EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 6
Issue: 4
327-337
Year: 2019

EVALUATION OF *IN VITRO* ANTIOXIDANT AND ANTI-UROLITHIATIC POTENTIAL OF VARIOUS FRACTIONS OF *CLITORIA TERNATEA* L. BLUE FLOWERED LEAVES

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Article Received on 10/02/2019 **Article Revised on 02/03/2019** **Article Accepted on 24/03/2019**

ABSTRACT
The present study comprises preparation of hot crude plant extract from *Clitoria ternatea* L. blue flowered leaves and successive fractionation of the filtered extract using various solvents. All the fractions used for preliminary phytochemical tests as well as for quantification some important secondary metabolites. In addition, *in vitro* antioxidant and anti-urolithiatic potentials of all the fractions were evaluated. During qualitative phytochemical analysis, all the fractions displayed all the phytocompounds tested such as alkaloids, flavonoids, phenols, tannins, sterols, saponins and terpenoids. All the fractions contained an adequate level of quantitatively determined secondary metabolites such as total phenols, flavonoids, terpenoids and tannins. All the fractions showed varied level of antioxidant activities assayed. Interestingly, among various fractions, n-butanol fraction only exhibited significant inhibition of nucleation, aggregation and growth of CaOx crystals. This may be due to significant free radical scavenging activities of this fraction for total antioxidant capacity, $ABTS^{\bullet+}$, nitric oxide and DPPH which would further strengthen its use to ameliorate urolithiasis induced oxidative stress.

KEYWORDS: *Clitoria ternatea*, Blue flowered variety, Phytochemical analysis, *In vitro* antioxidant activity, Anti-urolithiatic potential.

1. INTRODUCTION
Since prehistoric times, people have exploring the nature, particularly, medicinal plants in search of new drugs. These efficacy depends upon the current knowledge about taxonomic attribute of plant species, plant parts and property of medicinal plants which in turn depends upon the prevalence of primary and secondary metabolites.[1] Primary metabolites are essentially required for growth and development of plants, but secondary metabolites synthesized during secondary metabolism of plants are not involved directly in growth and act as biocatalysts and also as potential sources of many phytobased drugs. The most important secondary metabolites are saponins, alkaloids, tannins, flavonoids and cardiac glycosides.[2] Qualitative phytochemical screening will help to understand a variety of chemical compounds produced by plants and quantification of those metabolites will help to extract, purify and identify the bioactive compounds.[3]

Oxidative stress acts a major role in the development of chronic and degenerative ailments such as arthritis, aging, autoimmune disorders, neurodegenerative and cardiovascular disorders and cancer Oxidative stress acts a major role in the development of chronic and degenerative ailments such as arthritis, aging, autoimmune disorders, neurodegenerative and cardiovascular disorders and cancer.[4] Although cells are equipped with an remarkable stock of antioxidant enzymes as well as small antioxidant molecules, these agents may not be sufficient to normalize the redox status under oxidative stress caused due to adverse physicochemical, environmental or pathological conditions, when either the generation of free radicals is enhanced or their scavenging ability is inhibited. Under these circumstances, supplementation with exogenous antioxidants is necessary to restore the redox homeostasis in cells.[5] Many synthetic compounds like butylated hydroxytoluene (BHT) and butylated hydroxy anisole (BHA) are commercially obtainable as antioxidants. However, they are not suggested for usage due to the toxicity associated with them.[6] Therefore, many research groups have driven efforts to evaluate the antioxidant properties of natural products. These properties have been investigated through either chemical (*in vitro*) or biological (*in vivo*) methods or both.[7] The results of these researches suggest that the long-term consumption of food which are rich in antioxidants can retard or avoid the occurrence of chronic diseases.[8,9] Despite the large number of natural products that are currently consumed as antioxidant agents, the search for new chemical entities with antioxidant property still remains a growing field.

Urolithiasis is the presence of calculi or stones in the kidney or in any part of the urinary tract, including the ureters and bladder. Nearly about 80% of these calculi are comprised of calcium oxalate (CaOx, CaC_2O_4) and phosphate.[10] The recurrence rate of urolithiasis without any precaution or preventive treatment is approximately 10% per year.[11] Epidemiological studies indicated that the urolithiasis is more prone to men (12%) than women (6%) and is more common with increasing ages between 20 and 40 in both men and women.[12] Urolithiasis is a multifaceted step which includes crystal nucleation, aggregation and growth of insoluble particles.[13] It is assumed that when the urine becomes saturated with insoluble materials as a result of the extreme rate of excretions which leads to the formation of crystals and aggregates to form a stone.[14] Oxalic acid is biosynthesized from ascorbic acid, glycolate and glyoxylate in the metabolism of higher plants. A significant loss of minerals is more common in the body when it is consumed in large content of oxalate rich foods.[15] When calcium ions present in the body bind with free oxalic acid/oxalate and then they precipitate as insoluble calcium oxalate crystals and may lead to hypocalcaemia and urolithiasis.[16] Generally kidney stones are comprised of high concentration of calcium oxalate with subsequent minute amount of calcium carbonate and calcium phosphate.[17] Calcium oxalate is the predominant component of most stones, followed by struvite, cystine, uric acid and other compounds.[18] CaOx stones are mostly found in two different varieties, CaOx monohydrate (COM) or Whewellite and CaOx dihydrate (COD) or Weddellite. COM, the thermodynamically most stable form, is observed more frequently in clinical stones than COD and has a greater affinity for renal tubular cells, thus responsible for the formation of stones in the kidney.[19]

Several advancements have taken place in the methods for treating urolithiasis, but the costly expenditure and the reoccurrence of stone formation are some of the draw backs of treatment of this disease.[20] Extracorporeal shock wave lithotripsy is commonly used for treatment of urolithiasis. Its multiple sessions in recurrent stone formation may cause chronic deterioration of renal function.[21] Modern medicines cause side effects such as hemorrhage, hypertension, tubular necrosis and subsequently fibrosis of the kidney.[22] So searching of anti-urolithiatic drugs from natural sources could be of great help as drugs from plant origin are cheaper as well as they confer least side effects.

