Journal of Drug Design and Medicinal Chemistry
2022; 8(3): 34-40
http://www.sciencepublishinggroup.com/j/jddmc
doi: 10.11648/j.jddmc.20220803.11
ISSN: 2472-355X (Print); ISSN: 2472-3576 (Online)

Standardization, Optimization of Formulation and In-vitro Evaluation of Anti-urolithiasis Activity of *Citrus medica* Extract

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To cite this article:
Choudhary Dimple, Naik Archana. Standardization, Optimization of Formulation and In-vitro Evaluation of Anti-urolithiasis Activity of *Citrus medica* Extract. *Journal of Drug Design and Medicinal Chemistry*. Vol. 8, No. 3, 2022, pp. 34-40. doi: 10.11648/j.jddmc.20220803.11

Received: May 29, 2022; **Accepted**: June 22, 2022; **Published**: July 28, 2022

Abstract: *Citrus medica* is very popular in traditional system of medicine specially in China and India. The fruit possesses various medicinal activities. Many studies have been conducted on *Citrus medica* to authenticate its use as multipurpose medicinal agent. Aim of current study was to standardize the fruit of *Citrus medica* followed by evaluation of anti-urolithiasis activity and preparation of formulation. Flavonoid rich extract of *Citrus medica*was prepared by maceration followed by liquid liquid extraction. Qualitative analysis was carried by TLC. Extract was then subjected to spray drying process to get free following powder for formulation. Standardization of flavonoid rich extract of fruit of CM was carried out by HPTLC method using toluene: ethyl acetate: formic acid (6:4:0.1 v/v) asmobile phaseand Silica gel GF 254nm as stationary phase with biomarker i.e. naringenin. Detection was carried out at 281 nm. Total flavonoid content was estimated by UV spectroscopy by considering quercetin as standard. Anti-urolithiasis activity was evaluated by *in-vitro* model using cystone as standard agent used for kidney stone. Extract showed potent activity as compared to standard. Spray dried powder of flavonoid rich extract was then incorporated in tablet formulation. Evaluation of tablet was carried out as per guidelines and it passes as per the specifications. Hence *Citrus medica* fruits which are traditionally popular is proved as potent agent as well as standardization give assurity about quality of the herbal formulation and can be used as potent anti-urolithiatic agent in ayurvedic era.

Keywords: Urolithiasis, *Citrus medica*, Citron, Bajura, Bijapura, Kidney Stone, Standardization

1. Introduction

Citrus medica L. (Brain citron), locally known as Otroj in Saudi Arabia, is a fruit with pleasant fragrance. It is an important member in the genus *citrus*, belonging to the Rutaceae family. There are about 16 species and many horticulture varieties of citrus found in Arabian Peninsula [1, 2]. Almost all species and varieties of citrus are known to possess therapeutic benefits and used in traditional medicine of many countries [3-5]. Various parts of citrus plant not only used for medicinal purposes but for their chemical constituents are utilized in cosmetic, perfumery and in beverage industries [6, 7]. Various parts of the otroj plant including fruit, leaves, twigs, flowers, seeds are useful therapeutically [8, 9]. *Citrus medica* (CM) seed extract is proved as antidiabetic [9] and hypolipidemic activity in diabetic rats [5, 9]. The root of this plant is also reported to have anticancer activity [10, 11]. Some studies have revealed that flavonoids obtained from citrus, has suppressing effect on blood cholesterol and triglycerides [8, 12]. In somein-vitro assays *Citrus medica* peel extract has shown to possess antioxidant activity and reported to contain limonene and gamma-terpinene among others [6, 12]. The peel of *Citrus medica* being used in Indian, Arab and Chinese traditional medicine for the treatment of inflammation, to control increase urinary output and to treat kidney stones [13].

