Indian Journal of Natural Products and Resources
Vol. 12(2), September 2021, pp. 425-430

Preliminary phytochemical screening, thin layer chromatography profiling, and *in-vitro* antiurolithiatic activity of the leaves of *Ravenala madagascariensis* Sonn.

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Received 26 June 2020; Revised 12 April 2021

Urolithiasis, the third most common disorder of the urinary tract is bundled with highly complex and unpredictably varied etiological factors. The undesirable adverse effects with current medications and the recurrence rate pose a major challenge in combating the disorder. *Ravenala madagascariensis* Sonn. has been used traditionally in treating kidney stone problems. The present study was aimed at investigating the antiurolithiatic activity of different extracts of the leaves of *R. madagascariensis*. Phytochemical screening carried out on the methanolic, hydromethanolic, and decoction extracts revealed the presence of alkaloids, carbohydrates, glycosides, cardiac glycosides, steroids, proteins, flavonoids, and quinones. Thin layer chromatography profiling of all three extracts was established. The turbidity method was carried out to evaluate *in-vitro* antiurolithiatic activity and the herbal formulation cystone was used as the standard drug. The results of the study showed that the decoction of *R. madagascariensis* exhibited excellent antiurolithiatic potential with an IC_{50} value of 188.65 µg/mL in comparison with the methanolic (305.93 µg/mL) and hydromethanolic (306.83 µg/mL) extracts. Thus the findings of the study validate the claims of antiurolithiatic activity of *R. madagascariensis* that could be attributed to the presence of active phytoconstituents. Further studies are aimed at its formulation and development.

Keywords: Calcium oxalate, *In-vitro*, Kidney stone, *Ravenala madagascariensis*, Turbidity method.
IPC code; Int. cl. (2015.01)-A61K 36/00, A61K 127/00, A61P 13/00

Introduction

Urolithiasis is regarded as a multifactorial disease with complex and unpredictable ethiology[1]. Lithogenic factors include the patient's age, gender, diet, fluid intake, climate, occupation, genetic and metabolic abnormalities[2]. Kidney stones are formed due to the crystallization of ions and macromolecules present in the supersaturated urine[3]. This involves a sequential process of supersaturation followed by nucleation, growth, and aggregation leading to the retention of particles on the urinary tract[4]. The prevalence of kidney stone disease ranges from 7-13% in North America, 5-9% in Europe, and 1-5% in Asia[5]. In India, parts of Gujarat, Maharashtra, Punjab, Haryana and the states of the North East were remarked as the stone belts[6]. The crystalline components of kidney stones are mostly calcium oxalate and calcium phosphate crystals and to a lesser extent due to magnesium ammonium phosphate, uric acid, and cystine[7]. The current conventional treatments include medications and surgery using shock wave lithotripsy, ureteroscopic lithotripsy, digital endoscopy, and nephrolithotomy[8]. The major challenge in the treatment of urolithiasis is the recurrent renal stone formation[9,10]. Hence, an alternative approach from the natural origin in the treatment of kidney stones may pave way for further research in plants with promising antiurolithiatic claims.

Ravenala madagascariensis Sonn., (family Strelitziaceae), an endemic species of Madagascar is also regarded as an iconic symbol of this island. Besides being renewable and inexpensive, the plant has been a source of income in local populations of Madagascar. Also, *R.madagascariensis* is widely cultivated in tropical and subtropical region and often planted in the gardens of South India[11]. The plant is a palm-like tree with rhizomatous roots, hard and woody stem with alternate distichously arranged leaves holding circinnate flowers. The fruits are oblong, woody capsules containing seeds covered with blue arils[12].

R. madagascariensis Sonn., has been traditionally used in the treatment of diabetes and kidney stone problems[13,14]. The plant was also found to possess

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antiseptic properties[15]. *R. madagascariensis* is regarded as a monospecific plant and based upon its morphological characteristics, habit and habitat, four varieties including Hirana, Bemavo, Horonorona and Menafalaka have been identified[16]. In an ethnobotanical study conducted by Rakotoarivelo *et al*., the ethnobotanical uses and economic value of all the four varieties in the local population of Ambalabe rural community were reported with varied usage as food, medicine and tools. Additionally, the study has further demanded suitable measures for long term management and sustainable harvests of *R. madagascariensis*[17].

