

Full Length Research Paper

Antiuro lithiatic activity of selected plants extracts against calcium oxalate crystals

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The present study was designed to evaluate the antiuro lithiatic activity of selected plants extracts (*Achyranthes aspera*, *Lawsonia inermis*, *Ficus benghalensis*, *Raphnus sativus* and *Macrotyloma uniflorum*). The methanol extract of selected plants was analysed for *in-vitro* antiuro lithiatic activity using nucleation, aggregation and growth assay of calcium oxalate (CaOX) monohydrate crystals. The nucleation, aggregation and growth of CaOX crystals were significantly inhibited by all selected plant extracts. The highest inhibition of CaOX nucleation was shown by *R. sativus* (55.21±1.9%) followed by *M. uniflorum* (53.91±1.1%) and the least by *F. benghalensis* (43.63±0.8%) at 1.0 mg/mL. The highest inhibition of calcium CaOX aggregation was shown by *R. sativus* (61.6±1.6%) which was very close to Cystone (63.28±2.5%). The growth of CaOX was highly inhibited by *A. aspera* (42.17±1.0%) followed by *M. uniflorum* (40.27±1.4%) with lowest inhibition by *F. benghalensis* (31.44±1.4%) as compared to Cystone (43.35 ±0.9%). The study showed that the selected plants showed the significant antiuro lithiatic activity against CaOX crystals, which could be a potential source for the treatment of renal stone disease.

Key words: Antiuro lithiatic, calcium oxalate, nucleation, aggregation, methanol extracts.

INTRODUCTION

Urolithiasis is the process of formation or deposition of calculi in the urinary tract, which is considered as the third most common disorder estimated to occur in around 12% of the global population worldwide (Sharma et al., 2016; Khan, 2013). The formation of calcified renal stone is a physiochemical event leading to crystal nucleation, aggregation and its growth assisted by many biological processes including urine volume, pH, increased calcium oxalate or sodium oxalate, and urates (Ratkalkar and Kleinman, 2011; Khan et al., 2016). At present, over 90% of upper urinary tract stones patients have been treated according to the size, type, and position of the stones with a success rate of 68 to 86% (Bahmani et al., 2016).

Increased dietary protein intake has been reported to elevate the rates of developing kidney stones and approximately 75% of all renal stones are composed of calcium oxalate and/or calcium phosphate (Stamatelou et al., 2003).

Technological progress has been remarkable in the treatment/management of kidney stones, but the associated complexities such as their tendency to increase recurrence, hemorrhage, hypertension, tubular nephrosis are a major limiting factor (Chaudhary et al., 2010; Patel and Shah).

Therefore, alternative or complementary medicine with minimum side effect might be useful. There is a growing

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Table 1. Scientific names, voucher numbers, local names, parts used, reported medicinal uses and extractive yield of selected plants.

Scientific name [Herbarium No.]	Local name	Parts used in this study	Reported ethno medicinal use	Extractive yield (%)
<i>Achyranthes aspera</i> [UHS1804]	Datiwan	Whole plant	Used in dropsy, piles, stomach ache, skin eruptions, pyorrhoea, and cough (Nepal, 2004; Kunwar et al., 2009)	2.1
<i>Lawsonia inermis</i> [UHS1805]	Mehendi	Whole plant	pastes of leaves are used to relieve burning of palm and soles (Dhami, 2008)	3.5
<i>Ficus benghalensis</i> [UHS1803]	Bar	Bark	Locally applied for mumps, bruises, and rheumatism (Ghimire and Bastakoti, 2009)	4.0
<i>Raphnus sativus</i> [UHS1802]	Mula	Whole plant	Used in indigestion, liver and gall bladder troubles, urinary complaints and ear pain (Parajuli, 2012, 2013)	5.2
<i>Macrotyloma uniflorum</i> [UHS1801]	Gahat	Seeds	Used as pulses, medicine for curing stone (Upreti et al., 2016)	2.7

public interest in herbal medicine for the management of urolithiasis in developed as well as developing country because of their wide biological and medicinal values, low toxicity and lesser costs (Sharma et al., 2016; Aggarwal et al., 2010).