Many synthetic drugs used like diuretics and narcotic analgesics are being used in treatment of kidney stone but overuse of synthetic drugs, which produce higher incidence of adverse drug effect, have forced humans to return to nature for safe remedies herbal treatment [14, 15]. Also recurrence problem associated to kidney stone causes the prolongation in treatment and increases the duration of treatment, which lead to increase in cost as well as risk of side effects related to synthetic drug. Hence

herbal remedies may be very good option in such case where prophylactic or long-term therapy is required [16, 17].

Evidences prove that natural therapy is more valuable than other available treatments, with lesser side effects, economic nature, no risk of long-term fertility and reoccurrence [14-17].

Since the plant being reported to have antioxidant and increase urine output which are complementary to kidney functions, it was thought worthwhile to study the utility of the fruit peel of *Citrus medica* in treatment of kidney stone and would prove to be beneficial in offering protection against the kidney stone [17, 18]. Standardizing the extractwill help to improve quality of traditional medicine and deciding the dose regimen for treatment.

2. Materials and Methods

2.1. Plant Material

A fresh fruit of *Citrus medica* were procured from local market of Gujrat and Rajasthan and were authenticated by Dr. Harshad M Pandit (Formerly Head and Associates Professor of Botany). The fruit was identified as (specimen #: dnc p 87191526) the hesperidium of *Citrus medica* Linn. Of family Rutaceae.

2.2. Preparation and Standardization of Test Drug

The whole unripe fruits of *Citrus medica* Linn. was cut into small pieces and blended into fruit juicerand was subjected for various test as follows.

2.3. Pharmacognostical Evaluation

The dried powder of fresh fruit of *Citrus medica* was analysed for its pharmacognostic parameters including microscopic, macroscopic, histochemical analysis physicochemical evaluation, phytochemical screening, chromatographic study etc [5, 6].

2.4. Extraction and Isolation of Flavonoid

Approximate 100g offresh fruit peel of *Citrus medica* was cut into the small pieces and were blended into juicer. It was then immersed into 250 ml of 60% methanol and heated on electric water bath for around 4 hr with occasionally stirring at 60°C. the extract was cooled and filtered. This extract was then concentrated using electric water bath at 90°C. methanolic extract was then transferred to 250ml separating funnel with 20 ml diethyl ether and was shaken for 15 mints. Diethyl ether layer was discarded and aqueous layer was retained in separating funnel. Flavonoids were then isolated from methanolic extract by liquid-liquid extraction by using n-butanol as solvent. This flavonoid rich extract was then collected and concentrated. The presence of flavonoid was confirmed by Shinoda test and TLC study [16-18].

2.5. Standardization of Extract

Standardization of flavonoid rich extract of fruit of *Citrus medica* was carried out using naringenin as biomarker. Naringenin was isolated from flavonoid rich extract by column chromatography by using silica gel (#60-120) as stationary phase and toluene: ethyl acetate: formic acid-6:4:01 v/v as mobile phase at 2.5ml/min flow rate. Eluent or fractions were analysed by TLC and fractions yielding identical Rf value of 0.75 were pooled out and were concentrated to dryness. These fractions were confirmed for presence of naringenin chemically by Shinoda test and also spectroscopically by UV spectroscopy. Characterization was carried out by FT-IR, NMR and HR-LCMS methods [19, 20]. Quantitative estimation of naringenin was performed by HPTLC study.

2.6. In–vitro Evaluation of Anti-urolithiatic Activity

Extract was evaluated for anti-urolithiasis activity by *in-vitro* study. Study was performed by using semipermeable membrane which was achieved by decalcification of egg. Calcium oxalate and calcium phosphate crystals were prepared by chemical reaction.

Study was carried out in two sets (for calcium oxalate calcium phosphate) each containing four groups. The groups were as follows:

Group I: Negative Control (10 mg Calcium oxalate / Calcium phosphate).

Group II: 10 mg Calcium oxalate / Calcium phosphate + 100 mg standard (Cystone).

Group III: 10 mg Calcium oxalate / Calcium phosphate + 100 mg of butanolic extract.

Group IV: 10 mg Calcium oxalate / Calcium phosphate + 200 mg of butanolic extract.