Clitoria ternatea L. belongs to family Fabaceae and it is commonly known as 'Butterfly pea'. It is a perennial twining herb, found throughout India in tropical areas. Traditionally it is recommended for the treatment of snakebite, scorpion sting, chronic bronchitis, indigestion, constipation, fever, arthritis, eye ailments, sore throat, skin diseases, rheumatism, syphilis, eye and ear-diseases in India.[23,24,25,26] Beside this, it is a good source of forage legumes in India.[27] Ethno botanically, it is used in various urinary troubles like infection, burning sensation in urinary tract, lack of urination and frequent urination.[28] Pharmacologically it is reported for its antioxidant, hepatoprotective and antidiabetic activities.[29]

C. ternatea has two varieties, i.e., white flowered and blue flowered. The white-flowered one is found to be therapeutically more active and has been accepted as 'Shankhpushpi' by most of the South Indian vaidyas.[30] Various groups of phytochemicals such as phenolics[31,32], fatty acids[33,34] and lactones[35,36] have been reported from white variety. Blue flowered variety was found to have anthocyanins and delphinidin glucoside.[37] Recently, an attempt was taken to study in detail about various microscopical, physico and phytochemical parameters of these two varieties.[38] *C. ternatea* has been documented for its therapeutic effects in treating urinary complaints and antilithic activity.[39,40] Most of the previous studies of this species in various aspects such as phytochemical, antioxidants and others including antiurolithiatic activity, variety employed (whether blue or white flowered variety) is found lacking. Therefore, in the present investigation, an attempt was taken to study phytochemicals, *in vitro* antioxidant and anti-urolithiatic activities of various solvent fractions obtained from leaves belonging to blue flowered variety of *C. ternatea*.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

2,2-Diphenyl-1-picryl-hydrazyl, 2,2' azinobis (3-ethylbenzothiozoline-6-sulfonic acid) disodium salt, 2,4,6-Tris (2-pyridyl)-s-triazine, butylated hydroxytolune, gallic acid and rutin were purchased from Sigma Aldrich (Bangalore, India). Folin-Ciocalteu reagent was obtained from SD Fine Chemicals Pvt. Ltd, Mumbai. All other drugs and chemicals used in the study were obtained commercially and of analytical grade.

2.2. Plant material

The fresh physiologically mature and disease free leaves of *Clitoria ternatea* L. (Blue flower variety) were collected in Bharathiar university campus, Tamil Nadu, India. The plant material was authenticated by Prof. and Head, Dr.A.Rajendran, Department of Botany, Bharathiar University and voucher specimen (No. 007739) was prepared and deposited in the department for future reference. The leaves were thoroughly washed and cut into small pieces and dried under shade condition for two weeks and powdered using mixer grinder and stored at 4^{o}C.

2.3. Hot crude plant extraction and successive fractionations

For preparation of 100 ml crude hot plant extract, 100 g leaf powder was taken and mixed with 400 ml distilled water (1:4 w/v ratios) and boiled for 20 min. After cooling, the mixture was filtered using Whatman No.1 filter paper. Finally the filtrate was dried and 2.0 g crude

plant extract powder was reconstituted in 100 ml using distilled water. It was then fractionated successively with n-butanol (polar solvent), ethyl acetate, chloroform (mid polar solvents) and hexane (non-polar solvent) using separating funnel by liquid-liquid extraction (LLE) method.

2.4. Determination of extraction yield

All the fractions such as n-butanol, ethyl acetate, chloroform, hexane and residue containing fraction were dried in preweighed petriplate and % extraction yield was calculated using the following formula,

Extraction yield (%)={Weight of the dry extract (g) ÷ weight of the sample used for extraction (g) ÷}×100

2.5. Phytochemical analysis

All the fractions obtained through the technique of LLE were subjected to preliminary qualitative phytochemical tests such as alkaloids, flavonoids, phenols, tannins, sterols, saponins and terpenoids following the method of Harborne.[41] In addition to qualitative analysis, total phenolics, flavonoids, terpenoids and tannins of all the fractions were determined. The total phenolic content and tannins were determined by the method described by Makkar[42] and contents were calculated as gallic acid equivalents (GAE). The flavonoid content was determined using the aluminum chloride method with rutin as a reference.[43] The total terpenoid content was estimated and calculated from calibration curve of linalool and the results were expressed as linolool equivalent (mg/g).[44]

2.6. In vitro antioxidant activities

2.6.1. Ferric reducing antioxidant power (FRAP) assay

The ferric reducing activity of the plant fractions were estimated[45] based on the ability of the antioxidant to reduce ferric to ferrous ions in the presence of 2, 4, 6-Tris (2-pyridyl)-S-triazine (TPTZ), forming an intense blue ferrous–TPTZ complex with absorption maxima at 593 nm. A standard graph for ferrous sulphate in methanol at different concentrations was prepared. FRAP values of the fractions were expressed as µM of Fe (II)/g of extract.

