Antiurolithiatic activity of Boldoa purpurascens aqueous extract : an in vitro and in vivo study

Antiurolithiatic activity of *Boldoa purpurascens* aqueous extract: an *in vitro* and *in vivo* study

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Abstract

Ethnopharmacological relevance: Boldoa purpurascens Cav. (Nyctaginaceae) is a plant species used in traditional medicine in Cuba as antiurolithiatic.

Aim of the study: The aim of the present investigation was to evaluate the *in vitro* and *in vivo* antiurolothiatic activity of an aqueous extract from the leaves of *Boldoa purpurascens.*

Materials and Methods: The aqueous extract from leaves of *Boldoa purpurascens* was evaluated for antiurolithiatic activity *in vitro* and *in vivo*. *In vitro* crystallization of calcium oxalate (CaOx) was assessed using a nucleation, aggregation and growth assay. The effects of the extract and of Cystone®, used as a positive control, on the slope of nucleation and aggregation, as well as on the growth of CaOx crystals, were evaluated spectrophotometrically. The densities of the formed crystals were compared microscopically. *In vivo* activity was evaluated in an urolithiasis model in rats, in which kidney stones are induced by ethylene glycol (0.75%) and ammonium chloride (2%) in drinking water for 10 days. Three different experimental doses (100, 200 and 400 mg/kg, p.o.) of the extract and Cystone® were administered for 10 days. After 10 days, various biochemical parameters were measured in urine and serum, and histopathological analysis of the kidneys was carried out.

Results: The aqueous extract of *Boldoa purpurascens* inhibited the slope of nucleation and aggregation of CaOx crystallization, and decreased the crystal density. It also inhibited the growth and caused the dissolution of CaOx crystals. Cystone® exhibited similar effects. At a dose of 400 mg/kg the extract reduced the concentration of uric acid in urine, as well as the serum concentration of uric acid and creatinine. Histopathologic analysis of the kidneys of the same treatment group revealed reduced tissue damage; the results were almost similar to the untreated healthy control group.

Conclusion: This study indicates that an aqueous leaf extract of *Boldoa purpurascens* may be effective in the prevention of urinary stone formation, and substantiates the traditional claim.

Keywords

Boldoa purpurascens; *Nyctaginaceae;* kidney; urolithiasis; calcium oxalate

Abbreviations

CaOx: Calcium oxalate; COD: Calcium oxalate dihydrate; COM: Calcium oxalate monohydrate; OD: Optical density; ethylene glycol

1. Introduction

Kidney stones are hard, solid particles that can be formed in the urinary tract. In many cases, the stones are very small and can leave the body without any problem (Nirumand et al., 2018). However, if a stone (even a small one) blocks the flow of urine, excruciating pain may result, and prompt medical treatment may be needed. Recurrent stone formation is a common problem for many patients. Calcium containing stones, especially calcium oxalate monohydrate (COM), calcium oxalate dihydrate (COD) and basic calcium phosphate are the most common (Evan, 2010). The incidence of urolithiasis is very high in Cuba (Bacallao et al., 2014). *Boldoa purpurascens* (Nyctaginaceae) is a bush, which is wild to most parts of Cuba, and which is also known as "nitro" or "toston". In traditional medicine, the leaves are used in the treatment of urinary disorders as diuretic and antiurolithiatic (Roig, 1988). It is also involved in lowering the level of blood sugar (González et al., 2013). The aim of this study was to evaluate *in vitro* as well as in an animal model, the ability of different concentrations of the aqueous extract of the leaves of *Boldoa purpurascens* and Cystone®, used as a positive control, to dissolve experimental kidney stones. The use of Cystone® as a positive control is based on the study of Kumar et al. (2016).

2. Materials and Methods

2.1. Plant Material

Leaves of *Boldoa purpurascens* were collected in January–February 2018 from its natural habitats in the Botanical Garden of the Central University "Marta Abreu" of Las Villas, Cuba. The plant material was identified and authenticated by Dr. C. Idelfonso Noa. The voucher specimen No. 3012 is kept at the herbarium for further reference.

2.2. Extraction

The leaves were dried and pulverized, and 273 g of powder was extracted with water in a Soxhlet apparatus. The extract was concentrated under reduced pressure. The phytochemical characterization of the aqueous leaf extract was reported before (González et al., 2008; Hernández et al., 2016). This extract was used for further pharmacological evaluation according to Sheng et al. (2005).