Calcium oxalate/ calcium phosphate crystals, cystone and butanolic extract as mentioned above were weighed accurately and were packed in semi permeable membrane by suturing. This was allowed to suspend in conical flask containing 100ml 0.1 M of tris buffer. The conical flask of all groupswas placed in incubator, pre heated to 37°C for 2 hours, for about 7-8 hours. The content of each semi permeable membrane was then taken in separate test tube. % Dissolution of calcium oxalate and calcium phosphate was then obtained by titrimetric method and optical density method respectively. Evaluation was carried out using method given by JineetkumarGawad [21].

Figure 1. *Decalcification of egg.*

Figure 2. *In-vitro experimental model set-up to evaluate antiurolithiatic activity.*

2.7. Spray Drying

The flavonoid rich extract obtained was very sticky for preparing tablets hence the extract was subjected for spray drying processto obtain extract in free-flowing powder form. While spray drying process the inlet temperature was set to 100°C whereas the out let temperature was set to 65°C. Feed flow rate was 25ml/mins and blower speed was set to 45rpm.

Figure 3. *Spray drying of extract.*

2.8. Optimization of Formulation and Evaluation

Optimization of formulation was required to make the crude extract more palatable and to achieve dose accuracy. Tablets were prepared by wet granulation method by using starch grain as binder and lactose as diluent. After formulation the tablets were evaluated for various parameters such as colour, shape, diameter, thickness, surface texture, hardness, friability, weight variation, content uniformity and disintegration time as per standard guidelines.

3. Result and Discussion

3.1. Pharmacognostic Evaluation

Citrus medica fruit were studied for its pharmacognostical features and physicochemical parameters [5, 6]. The results obtained for physicochemical parameters are depicted in table 1.

Table 1. *Physicochemical parameters of powder of Citrus medica fruit.*

S. No.	Physicochemical Parameter	Powder of *Citrus medica* fruit (Fresh fruit)
1	Moisture content of fresh peel	62.14 ±0.745 %W/W
2	Moisture content of dried peel	15.14±0.567 %W/W
3	Total Ash value	1.244 ± 0.236 %W/W
4	Water soluble ash value	0.336 ±0.156 % W/W
5	Acid insoluble ash value	0.09±0.015 %W/W

Also the xtract from various solvents were subjected for phytochemical analysis out of which methanolic, ethyl acetate and butanolic extract was found to contain flavonoid which was confirmed by shinoda test.

3.2. Extraction and Isolation of Flavonoid

Reproducibility of the extract was maintained by standardizing the extract with reference to parameter like total flavonoid content and naringenin concentration each time. The calculated yield of the flavonoid rich extract was 50mg/1kg of the fruit. To the aliquots (2ml) of extract of fraction magnesium turnings was added and 1 ml of conc. HCl were added. The change in the colour of the sample from reddish brown to cherry red colour indicated the presence of flavonoid. The presence of flavonoid was also confirmed by thin layer chromatography using mobile phase Toluene: Ethyl acetate: Formic acid (6:4:0.1v/v) which showed a spot at Rf0.75. Naringenin (used as biomarker) was isolated by column chromatography for standardization of the extract. Qualitative analysis of the naringenin was carried HR-LCMS, H-1 NMR [19], C-13 NMR [20] and FTIR and results were confirmed with standard database.