2.6.2. Phosphomolybdenum assay or Total Antioxidant Capacity (TAC)

The antioxidant activities of fractions were evaluated using the green phosphomolybdenum complex formation.[46] The absorbance of the mixture was measured at 695 nm and mean values of the results are expressed as milligrams of ascorbic acid equivalents/g extract.

2.6.3. Free radical scavenging activity on DPPH

The scavenging effect of plant fractions on stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was studied, employing the spectrophotometric method.[47] The radical scavenging activity of the extracts was expressed as IC50 (the concentration of the sample required to inhibit 50% of the DPPH concentration).

2.6.4. Antioxidant activity by the ABTS$^{\bullet}$+ assay

The total antioxidant activity of the fractions was measured using 2,2' azinobis (3-ethylbenzothiozoline-6-sulfonic acid) disodium salt radical (ABTS$^{\bullet}$+) decolorization assay.[48] A trolox calibration curve was constructed by measuring the reduction in absorbance of the ABTS$^{\bullet}$+ solution in the presence of different concentration of trolox (0-2000 µM). Results were expressed as mM trolox equivalent (TE) antioxidant capacity per gram of sample extracts.

2.6.5. Nitric oxide (NO) scavenging assay

The scavenging activity of fraction was estimated by the inhibition of nitric oxide which was generated from the sodium nitroprusside solution that can be estimated using Greiss reagent.[49] The absorbance of the chromophore formed was read at 546 nm and percentage of nitric oxide inhibition was calculated.

2.7. In vitro anti-urolithiatic activities

The effect of the various solvent fractions as well as cystone (standard) on nucleation, aggregation and growth of CaOx crystals were measured as mentioned below.

2.7.1 Preparation of artificial urine

The artificial urine was prepared with the following composition: sodium chloride 105.5 mmol/l, sodium phosphate 32.3 mmol/l, sodium citrate 3.21 mmol/l, magnesium sulfate 3.85 mmol/l, sodium sulfate 16.95 mmol/l, potassium chloride 63.7 mmol/l, calcium chloride 4.5 mmol/l, sodium oxalate 0.32 mmol/l, ammonium hydroxide 17.9 mmol/l, and ammonium chloride 0.0028 mmol/l. The synthetic urine was prepared each day fresh and pH adjusted to 6.0.[50]

2.7.2. Nucleation assay

The inhibitory activity of the plant extracts and standard cystone on nucleation of CaOx crystals was determined based on the spectrophotometric assay of.[51] Crystallization was initiated by adding calcium chloride ($CaCl_2$) and sodium oxalate ($Na_2C_2O_4$) solutions to artificial urine. The calcium chloride solution (4 mmol/l) and sodium oxalate solution (50 mmol/l), respectively, were prepared in a buffer containing tris 0.05 mol/l and NaCl 0.15 mol/l at pH 6.5 at temperature of 37ºC. Various extractions ranging from 200,400,600, 800 and 1000 µg/ml were taken and 1 ml of each concentration was mixed with 3 ml $CaCl_2$ solution followed by the addition of 3 ml $Na_2C_2O_4$ solution. Final mixtures were incubated for 1h, 2h and 3 h at 37ºC. The optical density (OD) of the mixtures was then measured at 620 nm wavelength for three different hours. Percent inhibition of nucleation by each fraction was calculated using the following formula.[52]

% inhibition = [1-(OD test / OD control)] x 100

2.7.3. Aggregation assay

Effect of various solvent fractions on CaOx crystal aggregation was determined by means of aggregation assay. $CaCl_2$ and $Na_2C_2O_4$ solutions (50 mmol/l each) were mixed together, heated to 60ºC in a water bath for 1 h and then incubated overnight at 37ºC to prepare seed CaOx crystals. After drying, CaOx crystal solution (0.8 mg/ml) was prepared in a 0.05 mol/l Tris-HCl and 0.15 mol/l NaCl buffer (pH 6.5). One milliliter of aliquots (200-1000 µg/ml) of each fraction were added to 3 ml CaOx solution, vortexed and then incubated at 37ºC for 3 different hours (1h, 2h and 3h). OD of the final mixtures was then read at 620 nm wavelength and percent inhibition of aggregation was then calculated as described for nucleation assay.[52]

2.7.4. Growth assay

Effect of different solvent fractions on the growth of CaOx crystals was determined by oxalate depletion assay. Varying concentrations of each solvent fraction (200-1000 µg/ml) were prepared in distilled water. CaOx crystal slurry at a concentration of 1.5 mg/ml was prepared in a 50 mM sodium acetate buffer (pH 5.7). 4 mM $CaCl_2$ solution and 4 mM $Na_2C_2O_4$ solution (1 ml each) were added to 1.5 ml Tris-HCl (10 mM) and NaCl (90 mM) buffer (pH 7.4). To this, 30 µl of CaOx crystal slurry was added. The reaction of $CaCl_2$ and $Na_2C_2O_4$ with crystal seed led to deposition of CaOx (CaC_2O_4) on the crystal surfaces, thereby decreasing free oxalate that is detectable by spectrophotometer at λ 214 nm. When solvent fractions at different concentrations (200-1000 µg/ml) for 3 different hours (1h, 2h and 3h) is added into this solution, depletion of free oxalate ions will decrease if the test sample inhibits CaOx crystal growth. Percent inhibition of crystal growth was then calculated as described for nucleation assay.[52] All the three assays were viewed under binocular compound microscope and photographs were captured using ultrascope (Scope image 9.1).

