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In vitro Anti-Urolithiatic Activity & Determination of Flavonoid Contents of Different Extracts of Citrus Sinensis Leaves

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Abstract

The most typical urinary tract condition is urolithiasis. Despite enormous medical advancements, there is still no truly effective therapy to treat renal calculi, despite the large incidence of urolithiasis in the world. Pharmacologists should pay particular attention to Citrus Sinensis' widespread use in treating kidney stones & other problems of the urinary tract. However, it is challenging to find studies that explain Citrus Sinensis' antiurolithiatic effect. It has a traditional claim for diuretic & kidney disorders. Its usage in urolithiasis has not been well supported scientifically. Therefore, the current study was conducted to investigate Citrus Sinensis' antiurolithiatic characteristics. The Methanolic Extract of Citrus Sinensis (MECS) leaves were investigated for their *in vitro* antiurolithiatic activities in the current study employing a crystal aggregation assay. In the current investigation, MECS significantly decreased crystal aggregation. In the current work, a total flavonoid content assay was used to confirm the presence of flavonoids in different extracts of Citrus Sinensis.

Keywords: Urolithiasis, citrus sinensis, flavonoid, MECS

Introduction

Urolithiasis is a term used to describe calculi or stones that form the urinary tract. This condition involves the formation of calcifications in the urinary system, usually in the kidneys or ureters, but may also affect the bladder and/or urethra. Urolithiasis is a common condition that with various risk factors & causes, including lifestyle habits & other practices. Renal stones are a common health condition; in fact, it is estimated that up to 10% of all individuals will develop a kidney stone throughout their lifetime, although some individuals do not experience symptoms. Each year, approximately 1 in every 1,000 people is hospitalized due to urolithiasis. Men are more likely to have urolithiasis than women, with a risk ratio of 3:1, although this gap appears to be narrowing over time. People between the ages of 20 & 40 years are at the highest risk of developing stones [1].

The risk of urolithiasis increases as a result of any factor that leads to urinary stasis due to a reduction or obstruction of urinary flow. Certain risk factors contribute to a higher incidence of stone formation. For example, men excrete less citrate & more calcium than women, which is thought to be linked to the higher incidence of urolithiasis in men. In addition to sex, an individual's ethnic background can be considered a risk factor, as individuals with a Native American, African, or Israeli background are more likely to be affected by the condition. Dehydration or reduced fluid intake may increase the risk of stone formation, in addition to a diet that is high in sodium, oxalate, fat, protein, sugar, unrefined carbohydrates, & vitamin C. In addition to diet, certain regions of the world are associated with an increased risk of urolithiasis, such as tropical climates, mountainous or desert terrain. Certain medications like ephedrine, guaifenesin, thiazide, indinavir, & allopurinol may lead to the development of stones [14].

Approximately 85% of stones are composed predominantly of calcium compounds. The most common cause of calcium stone production is excess calcium in the urine (hypercalciuria). Calcium stones are composed of calcium oxalate (CaOx) or calcium phosphate (CaP). Calcium oxalate stones are much more frequent than calcium phosphate stones. Nearly seven decades ago, it is described that plaque-like lesions were present in the renal papillae, which were invariably present in patients with calcium oxalate stones, although sometimes also present in individuals who did not form stones [1].

The most important pathophysiological factor for calcium nephrolithiasis is hypercalciuria. A more pathophysiology-oriented classification partitions hypercalciuria by defects in one or a combination of three organs kidney (renal leak), bone (resorptive), and gut (absorptive). These three categories can co-exist as a primary disorder and can simultaneously affect more than one organ. Less common than primary gut hyperabsorption are primary resorptive and renal hypercalciuria. An example of a primary resorptive defect is primary hyperparathyroidism, although the secondary enhancement of calcitriol synthesis leads to amplified

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intestinal absorption, contributing to the hypercalciuria. Increased resorption from acid load will be discussed. High dietary salt is associated with a high rate of nephrolithiasis and salt restriction diminishes the risk of kidney stones [2].

Citrus Sinensis belonging to the Rutaceae family, is the most widely grown and commercialized citrus specie. Citrus is widely grown in Nigeria and many other tropical and subtropical regions. In terms of volume in production, citrus ranks after banana as the world second fruit crop with more than 108 million tons. Sweet orange (*Citrus Sinensis* L. Osbeck) commonly called orange is a member of this family and a major source of vitamins, especially vitamin C, sufficient amount of folacin, calcium, potassium, thiamine, niacin and magnesium [6].