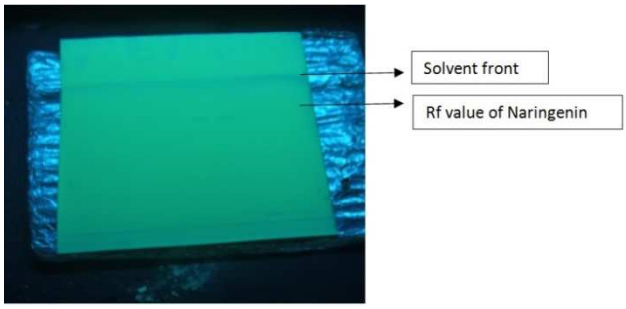

Figure 4. *Developed HPTLC plate for standardization of extract.*

3.3. Standardization of the Extract

The flavonoid rich extract from *Citrus medica* was standardized for the content of naringenin by HPTLC methods. The extract was chromatographed on HPTLC silica gel GF-254 plates using CAMAG TLC Scanner 3 using toluene: ethyl acetate: formic acid- 6: 4: 0.8 v/v as mobile phase and detection was carried out at 281nm (figure 4). Standardization of flavonoid rich extract was carried out using naringenin as biomarker. The LRA equation ofnaringenin was found to be Y=4597.7x - 516.12, and R^2 value was found to be 0.9993 (figures 5, 6 and 7). The content naringenin was found to be 2 % w/w (table 2).

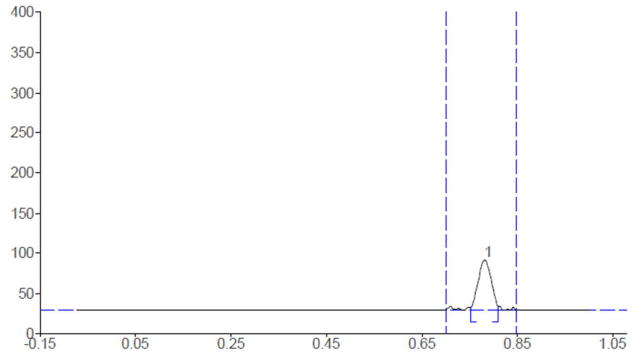

Figure 5. *Chromatogram of standard naringenin- 0.4ppm.*

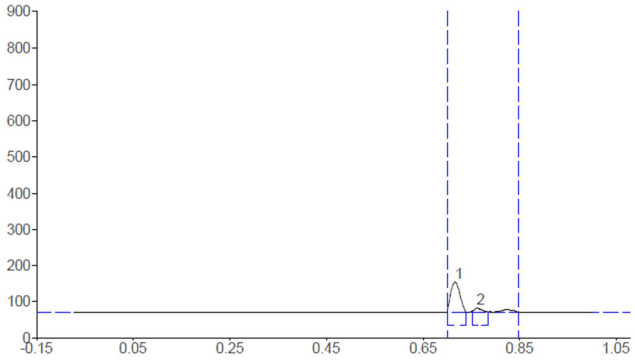

Figure 6. *Chromatogram of Butanolic extract.*

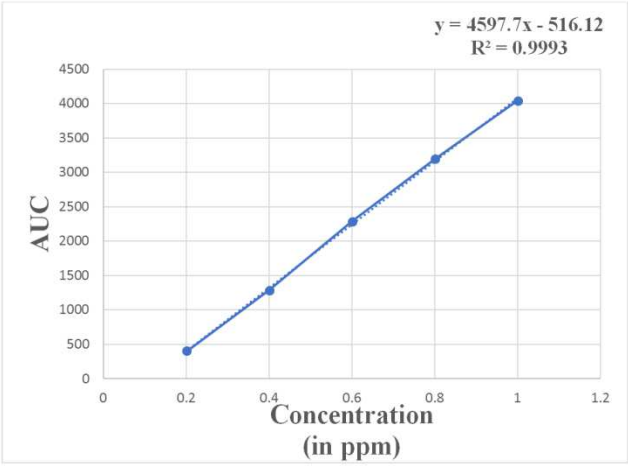

Figure 7. *Calibration curve of standard Naringenin.*

Table 2. *Determination of naringenin content from butanolic extract by HPTLC method.*

Track No.	Concentration in ppm	R_f value	Height	Area
	0.2	0.80	21.5	393.7
	0.4	0.78	61.6	1291
	0.6	0.78	112.7	2288.5
	0.8	0.77	158.5	3192.8
	1	0.76	199.6	4043.5
	Butanolic extract	0.72	84	1219.2
		0.76	12.9	501

3.4. In –vitro Evaluation of Anti-urolithiatic Activity

The aim of current studies was to evaluate the effect of flavonoid rich extract of *Citrus medica* with known anti-urolithiatic activity by *in-vitro* model [21, 22]. During study it was observed that group 4 (200mg extract) showed maximumi.e. 66.33% and 42.8%dissolutionof calcium oxalateand calcium phosphate crystals respectively followed by group 2 (100mg or standard drug) i.e. 64.73% and 42.6% dissolution. Group 3 i.e. 100mg of flavonoid rich extract showed 58.32% and 31.5% dissolution of calcium oxalate and calcium phosphate crystals respectively as shown in tables 3 and 4, figures 8 and 9.