2.8. Statistical analysis

Each assay was done three times using the same fraction in order to determine their reproducibility. The data (means of three replicate determinations ± standard deviation) were subjected to a one-way analysis of variance (ANOVA) and Duncan's new multiple range test to determine significant differences. The P values <0.05 was considered as level of significance.

3. RESULTS

3.1. Extractive yield percentage of fractions

The yield percent of all fractions were calculated and observed that residue containing fraction contained maximum extract recovery i.e 17.1%, whereas hexane fraction had low yield or extract recovery i.e 3.9%.

3.2. Qualitative phytochemical analysis

The results of preliminary qualitative analysis indicated that the presence of all the phytocompounds tested were present in all the fractions.

3.3. Quantitative phytochemical analysis

Results of quantitative analysis are presented in Table 1 which showed that chloroform and final residue containing fractions are rich in phenolics (chloroform fraction contained 239.0 ± 2.0 mg GAE/g extract and final residue containing fraction contained 237.0 ± 2.1 mg GAE/g extract) as compared to all other three fractions. Chloroform fraction showed higher level of flavonoid i.e 802.0 ± 1.6 mg RU/g extract. Fractions of chloroform, hexane and final residue showed almost same level of terpenoids content (174.3 ± 2.0 mg LIN /g extract, 172.6 ± 2.4 mg LIN /g extract and 168.0 ± 1.6 mg LIN /g extract, respectively). Tannin content was found to be higher in hexane fraction compared to other fractions (190.0 ± 3.3 mg TAN /g extract).

3.4. In vitro antioxidant activities

The results of FRAP, phosphomolybdenum reduction activity, $ABTS^{\bullet+}$, NO, DPPH scavenging activities are given in Table 2. The highest FRAP activity was noted for residue containing fraction i.e 24.6 ± 0.42 µM Fe (II) E/mg extract. Other extracts had significantly ($p<0.05$) lower FRAP activity. Compared to other fractions, n-butanol showed significant ($p<0.05$) higher phosphomolybdenum reduction (50.78 ± 4.4 mg AAE/g extract). All fractions displayed almost same level of $ABTS^{\bullet+}$ radial scavenging activity except residue containing fraction which showed very lower level of this activity (0.47± 0.03 mM TE/g extract). In the present study, all the fractions exhibited almost higher level of nitric oxide free radical inhibition. Among the five different concentrations (50-250 µg/ml), concentration of 250 µg/ml showed the highest scavenging activity i.e 170.38% for n-butanol fraction and 200 µg/ml showed the lowest scavenging activity i.e 7.41% for ethyl acetate fraction. Thus, n-butanol fraction exhibited an excellent DPPH radical scavenging activity with IC_{50} values i.e 168.2 µg/ml which indicates higher DPPH scavenging activity. On the other hand, ethyl acetate extract showed very weak antioxidant behavior with an IC50 value i.e 707.4 µg/ml which in turn shows the lower DPPH scavenging activity.

3.5. In vitro anti-urolithiatic activities

Results of the various solvent fractions as well as cystone (standard) on nucleation, aggregation and growth of CaOx crystals were presented in Table 3 and Figures1-3.

3.5.1 Nucleation assay

The *in vitro* inhibitory effect of various solvent fractions of *C. ternatea* during CaOx crystallization was determined by the time course of turbidity measured in synthetic urine at different fraction concentrations of 200, 400, 600, 800 and 1000 µg/ ml. Among the various fractions, n-butanol fraction showed maximum percentage of inhibition (82.8± 1.3) and final residue containing fraction showed minimum percentage of inhibition (57.1± 2.3) for CaOx nucleus formation at 1000 µg/ ml during 3h (Table 3).

Induction of CaOx nucleus formation and its inhibition by n-butanol fraction (1000 µg/ ml) for different time course turbity i.e 3 h are given in the Fig.1 along with standard drug cystone. The light micrographs at 3 h in the control system showed the formation of both types of CaOx crystals, as oval shaped COM and octahedral COD. At the end of the 3 h, n-butanol fraction at 1000 µg/ ml showed very fewer number of these crystals as shown by the arrows.

3.5.2. Aggregation assay

In this assay, at the end of the 3 h, after treatment, n-butanol fraction showed a significant reduction ($p<0.05$) in aggregation of preformed CaOx crystals compared to other fractions. Percent reduction in aggregation produced by this fraction was found to be 71.9±3.8% almost on par with that of standard drug cystone (80.5±2.7%) at 1000 µg/ ml (Table 3 and Fig. 2).

3.5.3. Growth assay

For this assay, n-butanol fraction showed the maximum inhibitory effect at the end of the 3 h. Percent reduction in growth in the presence of this fraction was found to be 79.4±2.2% comparable to that of cystone (82.7±2.6%) at 1000 µg/ ml (Table 3 and Fig. 3).