Pharmacology/Biological Studies:

i). **As Antioxidant:** A high quality orange is one that is mature with good color intensity uniformly distributed over the surface. Such oranges must be firm with a fairly smooth texture and shape that is characteristic of the variety, free from decay, defects and other blemishes. The biological activity and the healthy effects of citrus flavonoids as antioxidants have been reported. These group of pigments as found in plants and together with anthocyanin play a role in flower and fruit colouration. Also, they are present in dietary fruits and vegetables and exercise their antioxidant activity in several ways, including the activities of metal chelation. Studies indicate that flavonoids are excellent radical-scavengers of the hydroxyl radical due to their t ability to inhibit the hydroxyl radical and donate hydrogen atom. Oranges as excellent source of vitamin C, contain powerful natural antioxidant, folate, dietary fibre and other bioactive components, like carotenoids and flavonoids that prevent cancer and degenerative diseases. Consumption of foods rich in vitamin C improves body immunity against infectious agents and scavenging harmful, pro-inflammatory free radicals from the blood. Sweet orange contains a variety of phytochemicals like hesperetin and naringenin. Naringenin has a bioactive effect on human health as antioxidant, free radical scavenger, anti-inflammatory, and immune system modulator [3, 4].

ii). **Anti-Cancer & Anti-Arteriosclerosis:** Citrus flavonoids can prevent cancer through selective cytotoxicity, antiproliferative actions and apoptosis. Flavonoids are antimutagenic, thus protects the DNA from damage by their ability to absorb ultraviolet light. They neutralize free radicals that promote mutations when they are generated near DNA. This has been shown in mice body irradiated with c-ray. Flavonoids can also protect the DNA by interacting directly with the tumoral agents, as in the induced chromosomal aberrations by bleomycin. The inhibitory effect of citrus flavonoids on tumoral development and cell proliferation by rat malignant cells, in cardiac and hepatic tissue of syngenetic rats have been reported. The ability to function as such by citrus flavonoids are based on cell mobility inhibition. Oranges are also rich in iron, chlorine, manganese, zinc, sodium, phosphorous, iodine, calcium, folic acid, potassium, pectin, beta-carotene and amino acids and fibre. A single orange is said to have about 170 phytonutrients and over 60 flavonoids with anti-tumor, anti-inflammatory, blood clot inhibiting and antioxidant properties. All these properties help to promote overall health [22].

iii). **Anti-inflammatory:** Citrus flavonoids contain compounds with anti-inflammatory activity due to the presence of regulatory enzymes (protein kinase C, phosphodiesterase, phospholipase, lipoxygenase, and cyclooxygenase) that control the formation of the biological mediators, responsible for the activation of endothelial cells and specialized cells involved in inflammation. Flavonoid inhibition of the immune and inflammation responses can be associated with their inhibition of these enzymes. Indeed, citrus flavonoids are able to inhibit the kinases and phosphodiesterase essential for cellular signal transduction and activation. They also affect the activation of a number of cells involved in the immune response, including T and B lymphocytes.

Citrus flavonoids also prevent atherosclerosis, inhibiting the formation of atheroma reported that hesperidin obtained from citrus cultures may have a potential therapeutical use as a mild anti-inflammatory agent, being also useful as a precursor of new flavonoids endowed with this activity. Studies using mouse macrophage cells also show that hesperidin has an inhibitory effect on lipopolysaccharide-induced over expression of cyclooxygenase-2, inducible nitric oxide synthase, over-production of prostaglandin E2 and nitric oxide [5].

iv). **Anti-Obesity:** Sweet oranges contain low calories and no saturated fats or cholesterol, but is rich in dietary fibre, pectin which is very effective in persons with obesity. Pectin as bulk laxative protects the mucous membrane from exposure to toxic substances, as well as by binding to cancer causing chemicals in the colon. Pectin has also been shown to reduce blood cholesterol levels by decreasing its re-absorption in the colon by binding to bile acids in the colon. Orange peels contain the alkaloid synephrine, which reduces the production of cholesterol in the liver. The antioxidant elements in oranges combat oxidative stress that oxidizes the LDL (low-density lipoprotein) in the blood [5].