Table 3. *Percent of Calcium oxalate dissolution by various groups under study.*

Groups as per Experimental Design	Vol. of Standard $KMnO_4$	Wt. of Calcium Estimated (mg)	Wt. of Calcium Reduced (mg)	% Dissolution
1	3.5	5.6105	4.3895	43.895
2	2.2	3.5266	6.4734	64.734
3	2.1	4.1678	5.8322	58.322
4	2.6	3.3663	6.6337	66.337

Table 4. *Dissolution of Calcium phosphate by various groups under study.*

Groups as per Experimental Design	Optical Density	Wt. of Calcium Reduced (mg)	% Dissolution
1	0.75	1.58	15.8
2	0.48	4.62	42.6
3	0.61	3.15	31.5
4	0.51	4.28	42.8

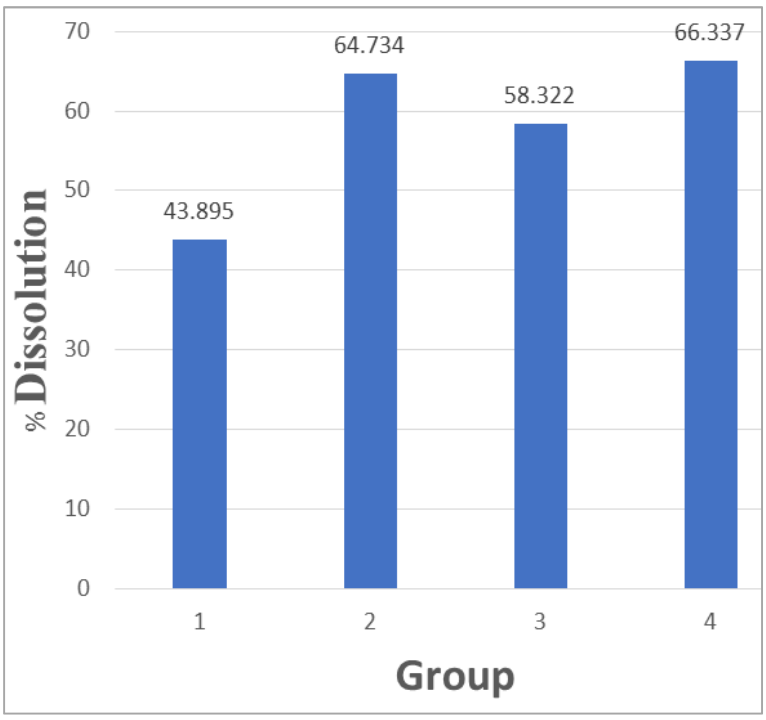

Figure 8. *Percentage dissolution of Calcium oxalate by various groups under study.*

Group -1 10mg calcium oxalate;
Group -2 10 mg calcium oxala64.73te + 100 mg Cystone;
Group -3 10 mg calcium oxalate + 100mg butanolic extract;
Group -4 10 mg calcium oxalate + 200 mg butanolic extract.