To date, most Nepalese people are unable to benefit from modern health care facilities for urinary stone treatment due to socio-economic factors (Lamichhane et al., 2017). Consequently, for the management of renal stone disease, they are relying on regionally available traditional herbal medicinal products (Gewali and Awale, 2008). The medicinal plants were used as principal ingredients in traditional medicine, available through tribal, home herbal remedy, and the Baidhya, Ayurveda and Amchi systems (Kunwar et al., 2010). Most of the plant-based medicines were used for the treatment of renal diseases (Bhattarai et al., 2010). Literature survey reveals that herbal formulation of plant-based medicine has been found to be successful in management of kidney stones in Nepal (Kumaran and Patki, 2011; Brardi et al., 2012).

Although these herbal medicines are popular in folk culture, the rationale behind their pharmacological and physiochemical antiurolithiatic efficacy has not been well elucidated. Only a few studies have suggested some evidence for the efficacy. In view of traditional and ethnomedicinal use, this study was designed to evaluate antiurolithiatic effects of selected plants, using methanol extracts in the *in-vitro* crystallization of calcium oxalate model.

MATERIALS AND METHODS

Preparation of methanol extracts

Five fully mature plants (Table 1) were collected from the Rupandehi, Nepal and further authenticated by Professor Subodh

Khanal, a Botanist at the Institute of Agriculture and Animal Sciences, Tribhuvan University. The voucher specimens of collected plants were deposited in the Pharmacognosy Laboratory, Department of Pharmacy, Universal College of Medical Sciences, Tribhuvan University, Nepal. The collected plants were dehydrated and pulverized. The grounded coarse powders (70 g) were immersed in methanol (350 mL) for 7 days at room temperature with frequent agitation. The extracts were filtered using a Buckner funnel and Whatman No. 1 filter paper. Each filtrate was concentrated to dryness in a rotary evaporator (Büchi Labortechnik, Germany) under reduced pressure and controlled temperature (40-50°C) to give final extracts, which was stored at 4°C in an airtight container until further use.

Experimental procedure

The effect of extract on CaOx crystallization was determined by the time course measurement of turbidity changes due to the crystallization in artificial urine on addition of 0.01 M sodium oxalate solution. The precipitation of calcium oxalate at 37°C and pH 6.8 was studied by the measurement of absorbance at 620 nm using UV/Visible spectrophotometer (Sharma et al., 2016).

Preparation of artificial urine

The artificial urine (AU) was prepared as per previously described method (Sharma et al., 2016) using the following composition: Calcium chloride dehydrates solution (10 mM) and sodium oxalate solution (1.0 mM), sodium chloride (200 mM) and sodium acetate trihydrate (10 mM). The AU was prepared fresh each time and pH adjusted to 5.7.

Nucleation and aggregation assay of CaOX crystals

The spectrophotometric assay method was used to determine the inhibitory activity of the extracts in the nucleation and aggregation of CaOx crystals (Sharma et al., 2016; Hess et al., 2000). Briefly, the calcium chloride dehydrate aqueous solution (10 mM) and sodium oxalate solution (1.0 mM), was diluted by a buffer of sodium chloride (200 mM) and sodium acetate trihydrate (10 mM) at pH 5.7 maintaining temperature at 37°C with circulating water bath. For