v). **Wholesome Health:** Oranges also contain very good amount of vitamin A, and other flavonoid antioxidants such as alpha and beta carotenes, beta-cryptoxanthin, zeaxanthin and lutein, compounds that have antioxidant properties. Vitamin A is necessary for maintaining healthy mucus membranes, skin and essential for vision. It is also a very good source of B-complex vitamins such as thiamin, pyridoxine and folates. These vitamins are essential in the sense that body requires them from external sources to replenish. Orange fruit also contains a very good amount of minerals like potassium and calcium. Potassium in an important component of cell and body fluids helps control heart rate and blood pressure. Vitamin A also required for maintaining healthy mucus membranes and skin and is also essential for vision. Consumption of natural fruits rich in flavonoids helps body to protect from lung and oral cervical cancers. Orange fruit also contains a very good amount of minerals like potassium and calcium. Potassium is an important component of cell and body fluids and helps to control heart rate and blood pressure. The alkaline properties in the orange stimulate the digestive juices, thus, reliving constipation. Regular intake of orange juice reduces the chances in the formation calcium oxalate which causes kidney stones. Polyphenols present in oranges prevents viral infections. Oranges protect the skin from damage caused by free radicals, thereby

helping you look young and keeps the skin fresh and glowing [5].

Material and Methods

Collection of Plant Material

The leaves of *Citrus Sinensis* were collected from Kolhapur region of Maharashtra.

Preparation of Aqueous Extract of *Annona Squamosa* Leaves: Leaves of *Citrus Sinensis* were shade dried & coarsely powdered by using grinder mixer. Soxhlet extraction was used to make methanolic extracts of *Citrus Sinensis*. Powdered drug was charged into Soxhlet apparatus & extraction was carried out with different solvents successively [23]. The extract was then preserved in the desiccator & then used for phytochemical & pharmacological studies.

Evaluation of *In vitro* Antiurolithiatic activity of Methanolic Extract of *Citrus Sinensis* (MECS) leaves by Crystal Aggregation Assay: The crystal aggregation test was used to assess the rate of aggregation of CaOx crystals. The COM crystals were made by combining 50 mmol/L calcium chloride & sodium oxalate solutions. In a water bath, the solutions were equilibrated to 60° C, then cooled to 37° C & preserved overnight. The solution was then centrifuged & evaporated at 37°c. CaOx crystals were used at a final concentration of 0.8 mg/mL in a pH 6.5 buffered solution containing 0.05 mol/L Tris-HCl & 0.15 mol/L sodium chloride. The experiment was carried out at 37°C with & without plant extract at concentrations of 100, 200, 400, 600, 800, & 1000 µg/mL.

For every 10-minute time interval, the absorbance was measured at 620 nm for one hour. All samples were tested three times. As a positive control, Cystone was used. The following formula was used to estimate the percentage inhibition of aggregation rate by comparing the turbidity slope of different concentrations of Cystone /MECS to the turbidity slope of the control.

$$[1-(Tsi/ Tsc)] \times 100$$

Where Tsi was the turbidity slope of aggregation in the presence of inhibitor sample i.e., Cystone/ MECS & Tsc was the turbidity slope of aggregation in the absence of inhibitor [24].

Estimation of Total Flavonoid Content

The aluminium chloride colorimetric test was used to determine total flavonoid concentration. In a 10 ml volumetric flask, the reaction mixture comprises 1 ml of extract & 4 ml of distilled water. 0.30 mL sodium nitrite (5%) was added, followed by 0.3 mL aluminium chloride (10%) after 5 minutes. 2 ml of 1M sodium hydroxide was treated & diluted to 10 ml with distilled water after 5 minutes. A similar set of quercetin reference standard solutions (200, 400, 600, 800, & 1000µg/ml) were prepared. An UV-Visible spectrophotometer was used to measure the absorbance of the test & standard solutions against the reagent blank at 510 nm. The total flavonoid content of the extract was measured in mg of quercetin equivalents (QE) per g of extract. The absorbance of the test sample was measured three times [25].

Results

Phytochemical Analysis of Methanolic Extract of *Citrus Sinensis* (MECS) Leaves: The preliminary phytochemical screening of the Methanolic Extract of *Citrus Sinensis* (MECS) revealed the presence of carbohydrates, saponins, tannins, anthraquinone glycosides & flavonoids.