4. DISCUSSION

4.1. Yield recovery percent

In the present study other than four fractions, the remaining residue of water extract was also evaporated to get aqueous extract and extract yield was calculated. Each fraction yielded various quantities of extract yield. The yield recovery percent is generally tended to increase with the increasing polarities of the solvents used for plant extraction as suggested by Sowndhararajan and kang.[53] The yields of n-butanol, ethyl acetate, chloroform, hexane and final residue were found to be 12.1%, 4.61%, 4.03%, 3.9% and 17.1%, respectively. When compared to other fractions, the last residue containing fraction exhibited the maximum extractive yield i.e 17.1%. Possibly, this could be due to the higher polarity of water. This is in agreement with the findings of Elangomathavan *et al*.[54] who investigated that water is more efficient to give higher extractive value from *cleistanthus collinus*.

4.2. Phytochemical analysis

Any part of the medicinal plant contains disease curative phytocompounds that can be used as precursors for the synthesis of useful drugs. The crude extracts or purified form of phytocompound/s have been used as medicines.[55] The medicinal value of the plants lies in bioactive phytochemical constituents or secondary metabolites that produce definite physiological action on the human body.[56]

4.2.1. Qualitative phytochemical analysis

Phytochemical screening of medicinal plants is very important in identifying new sources of therapeutical and industrial importance.[57] In the present study, using various solvent fractions of *C. ternatea* leaves were tested for the presence of alkaloids, flavonoids, phenols, tannins, sterols, saponins and terpenoids. The results indicated that the presence of all the phytocompounds tested were present in all the fractions. The same findings were reported by Chakraborty *et al*.[58] for this plant variety where they followed cold extraction method using aqueous leaf extract. It is reported that flavonoids, triterpenoids and saponins from different plants exhibited anti-urolithiatic and diuretic activity. Many plant extracts and different fractions possessing these active components have been screened for anti-urolithiatic activity.[59,52,60,61] Medicinal plants with proven anti-urolithiatic activity were found to contain alkaloids, tannins, steroid and terpenoids as chief principles.[62,63,64] The anti-urolithiatic mechanism is mediated possibly through diuretic and nephroprotective actions of the phytocompounds including phenols present in rhizomes of *Acorus calamus*.[65,66]

4.2.2. Quantitative phytochemical analysis

In the present investigation, four secondary metabolites such as total phenols, flavonoids, terpenoids and tannins were quantitatively determined and results showed that chloroform and final residue contained high level of phenolics compared to other three fractions. These indicate that the leaf is a good source of phenolics as evidenced by Chakraborty *et al*.[58] All the fractions contained a moderate level of flavonoid content. Flavonoids are essential in human diet and are present in plant extracts that have been used for medicinal purpose.[67] Even though all the fractions contained adequate level of terpenoids, the highest content was present in choloform fraction only. Terpenoids are natural secondary metabolite found in plant species which is providing flavour and fragrance. It prevents the development of chronic joint swelling.[68] Compared to other fractions, hexane fraction displayed a good level of tannin content which play a major role in plants to protect them from predators and also in growth regulation.[69]

4.3. In vitro antioxidant activities

Oxidation reactions can produce free radicals which in turn they start chain reactions and lead to damage or death of the cell. Antioxidants terminate these chain reactions by removing free radicals and inhibit oxidative reactions.[70,71]

4.3.1 Ferric reducing antioxidant power (FRAP)

Results of FRAP obtained are highly reproducible and related linearly with the molar concentration of the antioxidants present. This result is contrary with the results reported by Jadhav *et al.*[66] who determined FRAP assay using methanolic extracts of leaves, stem and root of both the variety of *C. ternatea* (white and blue coloured flowers) and found methanolic extract of white variety leaves had exhibited the highest FRAP activity i.e 2.132±0.037 mg of AAE per 100 g in FRAP. This result is also contrast with the results of Suganya *et*

al.[72] who have used leaves and flowers of *C. ternatea* without mentioning variety and found that flowers had the maximum FRAP activity compared to leaves. In the present study, only leaves of blue coloured flower variety have been used for obtaining various fractions such as n-butanol, ethyl acetate, chloroform, hexane and residue containing fractions. When determined the FRAP activity, the residue containing fraction showed the highest activity i, e 24.6 ± 0.42 µm Fe (II) E/mg extract compared to other fractions.

4.3.2. Phosphomolybdate assay or total antioxidant capacity (TAC)

In the present investigation, n-butanol fraction showed higher TAC activity compared to other fractions. Owing to the presence of phenols and flavonoid compounds, ethyl extract of the the plant *Lepidagathis prostrata* showed significant total antioxidant capacity compared to other extracts which would further strengthen its use to ameliorate urolithiasis-induced oxidative stress.[73]

4.3.3. ABTS$^{\bullet}$+ radical scavenging activity

ABTS$^{\bullet}$+ assay is based on the ability of antioxidants to reduce the ABTS$^{\bullet}$+ radical cation.[48] In the present study, n-butanol fraction displayed the highest ABTS$^{\bullet}$+ radial scavenging activity and residue containing fraction showed the lowest ABTS$^{\bullet}$+ radial scavenging activity. The same result was obtained by Labiad *et al*.[74]

4.3.4. Nitric oxide scavenging assay

Nitric Oxide, is an important bio regulatory molecule, which has a number of physiological effects including control of blood pressure, neural signal transduction, platelet function, antimicrobial and antitumor activity.[75] NO also show toxic property after reaction with oxygen and superoxide radicals. The reaction products are able to cause much cellular damage.[76] Scavenging of nitric oxide radical is based on the generation of nitric oxide from sodium nitroprusside in buffered saline, which reacts with oxygen to produce nitrite ions that can be measured by using Griess reagent. In the present research, different fractions of *C. ternatea* proved to decrease in amount of nitrite generated from the decomposition of sodium nitoprusside *in vitro*. However, n-butanol fraction recorded maximum percentage of NO activity.