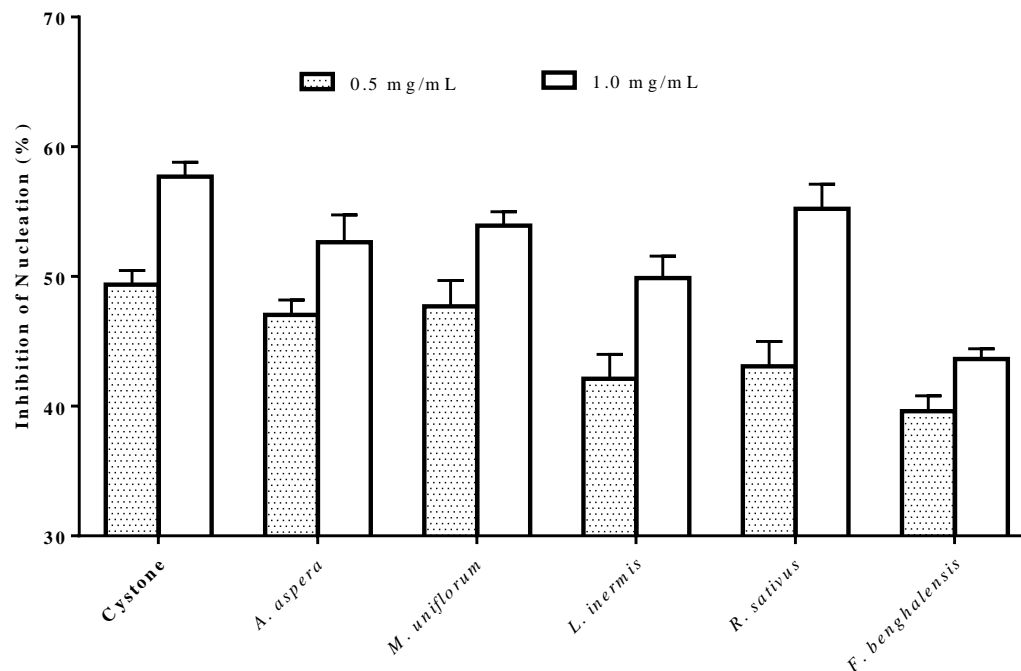


Figure 1. Nucleation assay of all five plant extracts at two different concentrations. The concentration of each plant extracts and standards are in mg/mL. Data expressed as mean value \pm standard deviation (n=3) and the concentration of the extract is provided in terms of mg/mL.

crystallization experiments, cystone or extract test solutions (1 mL) at 0.5 or 1.0 mg/mL in water were added to the stirred sodium oxalate solution (25 mL) at 37°C followed by the addition of calcium chloride solution (25 mL). The absorbance was recorded at 620 nm by spectrophotometer using the control with no test solution and the percentage inhibition was calculated as follows:

$$\text{Percentage inhibition (\%)}: [1 - (\text{Tsi} / \text{Tsc})] \times 100 \quad (1)$$

Where Tsc = turbidity slope of the control and Tsi = turbidity slope in the presence of the inhibitor. All the experiments were performed in triplicate.

Growth assay

The inhibitory activity of the extracts on the CaOx crystals growth was determined by a spectrophotometric assay (Sharma et al., 2016; Aggarwal et al., 2010; Chaudhary et al., 2010). Briefly, calcium chloride solution (4 mM, 20 ml) and 4 mM sodium oxalate (4 mM, 20) mL were added to a buffer solution (30 mL) of sodium chloride (90 mM) and Tris HCl (10 mM) at pH 7.2 followed by the addition of calcium oxalate monohydrate (COM) crystal slurry (600 μ l) prepared in acetate buffer (1.5 mg/mL). Consumption of oxalate commences immediately after the addition of the COM slurry, which was monitored at 214 nm for 600 s absorbance disappearance. Further, cystone or extract test solution (1 mL) at 0.5 or 1.0 mg/mL in water was added separately into the above solution and depletion of free oxalate ions was calculated by spectrophotometer as inhibition in calcium oxalate crystals growth using the following.

$$\text{Inhibitory of crystal growth (\%)} = (C - S) / C \times 100 \quad (2)$$

Where C = reduction of free oxalate without any extract; and S =

reduction of free oxalate with inhibitor. All the experiments were performed in triplicate.

Statistical analysis

The data were reported as mean \pm standard deviation. Statistical significance of the observations from nucleation aggregation and growth assay was calculated using one way analysis of variance test (ANOVA), followed by Dunnett's t-test, using GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA, USA). P<0.05 is considered as statistically significant level.

RESULTS

Nucleation and aggregation assay

The changes in the turbidity of different solutions (control, cystone and all five selected extracts at 0.5 and 1 mg/mL), were plotted at different time intervals. All the plants extracts at tested concentration showed the significant reduction (P<0.01) in nucleation and aggregation of CaOx crystals when compared with cystone. The methanol extract of *R. sativus* (55.21 \pm 1.9%) showed highest inhibition compared to cystone (57.7 \pm 1.1%) and *F. benghalensis* (43.63 \pm 0.8%) showed lowest inhibition of at 1 mg/mL (Figure 1). In aggregation assay, the extract of *R. sativus* (61.6 \pm 1.6%) showed highest inhibition compared to cystone (63.28 \pm 2.5%) and the extract of *F. benghalensis* (45.18 \pm 1.05%) showed