Table 1: Solvents, extraction methods & respective yield from *Citrus sinesis*.

Sr. No.	Extract	Solvents	Colour	Nature	% Yield
1	Successive	Pet. ether	Greenish brown	Semisolid	11.0
2		Chloroform	Greenish black	Semisolid	7.8
3		Ethanol	Brown	Solid	6.3
1	Individual	Aqueous	Dark brown	Solid	14.2
2		Methanolic	Dark brown	Semisolid	12.4

In vitro Antiurolithiatic Activity of *Citrus Sinensis* leaves

Table 2: Antiurolithiatic activity of Cystone by Crystal Aggregation assay.

Cystone (5mg/ml)	Absorbance	% Inhibition
Control	0.22	-
5min	0.10	54.54
10min	0.07	67.74
15 min	0.07	76.66
20min	0.07	76.66
25 min	0.09	76.00

Table 3: Antiurolithiatic activity of Methanolic Extract of *Citrus Sinensis* (MECS) by Crystal Aggregation assay.

MECS (10mg/ml)	Absorbance	% Inhibition
Control	0.22	-
5min	0.18	18.18
10min	0.17	22.72
15 min	0.15	31.81
20min	0.14	36.36
25 min	0.13	40.90

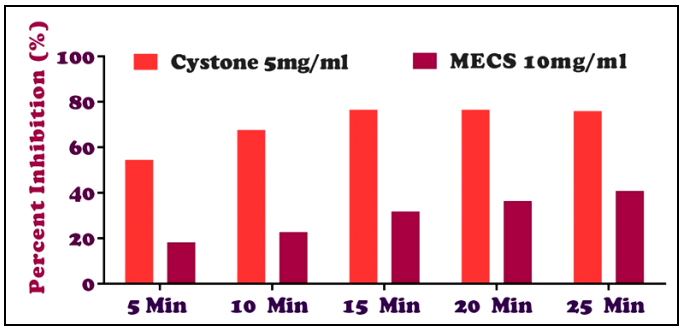

Fig 1: Antiurolithiatic activity of Methanolic Extract of *Citrus Sinensis* (MECS) by Crystal Aggregation assay.

Table 4: Absorbance of Quercetin as a reference standard.

Sr. no.	Quercetin (µg/ml)	Absorbance
1	200	0.086
2	400	0.232
3	600	0.358
4	800	0.450
5	1000	0.596

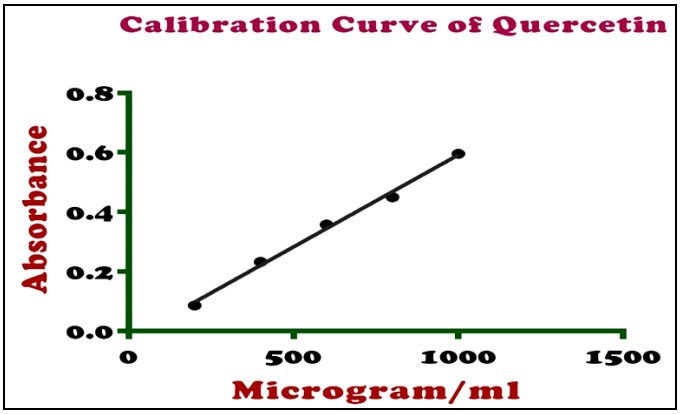

Fig 2: Quercetin Standard Calibration Curve.

Table 5: Total Flavonoid content of different extract of *Citrus Sinensis*.

Sr. No.	Samples at Conc (1000 µg/ml)	Absorbance	Total flavonoids content (mg QE/gm)
1	Aqueous Ext Citrus Sinensis (AECS)	0.39	673.66
2	Methanolic Ext Citrus Sinensis (MECS)	0.23	415.18

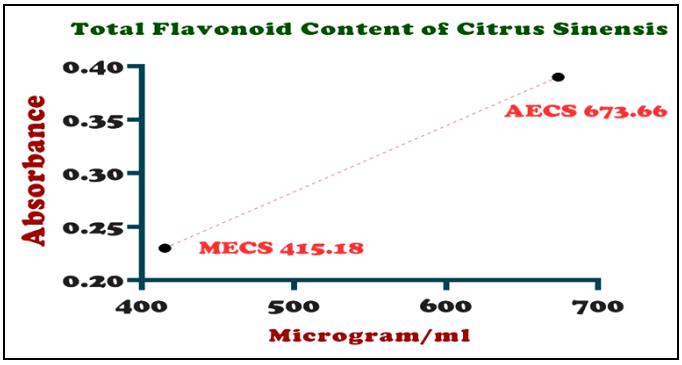

Fig 3: Total Flavonoid content of different extract of *Citrus Sinensis*.