2.3. Evaluation of the in vitro anti-urolithiatic activity

Preparation of experimental kidney stones (calcium oxalate (CaOx) stones) by homogenous precipitation: Solutions of $CaCl_2$ and $Na_2C_2O_4$ (50 mM) were mixed. The mixture was then heated at 60 °C in a water bath for 1 h and incubated overnight at 37 °C in an oven to form the CaOx crystals (Sujatha et al., 2015).

2.3.1. *Nucleation* assay

The effect of *B. purpurascens* aqueous extract on calcium oxalate (CaOx) crystal formation was determined by means of the nucleation assay according to the method described by Patel et al. (2012). Calcium chloride ($CaCl_2$) (5 mM) and sodium oxalate solution ($Na_2C_2O_4$) (7.5 mM) were prepared in Tris-HCl (0.5 M) and NaCl (0.15 M) buffer (pH 6.5). Dilutions of the extract and Cystone® (obtained from Himalaya Herbal Healthcare, India, batch no. 11700204) (100-1000 µg/mL) were prepared in distilled water. One milliliter of each concentration (100 µg/mL, 200 µg/mL, 400 µg/mL, 600 µg/mL, 800 µg/mL and 1000 µg / mL) of the extract and Cystone® were mixed with 3 mL of $CaCl_2$ followed by the addition of 3 mL of $Na_2C_2O_4$ solution. The mixtures were incubated for 30 min at 37 °C in an oven and cooled down to room temperature. Finally, the optical density (OD) of the mixtures was measured at 620 nm using a spectrophotometer (INESA L6S). The percentage inhibition of nucleation for the extract and Cystone® was calculated using the formula:

% Inhibition = $[1 – (OD_{test}/OD_{control}) x100]$

where OD_{test} is the optical density of the *Boldoa* extract and $OD_{control}$ the optical density of the negative control.

2.3.1.1. Microscopic evaluation

Number, size and morphology of CaOx crystals formed in absence or presence of extract and Cystone® were determined using a Leica DM 2500 LED microscope at 1000× magnification.

2.3.2. Aggregation assay

The effect of extracts and Cystone® on CaOx crystal aggregation was determined according to the assay describe by Bawari et al. (2018). $CaCl_2$ and $Na_2C_2O_4$ solutions (50 mM each) were mixed together, heated to 60 °C in a water bath for 1 h and then incubated overnight at 37 °C. After drying, a CaOx crystal solution (0.8 mg/mL) was prepared in a 0.05 M Tris-HCl and 0.5 M NaCl buffer (pH 6.5). One milliliter of each dilution (100-1000 µg/mL) of the *Boldoa* extract and Cystone® was added to 3 mL CaOx solution, vortexed and then incubated at 37 °C for 30 min. OD of the final mixtures was read at 620 nm and the percentage inhibition of aggregation was calculated as described for the nucleation assay.

2.3.3. Oxalate depletion assay

The effect of the extracts and Cystone® on the growth of CaOx crystals was determined by the oxalate depletion assay. Varying concentrations of *Boldoa* extract and Cystone® (100 µg/mL, 500 µg/mL and 1000 µg/mL) were prepared in distilled water. CaOx crystal slurry at a concentration of 1.5 g/mL was prepared in a 50 mM sodium acetate buffer (pH 5.7). A 4 mM $CaCl_2$ solution and a 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), and next 30 µL of CaOx crystal slurry was added. The growth of CaOx crystals was then determined by measuring the rate of oxalate depletion from the solution at 214 nm for 10 min. The effect of each concentration of *Boldoa* aqueous extract and Cystone® on crystal growth was determined by addition of 1 mL of the extract and Cystone® (100 µg/mL, 500 µg/mL and 1000 µg/mL) to the reaction mixture, and changes in OD were again recorded. The percentage inhibition of crystal growth was then calculated as described for the nucleation assay.

2.3.4. Titrimetric estimation of calcium oxalate

2.3.4.1. Preparation of a semi-permeable membrane from farm eggs

The shell was chemically removed from the eggs by placing the eggs in HCl 2 M overnight, which caused complete decalcification. After washing with distilled water, a hole was carefully made on the top, and the contents were completely squeezed out from the decalcified egg. Then the egg membranes were washed thoroughly with distilled water, placed it in ammonia solution, and stored in moistened condition at a pH of 7- 7.4 in a refrigerator (Fig. 1).