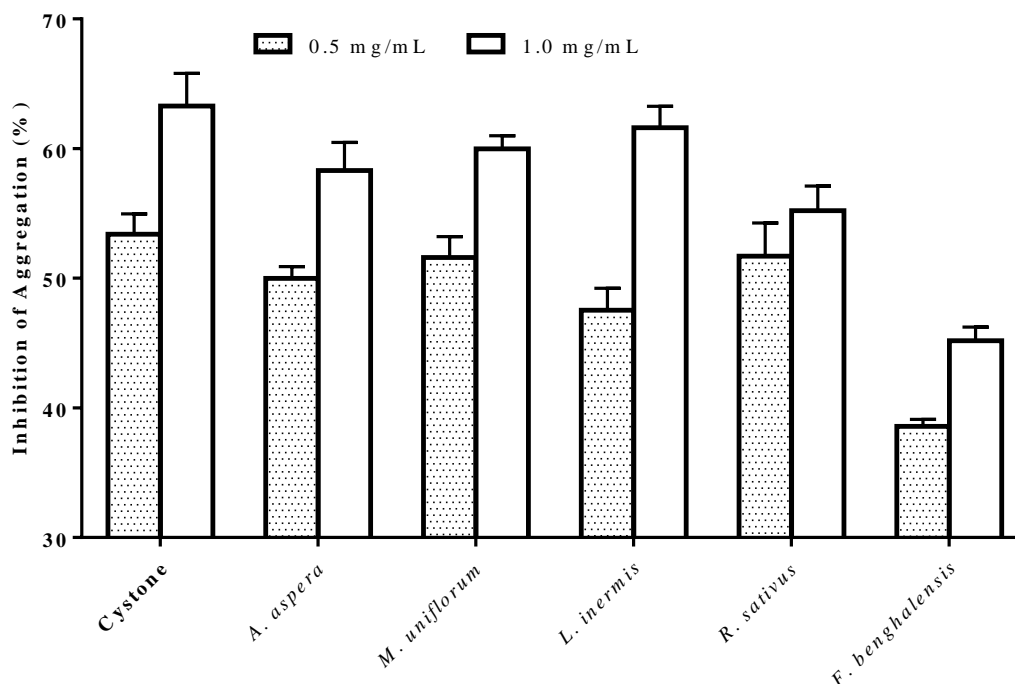


Figure 2. Aggregation assay of all five plant extract at two different concentrations. The concentration of each plant extracts and standards are in mg/mL. Data expressed as mean value \pm standard deviation (n=3) and the concentration of the extract is provided in terms of mg/mL.

lowest inhibition at 1 mg/mL (Figure 2). It was found that increasing the concentration of plant extracts resulted in the increase in percentage inhibition of calcium oxalate crystallization.

Growth assay

In calcium oxalate growth assay, all the plants extracts at tested concentration showed the significantly ($P < 0.01$) inhibited calcium oxalate monohydrate (COM) growth when compared with cystone. For checking inhibitory activity on COM crystal growth, it was observed that inhibition increases with increase in time; probably the secondary metabolites present in extract interfere with the growth of calcium and oxalate onto the slurry and thus inhibits the growth process. Out of the five methanolic extracts, the extract of *A. aspera* ($42.17 \pm 1.03\%$) showed highest inhibition compared to cystone ($43.35 \pm 0.95\%$) and the extract of *F. benghalensis* ($36.44 \pm 1.36\%$) showed lowest inhibition at 1 mg/mL (Figure 3). The increasing the concentration of plant extracts resulted in the increase in percentage inhibition of COM growth.

DISCUSSION

With effective ethnopharmacological value, *Achyranthes*

aspera, *Lawsonia inermis*, *Ficus benghalensis*, *Raphnus sativus* and *Macrotyloma uniflorum* are widely used by different tribes as an effective herbal medicine (Acharya, 2012; Tamang et al., 2017; Malla et al., 2014). The aim of this study was to evaluate and compare the antiurolithiatic activity of selected plants extracts, to ascertain scientific rationales behind its ethnomedicinal practice.

CaOx nucleation is a phase-change thermodynamic driven process in which dissolved substances spontaneously crystallize in a supersaturated solution. A comparable phase change and formation of CaOx crystals was observed during the nucleation assay, which can imitate the biological process. The nucleation of CaOx crystals was significantly inhibited in the presence of selected plants extracts, which was highly comparable to Cystone. This indicates the anti-crystallization property of selected plant extracts, which could be due to their ability to complex with free calcium and oxalate ions and prevent CaOx crystallization, as suggested for *Daucus carota* (Bawari et al., 2018).