4.3.5. Free radical scavenging activity on DPPH

The DPPH is a stable radical and it has been widely used to test the ability of compounds as free-radical scavengers or hydrogen donors and to evaluate the antioxidative activity of plant extracts and foods.[77,78] In the present investigation, n-butanol fraction possessed excellent DPPH scavenging activity compared to other fractions.

4.4 Anti-urolithiatic activity

In recent years, a great advancement has been made in using phytotherapy against urolithiasis condition and many researchers have been made detailed scientific studies to prove efficacy of various phytocompounds. Several medicinal plants have been used since ancient times to treat kidney stones though the rationale behind their use is not scientifically proved. One such unexplored plant is *C. ternatea* blue flowered variety. CaOx urolithiasis is the most prevalent type of all urinary stone diseases. Key events involved in its pathological biomineralization include crystal nucleation, growth and aggregation.[79] Present study was designed to address these key events involved in CaOx stone formation as a means to investigate the efficacy of various solvent fractions of *C.ternatea* blue flowered leaves as an antiurolithiatic.

4.4.1 Nucleation

Nucleation is a prerequisite in the pathogenesis of CaOx urolithiasis. Nucleation basically marks a thermodynamically driven event of phase change wherein dissolved substances in a supersaturated solution spontaneously crystallize.[79,11] In the present investigation, similar phase change and formation of CaOx crystals was witnessed while carrying out nucleation assay. Significant inhibition in the nucleation of CaOx crystals was observed in the presence of 1000 µg/ml n-butanol fraction at the end of 3 h comparable to that of standard drug cystone. This suggests the anticrystallization activity of n-butanol fraction against CaOx crystallization. One possible mechanism of anticrystallization activity of this fraction could be its ability to complex with free calcium and oxalate ions, thus preventing the formation of CaOx complexes, as has also been suggested for *Sarghassum wightti*[80] and *Daucus carota*.[81]

4.4.2 Aggregation

Aggregation of crystals marks the process wherein numerous crystals in the solution come together and adhere forming large crystal agglomerates which promote stone formation and also produce renal tubular obstruction.[79] CaOx polymorphism is a common phenomenon and of utmost significance in urolithiasis. COM and COD crystals are commonly found in CaOx uroliths.[82] Of the two polymorphs, COM is thermodynamically more stable with more aggregatory and adhesive tendency.[83] Therefore, a transformation from COM to COD is advocated as a crucial step in inhibition of calculi formation.[82] In present study, among the various fractions, n-butanol fraction only promoted transformation of pointy edged dendritic COM crystals to smoother edged COD crystals of extremely reduced size and number.

4.6.3 Growth

Growth of CaOx crystals marks the event of deposition of crystal forming ions present in the supersaturated solution on preformed CaOx crystal lattice.[79,84] This event of growth of CaOx crystals was also tracked in the present study. N-butanol fraction exhibited maximum growth inhibitory activity. Qualitative phytochemical estimation of n-butanol revealed the presence of

flavonoids, phenolic compounds, saponins and tannins. These phytoconstituents are of utmost significance for inhibiting urinary stone formation. Saponins possess antilithic properties[52] and are known to disintegrate mucoproteins that are crucial components of stone matrix.[85] Tannins and polyphenols inhibit CaOx crystal formation as well as dissolve the preformed CaOx crystals by aiding calcium complexation.[86] Therefore, the anti-crystallization, anti-aggregatory and crystal growth defying activity of n-butanol fraction would have been an outcome of these phytoconstituents present in n-butanol fraction.

Table 1: Quantitative phytochemical analysis of *C. ternatea* blue flowered leaves.

Name of the plant fraction	Phenol (mg GAE/g extract)	Flavonoids(mg RU/g extract	Terpenoids (mg LIN /g extract)	Tannin(mg TAN/g extract)
n-butanol	84.6±2.0 ^{d}	76.0±2.0^{e}	51.6±2.4^{e}	21.0± 2.0^{d}
Ethyl acetate	138±1.6^{c}	143.0±2.4 ^{d}	109.0±1.6^{d}	26.0±2.1^{c}
Chloroform	239.0±2.0^{a}	802.0±1.6 ^{a}	174.3±2.0^{a}	10.0±0.8 ^{e}
Hexane	36.6±2.4 ^{e}	164.0± 1.6^{c}	172.6±2.4^{b}	190.0±3.3^{a}
Final Residue containing fraction	237.0±2.1^{b}	172.0±1.6 ^{b}	168.0±1.6 ^{c}	54.0±1.6 ^{b}

Statistically significant at P<0.05 where ^{a}>^{b}> ^{c}> ^{d}>^{e}>; Means followed by the same letter in a column are not significantly different at P<0.05. Means ± SD (n=3).