In our previous studies, we performed an *in-vitro* antidiabetic activity which showed the ethanolic and aqueous extracts as potent inhibitors of glucose diffusion. Further, the ethanolic and aqueous extracts were found to be effective antidiabetic, hypolipidemic, renoprotective and antioxidant agents on alloxan induced diabetic rats[18-20].

Further, in a study reported by Onifade *et al.*, the ethanolic extract of *R. madagascariensis* was found to possess maximum antibacterial effect against *Proteus vulgaris, Klebsiella pneumonia, Serratia marcescens, Citrobacter freundii and P. mirabilis.* Additionally, the study has also reported the presence of tannins, phlobatannins, flavonoids, cardiac glycosides and anthraquinones and cyanogenic glycosides[21]. Also, toxicological studies carried out on different extracts of *R. madagascariensis* showed deleterious effects in the liver and heart of experimental rats which was suggested to be due to the presence of cyanogenetic glycosides[22].

Furthermore, a comparative study on the methanolic extracts of the pericarp, seed and aril of bemavo, a variety of *R. madagascariensis* fruits were reported to possess antimicrobial activity[23]. The study has also investigated the antioxidant activity of these extracts by DPPH assay and interestingly, the seed extract was found to be most active (about 12.9 fold) with an IC_{50} value of 0.57 µg/mL compared to the standard ascorbic acid (IC_{50} = 7.32 µg/mL).

Also, a recent report on the anti-tumour effects of *R. madagascariensis* revealed a promising cytotoxic effect exhibited by the ethanolic extract in PANC-1 and SW1990 pancreatic cell lines (IC_{50} value of 12.58 µg/mL and 18.9 µg/mL respectively). Further, the HPTLC results recorded in the study revealed an enriched presence of phytoconstituents in n-hexane, ethyl acetate and ethanol extracts[24].

Moreover, the phytocompounds including β-sitosteryl-D-glucoside, (2E, 7R, 11R)-phytyl-3, 7, 11, 15-tetramethylhexadec-2-enyl pentadecanoate, (24S, 31S)-cycloartan-31, 32-diol, cycloartanol were isolated and characterized[25,26]. However, the folklore claims of this plant on kidney stones still remain unvalidated. Hence the present study focused on evaluating the anti-urolithiatic activity of various extracts of leaves of *R. madagascariensis*.

Materials and Methods

Chemicals and Reagents

All the chemicals and reagents used in the study were procured from certified suppliers and were of the highest analytical grade. The polyherbal drug Cystone was purchased from The Himalaya Drug Company, Bengaluru.

Collection and authentication of plant material

Leaves of *R. madagascariensis*, were collected from Tambaram, Tamil Nadu in October 2019. The plant was identified and authenticated by Plant Anatomy Research Centre, Chennai, No. PARC/ 2020/4281. The shade dried leaves were coarsely powdered and used for further studies.

Extraction of plant material

The methanolic and hydromethanolic extracts were prepared by macerating 300 g each of the powdered plant material with methanol (100%) and methanol-water (50-50%) at room temperature for 24 h with occasional shaking. The extracts were then filtered and the filtrates were evaporated to dryness on a rotary evaporator under reduced pressure and stored in an airtight wide-mouthed bottle. A fresh decoction was prepared every time during the experiment by boiling 1 g of powdered plant material in 100 mL of distilled water for 5 minutes and evaporated to dryness[27]. The percentage yield was calculated using the formula,

$$\text{Percentage extraction yield} = \frac{W2 - W1}{W0}$$

where W2 is the weight of the extract and the bottle, W1 is the weight of the bottle alone, and W0 is the weight of the powdered plant sample subjected to extraction.

Preliminary phytochemical analysis

Preliminary phytochemical screening was carried out to identify the presence of various phytochemical constituents in all three extracts. The methanolic,

hydromethanolic, and decoction extracts of *R. madagascariensis* leaves were subjected to phytochemical analysis using different chemical tests provided in the standard protocols for the presence of alkaloids, carbohydrates, glycosides, cardiac glycosides, steroids, proteins, flavonoids, and quinones[28].