COM, a CaOx polymorph, is most commonly found in urolithiasis, which is more stable with a more aggregatory and adhesive ability (Sheng et al., 2005). Thereby, COM generates large crystal aggregates which deposits heavily into the renal epithelial tissue, causing injuries. In this study, all the plant extracts inhibited the aggregation of COM, which could be due to presence of flavonoids, phenolic compounds, saponins and tannins, which was

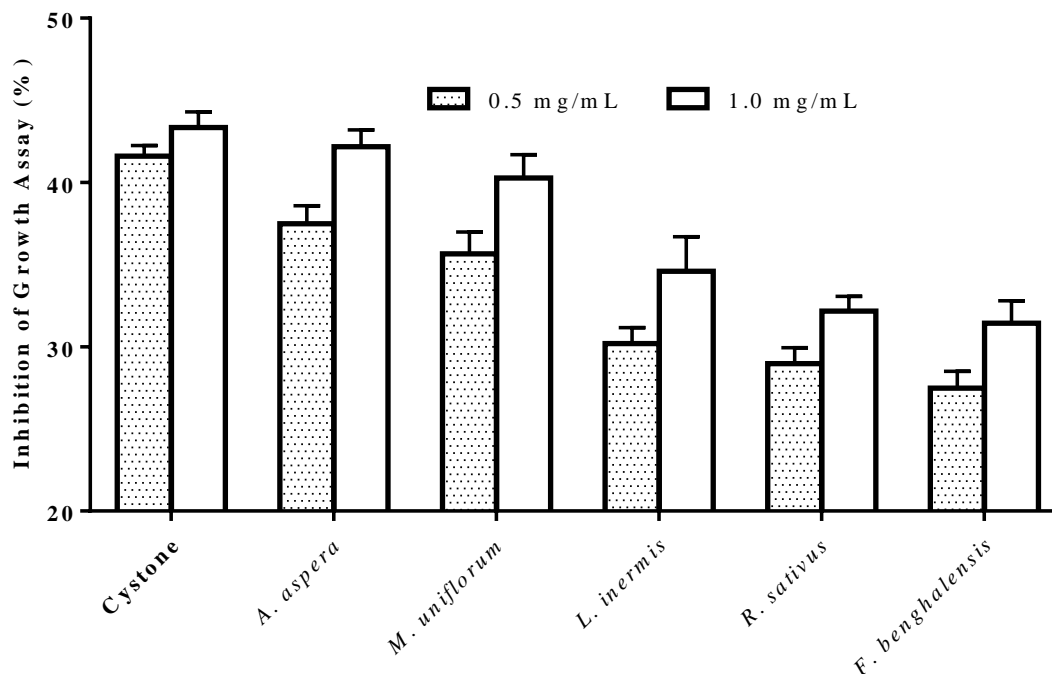


Figure 3. Growth assay of all five plant extract at two different concentrations. The concentration of each plant extracts and standards are in mg/mL. Data expressed as mean value \pm standard deviation (n=3) and the concentration of the extract is provided in terms of mg/mL.

reported to inhibit urinary stone formation studies (Vargas et al., 1999; Asif et al., 2014). Moreover, CaOx polymorph growth is a marker of deposition event for crystal forming ions in the supersaturated solution. This incidence of CaOx crystals growth was also monitored in the current study. All the selected plant extracts showed growth inhibitory activity.

Natural extracts shows a varied range of phytochemicals of which tannins, polyphenols, flavonoids alkaloids and saponins are most abundance. There are reports that flavonoids inhibit calcium oxalate crystallization in human urine as well as in animal models (Zhong et al., 2012) as well as crystal deposition (Noorafshan et al., 2013). Saponins showed anti-crystallization properties by disaggregating the suspension of mucoproteins, which are the promoters of crystallization (Gürocak and Küpeli, 2006). In this study, maximum antiurolithiatic activity may be apparent due to flavonoids, saponins, alkaloids, polyphenols that may be present in higher quantity and also by CaOx crystal inhibition, diuretic, hypermagneseuric and antioxidant effects which are reported by previous studies (Vargas et al., 1999; Asif et al., 2014). Many literatures report reveals that the physical (agitation, temperature, and supersaturation) and chemical (concentration of acidic or basic additives) conditions of urine are responsible for precipitation of calcium oxalates (Šter et al., 2018). The overall condition could be modified by phytochemicals from selected plant extracts to inhibit crystallization.