Table 2: *In vitro* antioxidant activities of *C. ternatea* blue flowered leaves.

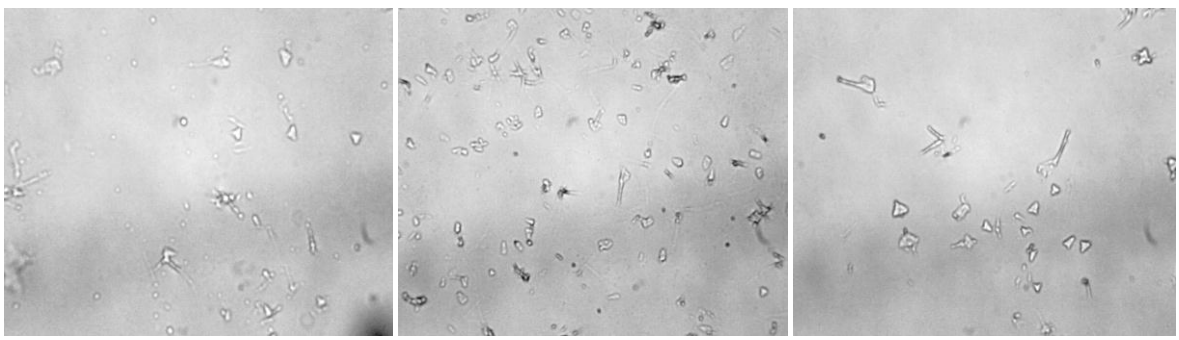
Name of the fraction	FRAP (µM Fe(II)E*/mg extract	PHOSPHO-MOLYBDENUM (mg AAE/g extract	ABTS$^{\bullet}$+ (mM TE/g extract)	NITRIC OXIDE (%inhibition)	DPPH (IC_{50}µg/ml)
n-butanol	3.31±0.33^{b}	50.78±4.4^{a}	3.68±0.12^{a}	62.4±0.4^{a}	168.2^{e}
Ethyl acetate	0.9±0.006^{e}	3.64±2.1^{e}	3.12±0.12^{d}	54.34±6.8^{e}	707.4^{a}
Chloroform	2.08±0.45^{c}	10.4±1.3^{c}	3.15±0.24^{c}	57.61±4.1^{d}	344.9^{b}
Hexane	1.33±0.21^{d}	7.78±2.6^{c}	3.46±0.02^{b}	61.49±5.6^{b}	235.1^{d}
Final residue	24.6±0.42^{a}	19.8±9.0^{b}	0.47±0.03^{e}	61.4±1.1^{c}	324.6^{c}

phenol (Gallic acid equivalents); Flavonoids (rutin equivalents); Terpenoids(linalool equivalent);Tannin (tannin acid equivalents); statistically significant at P<0.05 where ^{a}>^{b}> ^{c}> ^{d}>^{e}>; Means followed by the same letter in a column are not significantly different at P<0.05. Means ± SD (n=3).

Table 3: *In vitro* anti-urolithiactic activity of different fractions of *C. ternatea* blue flowered leaves.

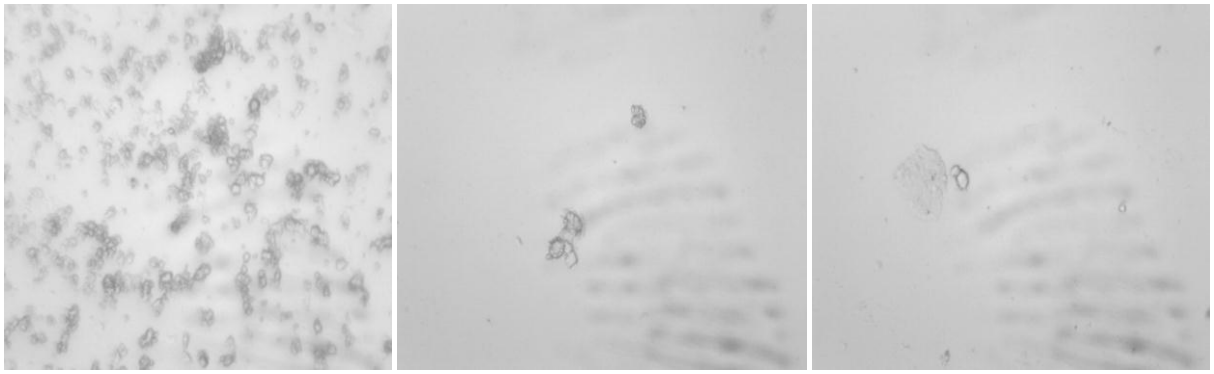
Solvent	Nucleation assay (% inhibition)			Aggregation assay (%inhibition)			Growth assay (% inhibition)		
	1h	2h	3h	1h	2h	3h	1h	2h	3h
Cystone (control)	60.5±3.6^{d}	72±3.18^{c}	85.5±3.2^{a}	50.4±3.8^{d}	60.4±3.6^{c}	80.5±2.7^{a}	52.0±3.5^{d}	65±3.2^{b}	82.7±2.6^{a}
n-butanol	77.8±2.8^{b}	79.3±1.4^{b}	82.8±1.3^{a}	51.8±3.7^{d}	58.3±4.5^{c}	71.9±3.8^{b}	58.1±2.7^{c}	62.2±2.2^{c}	79.4±2.2^{a}
ethyl acetate	70.1±3.8^{c}	72.8±1.1^{c}	75.2±2.1^{b}	30.5±4.2	41.8±5.2^{e}	50.5±3.3^{d}	55.3±3.1^{c}	60.2±2^{c}	70.7±1.9^{b}
Chloroform	65.1±2.1^{d}	67.6±1.7^{d}	70.2±3.4^{c}	22.7±4.6	33.2±4.2	45.8±3.9	42.3±2^{e}	57.3±2.2^{c}	65.3±2.0^{b}
Hexane	60.3±3.7^{d}	62.5±1.3^{d}	65.1±1.1^{d}	15.3± 3.0	26.1± 4.5	31.4± 4.4	39.2±0.7^{e}	48.7±2.1^{d}	53.4± 2^{d}
Final residue containing fraction	52.4±3.7^{e}	54.0±3.4^{e}	57.1±2.3^{e}	8.2±2.5^{f}	17.8±4.1	20.1±3.3	20.0±1.9^{f}	21.2±1.8^{f}	30.9±0.8^{e}