Figure 1: Semipermeable membrane from farm eggs

2.3.4.2. Determination of calcium oxalate

Titrimetric estimation of CaOx was determined according to Saso et al. (1998) with slight modifications. Calcium oxalate (1 mg) was weighed and 20, 30, 40 and 50 mg of the extract and standard Cystone® were packed separately in a semi-permeable membrane by suturing (Fig. 1). They were allowed to suspend in a conical flask containing 100 mL 0.1 M TRIS buffer. One group served as negative control (containing only 1 mg of calcium oxalate). All conical flasks were placed in an incubator, preheated to 37 ^{o}C for 2 h. Next, the content of the semi-permeable membrane from each group was transferred into a test beaker. Finally, 2 mL of 1 N sulphuric acid

were added and titrated with 0.9494 N $KMnO_4$ till a light pink color was obtained (end point). 1 mL of 0.9494 N $KMnO_4$ was equivalent to 0.1898 mg of calcium oxalate. The amount of undissolved calcium oxalate was subtracted from the total quantity at the start of the experiment, to determine which quantity of calcium oxalate could actually be dissolved by the test substances.

2.4. *Evaluation of the in vivo anti-urolithiatic activity*

The ethylene glycol and ammonium chloride-induced hyperoxaluria model was used to induce urolithiasis in rats (Touhami *et al*., 2007). The *in vivo* assay was carried out according to the Guide for the Care and Use of Laboratory Animals, 8th edition, and the Manual of Procedures for the Use of Laboratory Animals of the National Institute of Health. Euthanasia was carried out according to the AVMA (Leary et al., 2013).

2.4.1. *Animals*

Thirty-six healthy male Wistar albino rats (weighing 140–200 g) were divided into six groups of six animals each and housed in clean polypropylene cages under controlled temperature (25 °C ± 2 °C), humidity 45%–55%, and 12 h light-dark cycle throughout the experimental period. They were fed with standard pellet diet and water *ad libitum* throughout the study. However, food was withdrawn while collecting 24 h urine samples inside metabolic cages.

Animals and commercial pellet diet were provided by The National Center of Production of Laboratory Animals (CENPALB, Cuba). The experimental protocol was approved by the Institutional Ethic Committee of the Experimental Toxicology Unit of Villa Clara Medical School in accordance with institutional guidelines.

2.4.2. *Drugs and Chemicals*

Ethylene glycol was purchased from Merck Millipore. Ammonium chloride was purchased from QuimiNet. Diagnostic kits for calcium, creatinine and uric acid were purchased from HELFA Diagnostics (Cuba). All other chemicals and reagents used were analytical grade and procured from approved chemical suppliers.

2.4.3. *Ethylene Glycol–Ammonium Chloride-Induced Urolithiasis*

Three dose levels of aqueous extract of *Boldoa purpurascens* leaves (100, 200 and 400 mg/kg) were used for the evaluation of the *in vivo* antiurolithiatic effect. The treatment protocol for 10 days (Touhami et al., 2007; Jagannath et al., 2012) for each group was as follows:

Group I: *ad libitum* access to regular food and drinking water (healthy control).

Groups II, III, IV, V, and VI: *ad libitum* access to regular food and drinking water containing 0.75% [v/v] ethylene glycol (EG) and 2% [w/v] ammonium chloride (AC) in order to promote urolithiasis.

Group III (positive control): received Cystone® 750 mg/kg by oral gavage.

Groups IV, V, and VI received the aqueous extract of *Boldoa purpurascens* by oral gavage at the following doses: Group IV, 100 mg/kg; Group V, 200 mg/kg and Group VI, 400 mg/kg.

2.4.4. *Collection and analysis of urine and serum*

All animals were kept in individual metabolic cages and urine samples of 24 h were collected on the 10^{th} day. Animals had free access to drinking water during the urine collection period. Urine was observed under the microscope (magnification 10×) for knowing the size and shape of crystals, and analyzed for calcium, urea and uric acid content.

At the end of the experimental period of 10 days, rats were anaesthetized and blood was collected from the aortic vein and then centrifuged at 3000 rpm for 10 min. The serum levels of calcium, creatinine and uric acid were measured using the respective diagnostic kits. Creatinine clearance was also measured (Cockcroft and Gault, 1976).

2.4.5. *Histopathological analysis of kidney sections*

The rats were killed by a high dose of ether, the abdomen was cut open and the kidneys of each animal were removed. The extraneous tissue of isolated kidneys was cleaned off and preserved in 10% neutral formalin solution. By using conventional methods, one of the isolated kidneys was embedded in paraffin and was cut into thin sections of about 5 µm using a rotary vertical microtome. Then these sections were observed and photographed using an optical microscope under polarized light (magnification 4x and 10×) in order to detect changes in kidney architecture and calcium oxalate deposits. The stains used were hematoxylin and eosin for final analysis.