Conclusion

Out of selected plants, *R. sativus* possessed comparable antiurolithiatic activity to cystone in nucleation and aggregation and *A. aspera* in growth assay. The result of the study showed that the selected plants possess antiurolithiatic activity, which was directly proportional to the concentration of the extracts. These findings substantiate the traditional use of the plants in the treatment of urinary stones and kidney problems.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

- Acharya R (2012). Ethnobotanical study of medicinal plants of Resunga Hill used by Magar community of Badagaun VDC, Gulmi district, Nepal. *Scientific World* 10(10):54-65.
- Aggarwal A, Tandon S, Singla SK, Tandon C (2010). Reduction of

- oxalate-induced renal tubular epithelial (NRK-52E) cell injury and inhibition of calcium oxalate crystallisation in vitro by aqueous extract of *Achyranthes aspera*. *International Journal of Green Pharmacy* 4(3):480-489.
- Asif M, Jabeen Q, Atif M, Majid AMSA, Qamar-Uz-Zaman M (2014). Diuretic Activity of *Achyranthes aspera* Linn Crude Aqueous Extract in Albino Rats. *Tropical Journal of Pharmaceutical Research* 13(12):2039-2045.
- Bahmani M, Baharvand-Ahmadi B, Tajeddini P, Rafieian-Kopaei M, Naghdi N (2016). Identification of medicinal plants for the treatment of kidney and urinary stones. *Journal of Renal Injury Prevention* 5(3):129-133.
- Bawari S, Sah AN, Tewari D (2018). Antiurolithiatic Activity of *Daucus carota*: An in vitro Study. *Pharmacognosy Journal* 10(5).
- Bhattarai S, Chaudhary RP, Quave CL, Taylor RSL (2010). The use of medicinal plants in the trans-Himalayan arid zone of Mustang district, Nepal. *Journal of Ethnobiology and Ethnomedicine* 6:4-14.
- Brardi S, Imperiali P, Cevenini G, Verdacchi T, Ponchiotti R (2012). Effects of the association of potassium citrate and agropyrum repens in renal stone treatment: results of a prospective randomized comparison with potassium citrate. *Archivio Italiano di Urologia e Andrologia* 84(2):61-67.
- Chaudhary A, Singla S, Tandon C (2010). In vitro evaluation of *Terminalia arjuna* on calcium phosphate and calcium oxalate crystallization. *Indian Journal of Pharmaceutical Sciences* 72(3):340.
- Dhami N (2008). Ethnomedicinal uses of plants in Western Terai of Nepal: A case study of Dekhatbhuli VDC of Kanchanpur district. In: Jha PK, Karmacharya SB, Chettri MK, Thapa CB and Shrestha BB (Eds.), *Medicinal plants in Nepal: an anthology of contemporary research*. Kathmandu, Nepal: Ecological Society pp. 165-177.
- Gewali MB, Awale S (2008). Aspects of traditional medicine in Nepal. Japan: Institute of Natural Medicine. University of Toyama
- Ghimire K, Bastakoti RR (2009). Ethnomedicinal knowledge and healthcare practices among the Tharus of Nawalparasi district in central Nepal. *Forest Ecology and Management* 257(10):2066-2072.
- Gürocak S, Küpeli B (2006). Consumption of historical and current phytotherapeutic agents for urolithiasis: a critical review. *The Journal of Urology* 176(2):450-455.
- Hess B, Jordi S, Zipperle L, Ettinger E, Giovanoli R (2000). Citrate determines calcium oxalate crystallization kinetics and crystal morphology—studies in the presence of Tamm–Horsfall protein of a healthy subject and a severely recurrent calcium stone former. *Nephrology Dialysis Transplantation* 15(3):366-374.
- Khan SR (2013). Reactive oxygen species as the molecular modulators of calcium oxalate kidney stone formation: evidence from clinical and experimental investigations. *The Journal of Urology* 189(3):803-811.
- Khan SR, Pearle MS, Robertson WG, Gambaro G, Canales BK, Doizi S, Traxer O, Tiselius H-G (2016). *Kidney Stones*. *Nature reviews. Disease Primers* 2:16008.