Discussion

One of the most important processes in the pathophysiology of kidney stones is crystal aggregation. However, previous studies of crystal aggregation were rarely done and quantitative analysis of aggregation degree was restricted by a lack of the standard measurement. In order to create aggregation of calcium oxalate monohydrate (COM) crystals, we used an *in vitro* test. There are two forms of CaOx stones: monohydrate (COM) & dihydrate (COD). COM, the thermodynamically more stable form, is more frequent in clinical stones than COD, and has a stronger affinity for renal tubular cells, causing kidney stones to develop. CaOx crystallization process begins with an increase in urine supersaturation, followed by the development of solid crystalline particles in the urinary tract. This is followed by nucleation, by which stone-forming salts in supersaturated urinary solution combine into clusters, that then increase in size by the addition of new constituents. These crystals then develop & combine with other crystals in solution, finally accumulating in the kidney [12]. Renal damage encourages crystal retention & the formation of a stone nidus on the papillary surface of the kidney, as well as supporting crystal nucleation at lower supersaturation levels. Therefore, urinary supersaturation levels are related to the type of stone that forms, & lowering supersaturation can prevent possible stone recurrence. As a result, if the growth of crystallisation can be stopped, lithiasis can be avoided as well [24]. Cystone (5 mg/ml) & MECS (10 mg/ml) considerably reduced crystal aggregation in the current study. This effect might be attributed to the several phytoconstituents (alkaloid, flavonoid, saponin, etc.) found in MECS, which reduce urine supersaturation & thus kidney stone formation [7].

The presence of flavonoid in various extracts of *Citrus Sinensis* was validated in the current study using a total flavonoid content assay. Total flavonoids content measurement is a crucial step in evaluating bioactive substances & functional characteristics. Recent research has found that plant flavonoids can successfully prevent the development of CaOx stones *in vitro* & *in vivo*, which is linked to their diuretic, antioxidant, anti-inflammatory, antibacterial, & other protective effects [26, 27]. As a result, the presence of a wide variety of flavonoids & other phytoconstituents in *Citrus Sinensis* leaves may be responsible for their anti-urolithiatic effects.

References

1. Basavaraj DR, Biyani CS, Browning, AJ, Cartledge JJ. The role of urinary kidney stone inhibitors and promoters in the pathogenesis of calcium containing renal stones. European Association of Urology. 2007; 5:126-136.
2. Doddola S, Ranganayakulu D, Koganti B, Koganti VS, Prasad RG. Effect of Ethanolic extract of *Phyla nodiflora L. greene* against calculi producing diet induced urolithiasis. International Journal of Natural pproduct & resources. 2010; 1(3):314-321.
3. Tripoli EM, Giammanco D. Citrus flavonoids: molecular structure, biological activity and nutritional properties: A review. Food Chemistry. 2007; 104: 466-479.
4. Bombardelli E, Morazzoni P. The flavonoids: New perspectives in biological activities and therapeutics. Chimica Oggi journal, 1993, 25-28.
5. Sakata K, Hirose Y, Qiao, Z, Tanaka T, Mori H. Inhibition of inducible isoforms of cyclooxygenase and nitric oxide synthase by flavonoid hesperidin in mouse macrophage cell line. Cancer Letters, 2003.,199:139-145.
6. Kurian JC. Plant that heals. 1st ed., Oriental Watchman Publishing House, Pune, 2004, 299-310.
7. Touhami M, Laroubi A, Elhabazi K, Loubna, F, Zrara I, Eljahiri Y, Oussama A, Grases F, Chait A. Lemon juice has protective activity in a rat urolithiasis model. BMC Urology. 2007; 7:1-9.
8. Selvam R, Kalaiselve P, Govindaraj A, Murugan VB, Sathish Kumar A.S. Effect of *Aerva lanata* leaf extract and *Vediuppu chunnam* on the risk factors of calcium oxalate urolithiasis during experimental hyperoxaluria. Pcology Research. 2001; 43(1):89-93.
9. Dandia SD, Kalra VB, Pendse AK. The preventive action of cystone in oxamide induced urolithiasis and histochemical changes in the urinary tract: An experimental study in rats. The Indian Practitioner, 1975, 127-130.
10. Mitra SK, Gopumadhavan S, Venkataranganna MV, Sundaram R. Effect of Cystone: A polyherbal formulation on glycolic acid induced urolithiasis in rats. Phyto Research, 1998; 12:372-374.
11. Farooq SM, Asokan D, Kalaiselvi P, Varalakshmi P. Prophylactic role of phycocyanin: a study of oxalate mediated renal cell injury. Chem Bio Int, 2004; 149:1-7.
12. Solis RV, Perez-Gutierrez RM. Diuretic and urolithiatic activities of the aqueous extract of the fruit of *Randia*