3. Statistical analysis

Results are presented as mean ± SEM, using SPSS (version 22 (2013)). Statistical significance of data was assessed by analysis of variance (One way-ANOVA), followed by non-parametric techniques using the Kruskal-Wallis and Mann-Whitney tests for independent samples. Significance was considered at $P < 0.05$.

4. Results

4.1. *Evaluation of the in vitro anti-urolithiatic activity*

In the nucleation assay, addition of $Na_2C_2O_4$ solution to the reaction mixture consisting of $CaCl_2$ resulted in the formation of numerous CaOx crystals. Microscopic analysis showed that numerous large CaOx monohydrate

(COM) crystals of either rectangular habit or dendrites with sharp edges were predominant in the control group. The extract at higher concentrations (Fig. 2) and Cystone® at lower concentrations (Fig. 3) favored the formation of tetrahedral shaped calcium oxalate dihydrate (COD) crystals with smoother morphology. The extract and Cystone® also reduced the size and number of CaOx crystals. The percent reduction in size of CaOx crystals obtained for the extract (62.97%) was comparable to that produced by Cystone® (60.57%).

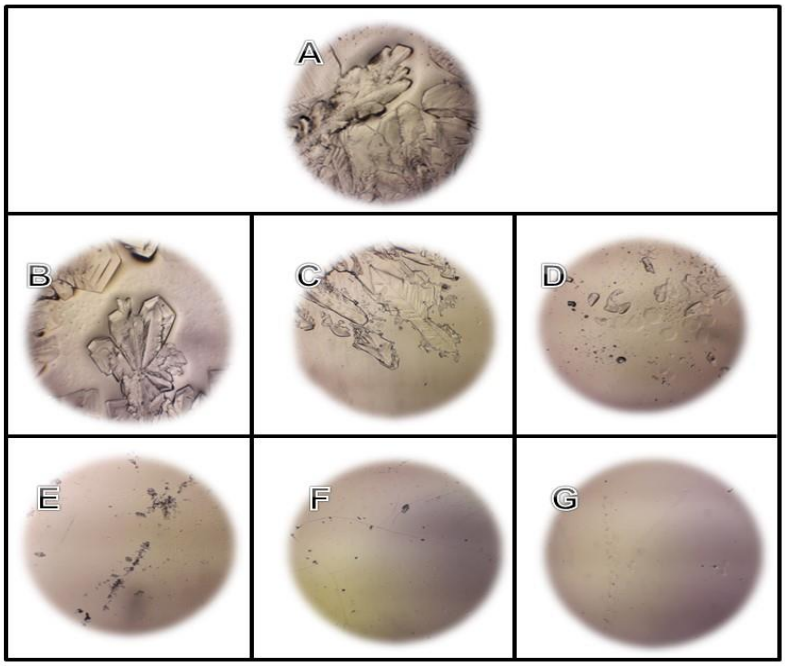

Figure 2: Representative photographs of CaOx crystals as observed under the light microscope in the absence of extract (A), and in the presence of extract: (B) 100 µg/mL, (C) 200 µg/mL, (D) 400 µg/mL, (E) 600 µg/mL, (F) 800 µg/mL and (G) 1000 µg/mL (magnification 10×).

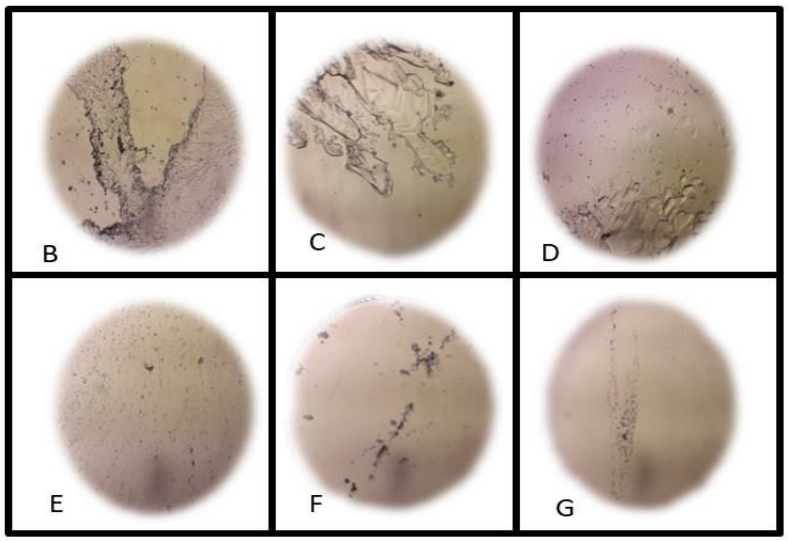

Figure 3: Representative photographs of CaOx crystals as observed under the light microscope in the absence of Cystone® (A) and in the presence of Cystone®: (B) 100 µg/mL, (C) 200 µg/mL, (D) 400 µg/mL, (E) 600 µg/mL), (F) 800 µg/mL and (G) 1000 µg/mL (magnification 10×).