- Kumaran MS, Patki PS (2011). Evaluation of an Ayurvedic formulation (Cystone), in urolithiasis: A double blind, placebo-controlled study. *European Journal of Integrative Medicine* 3(1):23-28.
- Kunwar RM, Shrestha KP, Bussmann RW (2010). Traditional herbal medicine in Far-west Nepal: a pharmacological appraisal. *Journal of Ethnobiology and Ethnomedicine* 6(1):35.
- Kunwar RM, Uprety Y, Burlakoti C, Chowdhary C, Bussmann RW (2009). Indigenous use and ethnopharmacology of medicinal plants in far-west Nepal. *Ethnobotany Research and Applications* 7:005-028.
- Lamichhane R, Zhao Y, Paudel S, Adewuyi EO (2017). Factors associated with infant mortality in Nepal: a comparative analysis of Nepal demographic and health surveys (NDHS) 2006 and 2011. *BMC Public Health* 17(1):53-53.
- Malla B, Gauchan D, Chhetri R (2014). Medico-ethnobotanical investigations in Parbat district of Western Nepal. *Journal of Medicinal Plants Research* 8(2):95-108.
- Nepal I (2004). National Register of Medicinal Plants Available at: <https://portals.iucn.org/library/node/8470>.
- Noorafshan A, Karbalay-Doust S, Karimi F (2013). Diosmin reduces calcium oxalate deposition and tissue degeneration in nephrolithiasis in rats: a stereological study. *Korean Journal of Urology* 54(4):252-257.
- Parajuli R (2012). Ethnomedicinal use of plants in rai community of maimajuwa and puwamajuwa VDCs of Ilam District, Eastern Nepal. *Bulletin of the Department of Plant Resources* 34:65-73.
- Parajuli RR (2013). Indigenous knowledge on medicinal plants: Maipokhari, Maimajhuwa and mabu VDCs of Ilam District, Eastern Nepal. *Journal of Department of Plant Resources Nepal* 35:50-58.
- Patel KM, Shah SK Evaluation of Antiurolithiatic Activity of *Lawsonia inermis* Linn. in Rats. Patel KM, Samir KS (2017). Evaluation of Antiurolithiatic Activity of *Lawsonia inermis* Linn. in Rats. *International Journal of Pharmaceutical Science and Nanotechnology* 10(1):3728-3735.
- Ratkalkar VN, Kleinman JG (2011). Mechanisms of Stone Formation. *Clinical reviews in Bone and Mineral Metabolism* 9(3-4):187-197.
- Sharma D, Dey YN, Sikarwar I, Sijoria R, Wanjari MM, Jadhav AD (2016). In vitro study of aqueous leaf extract of *Chenopodium album* for inhibition of calcium oxalate and brushite crystallization. *Egyptian Journal of Basic and Applied Sciences* 3(2):164-171.
- Sheng X, Ward MD, Wesson JA (2005). Crystal surface adhesion explains the pathological activity of calcium oxalate hydrates in kidney stone formation. *Journal of the American Society of Nephrology* 16(7):1904-1908.
- Stamatelou KK, Francis ME, Jones CA, Nyberg Jr LM, Curhan GC (2003). Time trends in reported prevalence of kidney stones in the United States: 1976-1994. *Kidney International* 63(5):1817-1823.
- Šter A, Šafranko S, Bilić K, Marković B, Kralj D (2018). The effect of hydrodynamic and thermodynamic factors and the addition of citric acid on the precipitation of calcium oxalate dihydrate. *Urolithiasis* 46(3):243-256.
- Tamang R, Thakur C, Koirala D, Chapagain N (2017). Ethno-medicinal Plants used by Chepang community in Nepal. *Journal of Plant Resources* 15(1):31-30.
- Uprety Y, Poudel RC, Gurung J, Chettri N, Chaudhary RP (2016). Traditional use and management of NTFPs in Kangchenjunga Landscape: implications for conservation and livelihoods. *Journal of Ethnobiology and Ethnomedicine* 12(1):19.
- Vargas SR, Perez GR, Perez GS, Zavala SM, Perez GC (1999). Antiurolithiatic activity of *Raphanus sativus* aqueous extract on rats. *Journal of Ethnopharmacology* 68(1-3):335-338.
- Zhong Y-S, Yu C-H, Ying H-Z, Wang Z-Y, Cai H-F (2012). Prophylactic effects of *Orthosiphon stamineus* Benth. extracts on experimental induction of calcium oxalate nephrolithiasis in rats. *Journal of Ethnopharmacology* 144(3):761-767.