Thin layer chromatography (TLC) profiling

Thin layer chromatography was carried out using readymade silica gel G (Merck) precoated TLC plates. The extracts were diluted with respective solvents, spotted on the TLC plates, chromatographed using various mobile phases at different ratios by trial and error method. The methanolic extract and hydromethanolic extracts were run in the mobile phase, Ethylacetate:Toluene:Formic acid (7:3:1) and detected under UV light (365 nm). The solvent system, n-butanol:acetic acid:water (4:1:5) was used for the TLC of leaf decoction and the spots were detected in the presence of iodine vapour. Finally, R_f values were calculated based on the developed spots[28].

***In-vitro* antiurolithiatic activity[10]**

About 1 mL of 0.025 M calcium chloride dehydrate was mixed with 2 mL of Tris-buffer (pH 7.4) and 1 mL (10 mg/mL solution) of the extract. The reaction was completed by adding 1 mL of 0.025 M sodium oxalate to each test tube. The turbidity of the sample was measured using UV-Visible Spectrophotometer (Shimadzu, Japan) at 620 nm after 10 minutes. The polyherbal formulation, cystone was used as the standard drug. The experiment was also carried out in the absence of an inhibitor (control). Each procedure was done in triplicates. The inhibition in the formation of a stone nucleus was calculated using the formula,

$$\text{Percentage inhibition} = \{1 - (\text{Absorbance of test sample}/\text{Absorbance of control})\} \times 100$$

Graphs were plotted with percentage inhibition at Y-axis and sample concentration at X-axis and the concentration required for a 50% inhibition (IC_{50}) was determined graphically.

Statistical analysis

All data were presented as the mean±standard error mean (SEM). The experiments were repeated in triplicates. The groups were analysed by linear regression using Graphpad Prism 7 software. Any value of $P < 0.05$ was considered statistically significant.

Results and Discussion

Preliminary Phytochemical analysis

The percentage yield of the methanolic, hydromethanolic extracts and decoction of the powdered leaves of *R. madagascariensis*, was found to be 11.7% w/w, 8.2% w/w and 7.4% w/w respectively. The decoction showed the presence of carbohydrates, glycosides, proteins, flavonoids, saponins and quinones whereas both the methanolic and hydromethanolic extracts showed the presence of alkaloids, carbohydrates, glycosides, flavonoids, phenols, tannins, proteins, saponins and quinones (Table 1). These phyto-constituents are of utmost significance in inhibiting urinary stone formation[29,30]. Saponins are known to disintegrate mucoproteins that are crucial components of stone matrix[31]. Also, tannins and polyphenols were found to inhibit calcium oxalate crystal formation as well as dissolve the preformed calcium oxalate crystals by aiding calcium complexation[32,33]. Therefore, the anti-aggregatory activity would have been an outcome of these phytoconstituents present in the extracts of *R. madagascariensis*.

TLC Profiling

Thin-layer chromatography profiling was performed to separate the presence of phytoconstituents in each extract. TLC plays a valuable role in the quality control of herbals, particularly, in drug authentication assuring its safety and purity[34-36]. Among various solvent systems used for the development of chromatogram at differing ratios, the mobile phase with the good resolution was

Table 1 — The percentage yield and preliminary phytochemical screening of leaves of *Ravenala madagascariensis* Sonn.

Extract	Solvent used	Physical nature	Colour	Yield (%w/w)	Phytoconstituents
Methanolic extract	Methanol (100%)	Semi-solid	Dark brown	11.7%w/w	Alkaloids, Carbohydrates, Glycosides, Flavonoids, Phenols, Tannins, Proteins, Saponins, Quinones
Hydro-methanolic extract	Methanol and water (50% - 50%)	Semi-solid	Greenish brown	8.2%w/w	Alkaloids, Carbohydrates, Glycosides, Flavonoids, Phenols, Tannins, Proteins, Saponins, Quinones
Decoction	Distilled water	Semi-solid	Light brown	7.4% w/w	Carbohydrates, Glycosides, Proteins, Flavonoids, Saponins, Quinones

Table 2 — TLC profile of various extracts of leaves of *Ravenala madagascariensis* Sonn.