In the aggregation assay, the extract produced a significant reduction ($P<0.05$) of aggregation of preformed CaOx crystals. The percentage reduction in aggregation produced by the extract at 1000 µg/mL was found to be

96.87%, compared to 62.2% for Cystone® (Fig. 4). In the oxalate depletion assay, the percentage reduction of crystals growth in the presence of extract was found to be 60.57% at 1000 µg/mL, and 62.67% for Cystone®. The CaOx crystal growth inhibitory effect of the extract was similar to Cystone® at all concentrations (Fig. 4).

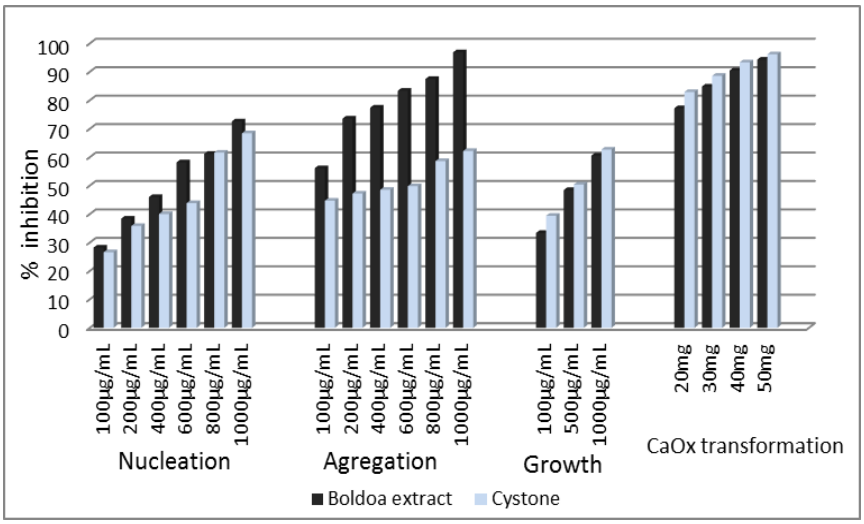

Figure 4: Effect of the aqueous extract of *Boldoa purpurascens* and Cystone® on nucleation, aggregation, growth and transformation of CaOx crystals.

4.2. Evaluation of the in vivo anti-urolithiatic activity

In this study the antiurolithiatic effect of the aqueous extract of leaves of *Boldoa purpurascens* examined in a rat model of experimentally induced urolithiasis. The pH of the urine of group I (healthy control) ranged between 6 and 7, and for group II (urolithiasis control) it was found to be 5. On the other hand, groups III, IV, V and VI showed an increase pH reaching values between 7 and 8.

Microscopy analysis (Fig. 5) revealed an increase in the number and size of the crystals in the urolithiasis control group when compared with groups treated with the extract of *Boldoa purpurascens* and Cystone®.

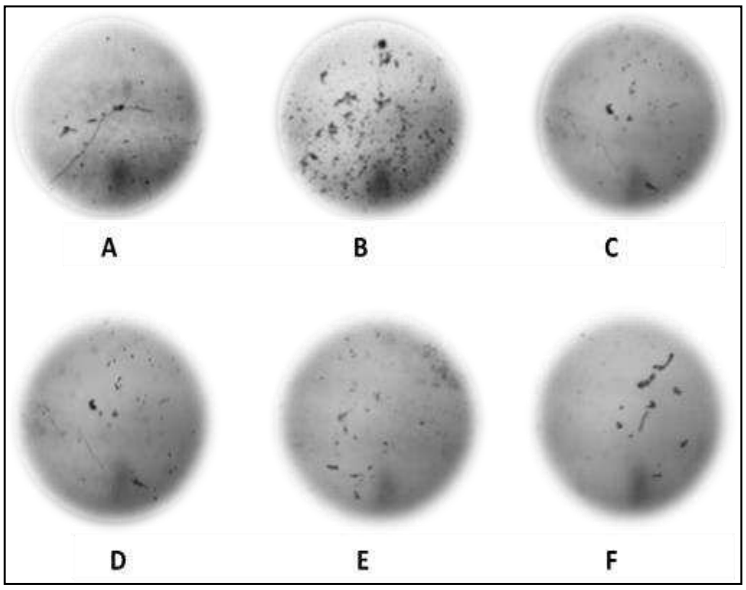

Figure 5: Microscopic images of (A) healthy control group (distilled water); (B) Urolithiasis control group; (C) group treated with Cystone®; (D) group treated with extract (100 mg/kg); (E) group treated with extract (200 mg/kg); (F) group treated with extract (400 mg/kg)) (magnification 10×)

The effect of the aqueous extract of *Boldoa purpurascens* on urine and serum biochemical parameters at the end of the experiment is shown in Table 1. Results revealed an increase in the excretion of these compounds or ions for group II, when compared to the healthy control group and with groups treated with the extract of *B. purpurascens*.