Data were presented as mean ± standard deviation (S.D); statistically significant at P<0.05 where ^{a}> ^{b}>^{c}>^{d}>^{e}>^{f}>.



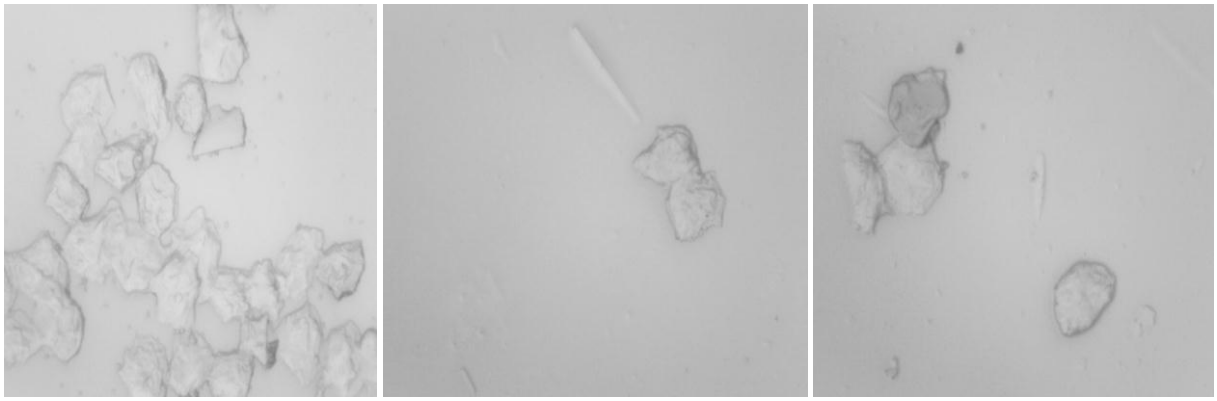
Control (A) **Cystone (B)** **n-butanol (C)**

Fig. 1: Plates showing light microscopic photographs of nucleation assay in control (A), standard (B) and n-butanol fraction (C) (X100).



Control (A) Cystone (B) n-butanol (C)

Fig. 2: Plates showing light microscopic photographs of aggregation assay in control (A), standard (B) and n-butanol fraction (C) (X100): photographs of B and C clearly show minimum aggregation of stones compared to control.



Control (A) Cystone (B) n-butanol (C)

Fig. 3: Plates showing light microscopic photographs of growth assay in control (A), standard (B) and n-butanol fraction (C) (X400): photographs of B and C clearly show minimum growth of stones compared to control.

5. CONCLUSIONS

In the present study, hot crude plant extract prepared from *Clitoria ternatea* blue flowered leaves was subjected to successive fractionation using various solvents. All the fractions used for preliminary phytochemical tests as well as for quantification some important secondary metabolites. In addition, *in vitro* antioxidant and anti-urolithiatic potentials of all the fractions were evaluated. During qualitative phytochemical analysis, all the fractions displayed all the phytocompounds tested andcontained an adequate level of quantitatively determined secondary metabolites. All the fractions showed varied level of antioxidant activities assayed. All the fractions obtained in this present study have been used to investigate their antiurolithiatic potential against calcium oxalate (CaOx) crystalization by employing three *in vitro* methods such as nucleation, aggregation and growth. N-butanol fraction showed prominent percent inhibition in all the three phases of CaOx stone formation viz. nucleation, growth and aggregation and favored the dissolution of COD crystals. It could be attributed to its saponins, tannins, flavonoids and phenolic contents. Also it may be due to significant free radical scavenging activities of this fraction for total antioxidant capacity, $ABTS^{\bullet+}$, nitric oxide and DPPH would further strengthen its use to ameliorate urolithiasis induced oxidative stress. Thus, this study concludes that n-butanol fraction of *C. ternatea* blue flowered leaves has prevention action or potential against CaOx crystallization. To our knowledge, this is the first study giving scientific evidence for anti-urolithiatic potential of this plant variety. However, further studies should be planned for *in vivo* and clinical explorations/trials to confirm the efficacy of this fraction as an antiurolithiatic.

ACKNOWLEDGMENTS

The authors are thankful to UGC-SAP and DST-FIST for financial support for department instrumentation to carry our this research work.

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