Different phases of kidney Bowman's capsule and tubule before and after treatment under Hematoxylin and Eosin (HE) stains. (A) Normal Bowman's Capsule; (B) Normal tubule; (C) Degeneration of bowman's capsule with ethylene glycol; (D) Degeneration of tubule with ethylene glycol; (E) Repaired Bowman's capsule with *L. macrophylla* extract; (F) Repaired tubule with *L. macrophylla* extract.

4. Discussion

The aim of the present study was to examine the anti-urolithiatic effects of the whole *L. macrophylla* ethanolic extract in an ethylene glycol induced urolithiatic model of rats. The data of this study showed that *L. macrophylla* has a potent anti-urolithiatic effect at least in this experimental condition.

Ethylene glycol is an intermediate in the synthesis of a number of commercial chemical products, including polyethylene terephthalate (PET) resins, unsaturated polyester resins and polyester fibers. It is also a constituent in antifreeze, surface coatings, heat transfer fluids and industrial coolants, surfactants and emulsifiers[25]. General population, or consumer, exposure occurs primarily from the use of ethylene glycol in automotive antifreeze. There have been a number of acute human poisonings from accidental or intentional ingestion of antifreeze, when the kidneys are the most sensitive target organ. Regimens for the treatment of acute ethylene glycol poisoning are designed to prevent kidney damage and to prevent metabolism to the toxic acidic metabolites in several previous studies[26-27]. Ethylene glycol has in itself a low toxicity, but *in vivo* it is broken down to four organic acids: glycoaldehyde, glycolic acid, glyoxylic acid and oxalic acid. The metabolites are toxic to cells that cause depression in central nervous system and cardiopulmonary and renal failure[28]. That is why ethylene glycol has been chosen as an appropriate material for the induction of urolithiasis in rats like many other previously developed models[29-30].

One of the ethylene glycol metabolites, oxalate is usually precipitated as calcium oxalate in the kidneys and other

tissues when glycolic acid causes severe acidosis[28]. Administration of 0.75% (v/v) ethylene glycol to young male albino rats for 14 d period forms renal calculi composed mainly of calcium oxalate. As mentioned above, the biochemical mechanisms for this process are related to an increase in the urinary concentration of oxalate. Stone formation in ethylene glycol fed animals is caused by hyperoxaluria, which is basically of two types: (1) acute, when the rat is challenged by a single, large dose of lithogen, (2) chronic, when the rat is continuously challenged with generally small doses of lithogen for a period of time. It has been reported that hyperoxaluria is usually measured by determining urinary oxalate, and crystal deposition and confirmed by examining paraffin sections of kidney[31]. It has been also reported that hyperoxaluria causes increased renal retention and excretion of oxalate[32].

In the present study, male Wistar albino rats were selected to induce urolithiasis because of the similarities of their urinary system with that of human. It has also been reported that the amount of stone deposition in female rats is significantly lower than that in male rats[33]. In our study, the administration of ethylene glycol to rats significantly increased the excretion of calcium, phosphate and oxalate and decreased the excretion of magnesium and creatinine in 24 h urine sample of the both preventive and therapeutic groups. After oral treatment with the ethanolic extract of *L. macrophylla* (500 mg/kg BW), the urinary excretion of calcium, phosphate and oxalate were significantly decreased and magnesium and creatinine levels were significantly increased in both preventive and therapeutic intervention trials although the therapeutic group was found more potent than the preventive group. Cystone was found more effective than *L. macrophylla* extract. However, comparable anti-urolithiatic effects of the *L. macrophylla* extracts with proven anti-urolithiatic drug authenticated the beneficial effect *L. macrophylla* in preventing calculi formation by supersaturated lithogenic substances in kidneys.

Hence, in the current investigation, the histopathological studies suggested that no microcrystalline deposition and kidney damage in the *L. macrophylla* extract treated groups (Figure 1) were left and the successful prevention of crystal deposition was obtained at the dose of 500 mg/kg, which may be due to the active compounds present in ethanolic extract of this plant. Additionally, the significantly lower kidney weights in the therapeutic *L. macrophylla* fed group suggested the reduction and excretion of kidney stones in this group. All these findings enabled us to confirm the inhibitory and curative potential of *L. macrophylla* on ethylene glycol induced urolithiasis in rats.

The results of the present study showed that the administration of ethylene glycol caused statistically significant decreases in the levels of calcium, phosphate and oxalate whereas increases in the levels of magnesium and urinary creatinine. In contrary, the administration of *L. macrophylla* extract to rats significantly reduced and prevented the growth of kidney stones and significantly improved the renal impairment in the same ethylene glycol induced urolithiatic model of rats. From the results of our study, it can be concluded that the supplementation of *L. macrophylla* extract has a potent beneficial effect on ethylene glycol induced urolithiasis model of rats. Further study is required to understand the detail mechanism of anti-urolithiatic action of *L. macrophylla* extract.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgements

The authors would like to thank the Department of Pathology, Chittagong Medical College, Chittagong, Bangladesh, for the histopathological study of kidney tissues. The authors are grateful to the Bangladesh Council of Scientific and Industrial Research (BCSIR), Chittagong, for providing all the necessary laboratory facilities of the research work.

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