Table 1. Biochemical parameters at the end of the experiment

	Urine			Serum		
Groups	**Uric acid (mg/dL)**	**Urea (mg/dL)**	**Calcium (mg/dL)**	**Uric acid (mg/dL)**	**Urea (mg/dL)**	**Creatinin (mg/dL)**
I	0.30±0.05	45.05±0.54	0.48±0.07	2.39±0.08	33.21±4.01	0.42±0.09
II	0.63±0.07***	102.72±1.05***	14.59±0.70 ***	5.04±0.40*	53.09±1.54*	0.95±0.02***
III	0.32±0.01	61.39±3.49*	4.01±0.37**	2.99±0.17	45.05±2.00	0.58±0.03
IV	0.55±0.03**	95.75±0.05***	10.90±0.80***	3.66±0.70	45.83±2.23	0.90±0.05***
V	0.47±0.01*	68.48±3.29*	9.14±0.43***	3.44±0.42	45.47±1.92	0.83±0.08**
VI	0.37±0.02	59.11±2.17	8.82±0.36***	2.71±0.64	39.50±3.43	0.43±0.01

Values are expressed as mean ± SEM and compared with healthy control: *p < 0.05, **p < 0.01, ***p < 0.001 (n=6)

The urine volume also played an important role in the formation of CaOx crystals; particularly in group II (urolithiasis control) a decrease in the production of urine excreted during 24 h as well as in the urine flow was observed, indicating an obstruction (Fig. 6))(Rathod et al., 2014).

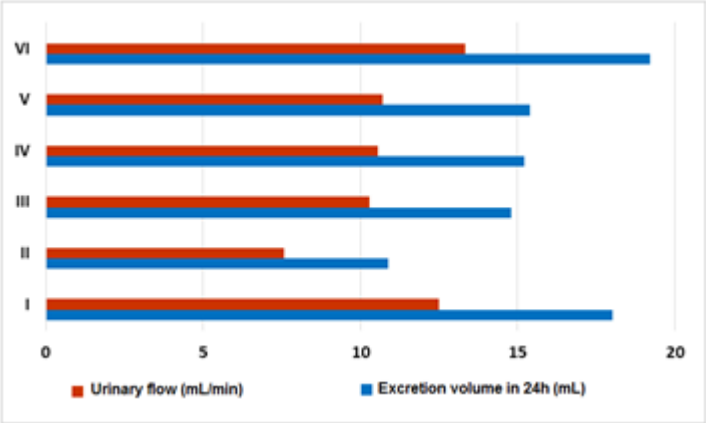

Figure 6: Urinary flow and excretion volume during 24 h

In addition, the deterioration of renal function of untreated rats was also evident from decreased values of creatinine clearance, another marker of glomerular and tubular damage (Fig.7).

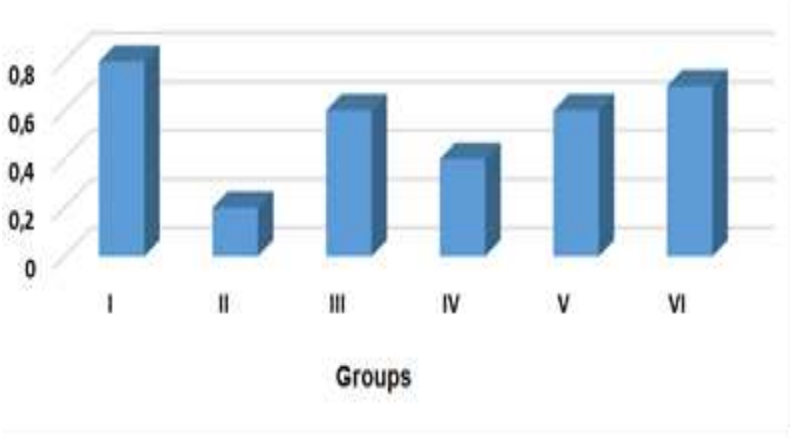

Figure 7: Creatinine clearance (mL/min)

Histopathological analysis of kidney sections (Fig. 8) showed changes in the morphology of the kidney with dilatation of the tubules and degeneration of the epithelium for the urolithiasis control group.