Extract	Solvent system	No. of spots	R_f values	Detecting agent
Methanolic extract	Ethylacetate:Toluene:Formic acid (7:3:1)	3	0.76, 0.83, 0.89	UV lamp
Hydro-methanolic extract		3	0.76, 0.83, 0.89	
Decoction	n-butanol:Acetic acid:Water (4:1:5)	3	0.17, 0.38, 0.63	Iodine vapour

used and R_f values were calculated (Table 2). Both the methanolic extract and hydromethanolic extract showed three (3) spots with R_f values 0.76, 0.83, 0.89 in the solvent system, Ethylacetate:Toluene: Formic acid (7:3:1) of which two (2) spots were UV active. Leaf decoction showed three (3) spots with R_f values 0.17, 0.38, 0.63 in the mobile phase system, n-butanol: acetic acid: water (4:1:5) in which iodine vapour was used as the detecting agent (Fig. 1).

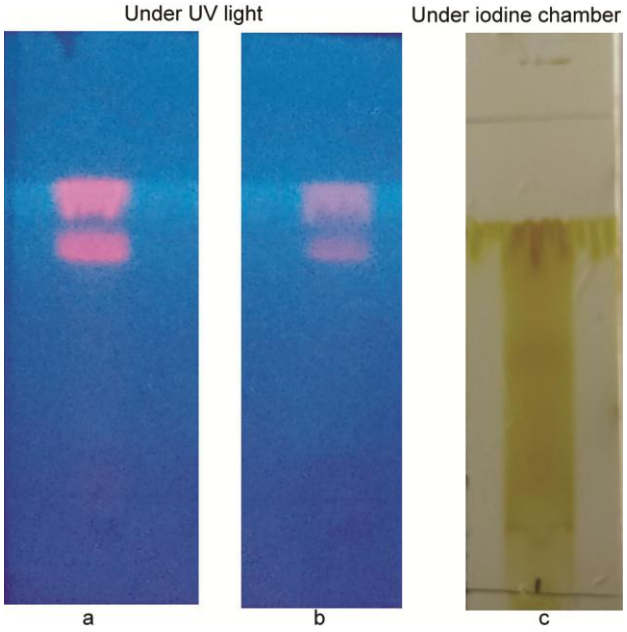

Fig. 1 — Thin layer chromatography of leaves of *Ravenala madagascariensis* Sonn. a) methanolic extract, b) hydromethanolic extract, and c) Decoction.

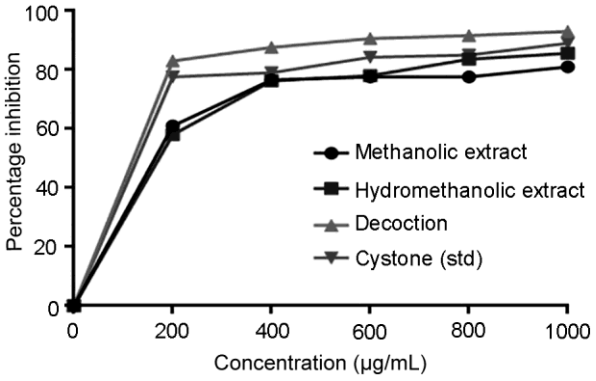

Fig. 2 — Percentage inhibition of stone nucleus formation of various extracts of leaves of *Ravenala madagascariensis* Sonn., by turbidimetry. Plot of percentage inhibition at various concentrations of methanolic, hydromethanolic and decoction extracts. Data are expressed as mean±SEM of triplicates.