echinocarpa on rats. *Journal of Ethnopharmacology*, 2002; 83:145-147.
13. Bashir S, Gilani AH. Antiurolithiatic effect of *Berenia ligulata* rhizomes: an explation of the underlying mechanisms. Journal of Ethnopharmacology, 2009; 122:106-116.
14. Moe OW. Kidney stone: pathophysiology & medical management. Lancet. 2006; 367:333-344.
15. Karadi RV, Gadge NB, Alagawadi KR, Savadi RV. Effect of *Moringa oleifera* Lam. Root wood on ethylene glycol induced urolithiasis in rats. Journal of Ethnopharmacology, 2006; 105:306-311.
16. Tostes V, Martinusso CA, Werneck CC, Mourao PA, Cadoso LR. Low molecular weight dextran sulfate prevents experimental urolithiasis in rats. Clinica Chimica Acta. 2004; 341:147-155.
17. Vidya L, Varalakshmi P. Control of urinary risk factors of stones by betulin & lupeol in experimental hyperoxaluria. Fitoterapia, 2000; 71:535-543.
18. Bahuguna Y, Rawat MS, Juyal V, Gupta V. Antiurolithiatic effect of flowers of *Jasminum auriculatum* vahl. International Journal of Green Pharmacy, 2009, 155-158.
19. Grases F, Ramis M, March JG. Effect of *Herniaria hisuta* & *Agropyron repens* on calcium oxalate urolithiasis risk in rats. *Journal of Ethnopharmacology.* 1995; 45:211-214.
20. Tsai CH, Chen YC, Chen LD, Pan TC. A traditional Chinese herbal antilithic formula, Wulingsan, effectively prevents the renal deposition of calcium oxalate crystal in ethylene glycol fed rats. Urology Research, 2008; 36:17-24.
21. Bracke ME, Vyncke B, Larebeke NA, Bruyneel EA. The flavonoid tangeretin inhibits invasion of MO4 mouse cells into embryonic chick heart *in vitro*. Clinical & Experimental Metastasis, 1989; 7:283-300.
22. Stapleton AE, Walbot V. Flavonoids can protect maize DNA from the induction of ultraviolet radiation damage. Plant Physiology, 1994; 105:881-889.
23. Azwanida N. N. A Review on the Extraction Methods Use in Medicinal Plants, Principle, Strength & Limitation. *Journal of Medicinal & Aromatic Plants,* 2015; 4:3-10.
24. Saha S., Verma R. Inhibition of calcium oxalate crystallisation *in vitro* by an extract of Bergenia ciliate. Arab Journal of Urology, 2013; 11:187-192.
25. Muchandi AA, Dhawale SC. Estimation of Total Phenolic Contents, Total Flavonoid Contents & Muscle Co-Ordination Activity of Ethanolic Extract of Stereospermum Suaveolens Dc. *International Journal of Research in Pharmaceutical & Nano Sciences*, 2017; 6(3):118 -124.
26. Vargas F., Romecín P. Flavonoids in Kidney Health & Disease. *Journal of Frontiers in Physiology*, 2018; 9:1-12.
27. Vargas SR, Perez GRM. Perez GS. Antiurolithiatic activity of Raphanus sativus aqueous extract on rats. *Journal of Ethnopharmacology*, 1999; 68:335-338.