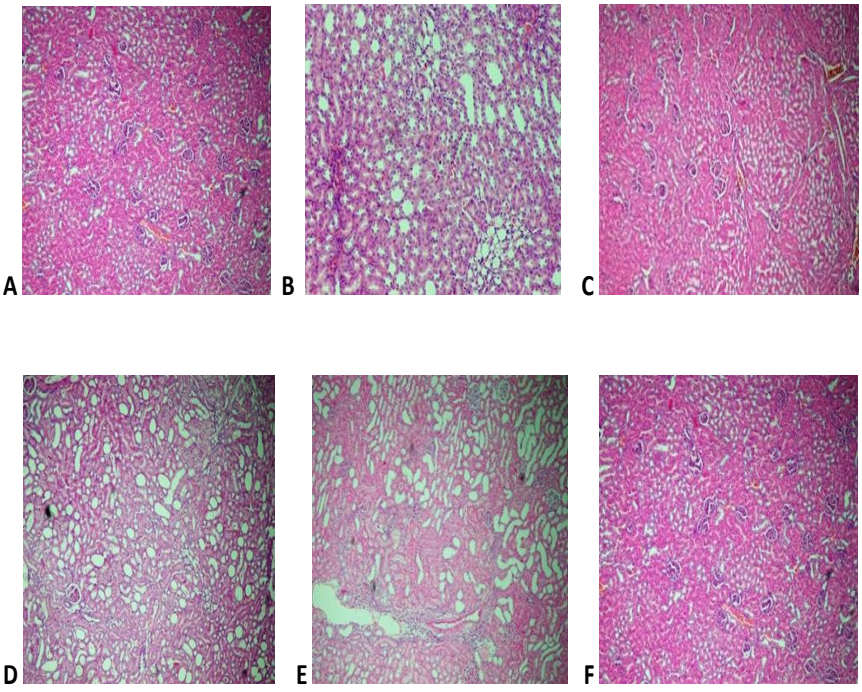

Figure 8: Histopathological analysis of kidney sections. (A) healthy control (I) (B) urolithiasis control (II) (C) group treated with Cystone® (III); (D) group treated with extract (100 mg / kg IV); (E) group treated with extract (200 mg / kg V); (F) group treated with extract (400 mg / kg VI) (magnification 4x and 10×).

5. Discussion

Urolithiasis is the most prevalent type of all urinary stone diseases. Key events involved in the pathological biomineralization include crystal nucleation, growth and aggregation (Lev et al., 2002; Aqil et al., 2003; Chaudhary et al., 2010; Patel et al., 2012; Sodimbaku et al., 2016). The *in vitro* study was designed to address these key events involved in CaOx stone formation as a means to investigate the efficacy of *Boldoa purpurascens* leaf extract as an antiurolithiatic. 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 (Chaudhary et al., 2010; Aggarwal et al., 2013). Significant inhibition in the nucleation of CaOx crystals was observed in the presence of extract, which was even better than in the presence of Cystone®. This suggests the anticrystallization activity of the extract in the CaOx crystallization assay. One possible mechanism of anticrystallization activity of the extract could be its ability to complex with free calcium and oxalate ions, thus preventing the formation of CaOx complexes. CaOx polymorphism is a common phenomenon and of utmost significance in urolithiasis. COM and COD crystals are commonly found in CaOx uroliths. Of the two polymorphs, COM is thermodynamically more stable with more aggregatory and adhesive tendency (Ratkalkar et al., 2007). Hence, COM tends to form large crystal aggregates and adheres strongly to renal epithelial tissue, injuring the same. Therefore, of the two polymorphs, COM significantly promotes crystal retention and eventual stone formation. The transformation from COM to COD is advocated as a crucial step in inhibition of calculi formation. In the present study, the extract also promoted transformation of pointy edged dendritic COM crystals to COD crystals of extremely reduced size and number. Reduction in size of CaOx crystals is critical, as smaller crystals tend to spontaneously pass out in urine (Sheng *et al*., 2005).