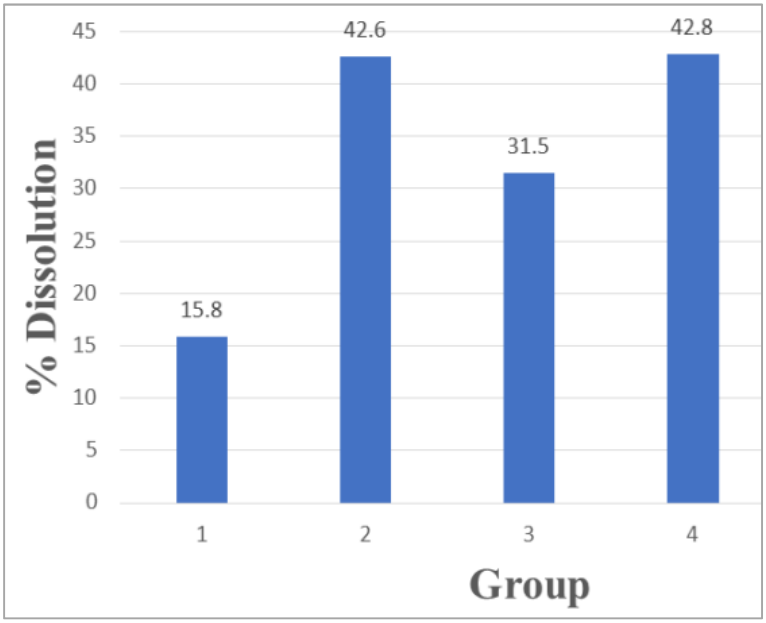

Figure 9. *Percentage dissolution of Calcium phosphate by various groups under study.*

Group -1 10mg calcium phosphate;
Group -2 10 mg calcium phosphate + 100 mg cystone;
Group -3 10 mg calcium phosphate + 100mg butanolic extract;
Group -4 10 mg calcium phosphate + 200 mg butanolic extract.

When compared to standard herbal formulation i.e., cystone. The efficacy of the 200mg extract was found almost equivalent to that of the 100 mg of the cystone. On the basis of the above results, it can be concluded that the extract has a potent anti-urolithiasis activity and hence the use of the *Citrus medica*can be an evolutionary step in the herbal

approach to treat the kidney stone in future.

3.5. Optimization of Formulation and Evaluation

Butanolic extract was very sticky and tablets prepared from the extract was showing chipping problem due to difficulty in ejection from punch while punching. Hence theextract was subjected for spray drying process to obtain extract in free-flowing powder form. The powder obtained after spray drying was less hydroscopic and free flowing which helped to overcome chipping problem as well as improved the stability of the tablets.

Tablets were prepared by wet granulation method was evaluated for various parameters and results obtained are recorded in tables 5 and 6.

Table 5. *Results of non-official tests.*

Sr. no.	Test	Specification	Result obtained	Inference
1	Colour	Brown	Brown	Passes
2	Shape	Standard convex	Standard convex	Passes
3	Diameter	12 mm	12.1mm	Passes
4	Thickness	-	3.2mm	-
5	Surface texture	Smooth and shiny	Smooth and shiny	Passes
6	Hardness	5-8 kg/cm^2	4.5 kg/cm^2	Passes
7	Friability	0.5 to 1 %	0.1343%	Passes

Table 6. *Results of official tests.*

Sr. no.	Test	Specification	Result obtained	Inference
1	Weight variation	649.2308-650.7692	No tablet deviate from this limit	Passes
	Content uniformity			-
2	Total flavonoid content	-	2.03 mg quercetin/1g dried extract	-
	Naringenin content	-	9.5mg/ tablet	-
3	Disintegration time	More than 15 mints	18 mints	Passes

All parameters were evaluated as per the standard guidelines of USP and promising results were obtained for each test.

4. Conclusion

Many synthetic drugs used like diuretics and narcotic analgesics are being used in treatment of kidney stone but overuse of synthetic drugs, which produce higher incidence of adverse drug effect, have forced humans to return to nature for safe remedies herbal treatment. Evidences prove that natural therapy is more valuable than other available treatments, with lesser side effects, economic nature, no risk of long-term fertility and reoccurrence. Since the plant being reported to have antioxidant and increase urine output which are complementary to kidney functions, it was thought worthwhile to study the utility of the fruit peel of Citrus medica in treatment of kidney stone and would prove to be beneficial in offering protection against the kidney stone.

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