***In-vitro* Antiurolithiatic activity**

Urolithiasis stays as the third most common disorder of urinary tract, characterized by the sedimentation of poorly soluble crystalloids of supersaturated urine[37]. About 2% of the world population suffers from renal stone disease with a male-female incidence ratio of 2:1 with a peak incidence during 2nd to 3rd decade of life[38]. In India, twelve per cent of the population is expected to have renal stones of which 50% of the cases may end up with kidney dysfunction[39]. *In-vitro* anti-urolithiatic activity of *R. madagascariensis* Sonn., leaf extracts were determined by turbidimetry method. The principle was based on the measurement of turbidity developed by the formation of calcium oxalate and their potential inhibition by the extracts of *R. madagascariensis* compared to the standard drug cystone. (Fig. 2) In the present study, all three extracts displayed dose-dependent inhibition of calcium oxalate crystallisation. The maximum percentage inhibition effect was exhibited by decoction (92%) followed by the hydromethanolic extract (85%) and methanolic extract (82%) compared to the standard cystone (93%) at the maximum concentration of 1000 µg/mL. The IC_{50} value of decoction of *R. madagascariensis* powdered leaves (188.65 µg/mL) showed the least and efficient IC_{50} value of 188.65 µg/mL than the methanolic (305.93 µg/mL), hydromethanolic (306.83 µg/mL) extracts and standard cystone (195.08 µg/mL).

Supersaturation of urine results in calcium oxalate crystallization within the urinary tract. During nucleation, stone forming crystals unite to form clusters with the addition of new constituents[32]. In the present study, decoction of *R. madagascariensis* exhibited stronger antiurolithiatic activity than the methanolic and hydromethanolic extracts. The study reveals the nucleation preventing property of the decoction of *R. madagascariensis* and the results were comparable with standard cystone. A*n ethnomedicinal data collected by Bahmani et al.,* during the period of July-September 2012, reported 18 medicinal plants by

the traditional healers of Shiraz in the treatment of kidney stones. Interestingly, the study revealed the decoction to be the most frequently (68%) prescribed mode of preparation[40]. Further, a study by *Vasanthi et al*., has reported the antiurolithiatic potential of a popular South Indian traditional decoction, Sirupeelai Samoola Kudineer, practiced in Siddha system of medicine against ethylene glycol-induced renal calculus in rats[41,42].

Furthermore, in a similar study by *Zarin et al*., the antiurolithiatic ability of four different types of Musa species including, *Musa acuminate* x *balbisiana Colla* cv. 'Awak Manis', *M. paradisiaca* cv. Nangka, 'Pisang Nangka', *Musa acuminate Colla* cv. 'Sucrier' and *Musa acuminate x balbisiana Colla* cv. 'Awak Legor' were investigated[43]. The results of the study revealed that the methanolic pseudo stem extracts of *Musa acuminate x balbisiana Colla* cv. 'Awak Legor' exhibited the highest percentage inhibition of calcium oxalate crystal nucleation of 55.39±1.01% at the end of 60min compared to standard cystone (30.87±0.74%). In our present study, we carried out a comparative antiurolithiatic activity in the methanolic, hydromethanolic and decoction of *R. madagascariensis* leaves and found that the decoction of *R. madagascariensis* to be highly active and showed a percentage inhibition of 92.09±0.15% compared to cystone (93.69±0.10%) at the concentration of 1000 µg/mL. Moreover, the IC_{50} values were found to be 305.93 µg/mL, 306.83 µg/mL, 188.65 µg/mL and 195.08 µg/mL for methanolic extract, hydromethanolic extract, decoction and cystone respectively.

Conclusion

In the present work, preliminary phytochemical screening, TLC profiling, and antiurolithiatic activity of various extracts of leaves of *R. madagascariensis* were carried out. Preliminary phytochemical screening revealed the presence of various alkaloids, carbohydrates, glycosides, phenols, proteins, saponins, flavonoids, and quinones. TLC profiling established the presence of phytoconstitiuents in the respective extracts. The *in-vitro* antiurolithiatic activity was carried out and the decoction was found to possess the maximum efficacy with IC_{50} value of 188.65 µg/mL. The study strongly suggests that the leaf decoction could be an effective alternative approach against urolithiasis. Thus the current work can further pave way for formulation development, evaluation, and transformation into a potent nutraceutical.

Conflict of interest

The authors declare no conflict of interest.

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