Growth of CaOx crystals marks the event of deposition of crystal forming ions present in the supersaturated solution on a preformed CaOx crystal lattice (Basavaraj et al., 2007; Aggarwal et al., 2013). The event of growth of CaOx crystals was also tracked in the present study. The extract exhibited growth inhibitory activity, which was also confirmed by the crystals of reduced size produced in the presence of extract. Aggregation of crystals marks the process wherein numerous crystals in the solution come together and adhere to form large crystal agglomerates. Aggregation is a key determinant of crystal retention, as large crystal agglomerates are the ones that produce renal tubular obstruction, thereby promoting stone formation (Aggarwal et al., 2013). *Boldoa* extract showed significant inhibitory effect on CaOx crystal aggregation. Flavonoids, which we have analyzed in the extract before, and their metabolites (phenolic compounds) formed after oral intake, may be of significance for inhibiting urinary stone formation (Gupta and Kanwar, 2018). Flavonoids and (poly) phenols may inhibit CaOx crystal formation and may dissolve preformed CaOx crystals by aiding calcium complexation. As COM crystals are

the most predominant form of all the polymorphs of CaOx found in kidney stones, formation of COM crystals was a mandatory requirement to test the efficacy of the extract against COM crystallization. Although *in vitro* data cannot simply be extrapolated to more complex *in vivo* systems, at least *in vitro* studies give an insight into the possible mechanism of action of tested compounds or extracts. The present study also demonstrated prominent inhibitory activity of the extract against CaOx crystallization.

The antiurolithiatic effect of the aqueous extract of leaves of *Boldoa purpurascens* was examined in experimentally induced urolithiasis in rats. Rat models of calcium oxalate urolithiasis induced by either ethylene glycol (EG) alone or in combination with ammonium chloride (AC) are most commonly used to study the pathogenesis of urolithiasis(Kalayan et al., 2009) . In this study an accelerated model was used, where rats are treated with 0.75% EG and 2% AC for 10 days (Jagannath et al., 2012). It is a minimally invasive method; different parameters can be evaluated such as renal function (pH, urinary density, determination of creatinine, urea and uric acid), oxalic acid in urine, and alteration of the morphology of the renal tissue as well as the presence-absence of kidney stones (Karadi et al.,2006).

The pH of the urine of group I (healthy control) was between 6 and 7, and for group II (urolithiasis control) it was found to be 5. Groups III, IV, V and VI showed a pH increase, reaching values between 7 and 8, which indicate the presence of calcium salts in this biological fluid that are being eliminated due to the treatment, but not for the urolithiasis control group.

It is known that if the pH is alkaline, calcium oxalate crystallization is reduced, due to increased calcium complexation by phosphate and citrate ions, preventing it from binding to oxalate; however, at pH greater than 6, the formation of calcium phosphate stones is favored (Lewandowski and Rodgers, 2004). In addition, the experimental groups showed a lower urinary density compared to the urolithiasis control group.

For the groups treated with the aqueous extract of *Boldoa purpurascens* at all doses, as well as for the animals treated with Cystone®, an increase in the volume of excreted urine and urinary flow was observed, being greater for the extract at doses of 400 mg/kg. This may be related to the potent diuretic action described for this species (González *et al*., 2008). The volume increase of urine decreases the saturation of oxalate and prevents the precipitation of CaOx at physiological pH. Diuresis also supports the mechanical expulsion of stones (Sridharan et al., 2016).

Microscopy analysis revealed that the quantity and size of calcium oxalate crystals was higher in the urine of the urolithiasis group (Fig. 5B), compared with the healthy control (Fig. 5A). On the other hand, the groups treated with aqueous extract showed a significant reduction in the quantity and size of calcium oxalate crystals in comparison with group II (Fig. 5D, E and F), as well as group III treated with Cystone® (Fig. 5C).

A significant increase in the excretion of calcium, uric acid and urea in urine was observed for group II, when compared to the healthy control (Table 1). In groups III and VI there was a slight increase, obtaining the lowest values for group VI, which suggests that at this dose the extract acts by inhibiting the formation of the stones or dissolving the crystals formed. The Kruskal-Wallis test revealed that there are significant differences between the treated groups and controls ($p < 0.05$) and that the effect was dose dependent.

The increase of calcium in urine is a factor that favors the nucleation and precipitation of calcium oxalate or apatite (calcium phosphate) from the urine and later, the growth of crystals. In addition, hypercalciuria decreases the inhibitory activity of urine against crystallization, by binding to natural inhibitors and inactivating them. On the other hand, uric acid interferes with the solubility of calcium oxalate and reduces the inhibitory activity of glycosaminoglycans. The predominance of uric acid crystals in calcium oxalate stones and the observation that uric acid binding proteins are able to bind calcium oxalate and modulate its crystallization also suggests its major role in stone formation (Aleling and Petros, 2018).

According to Boehm et al., (2007), for male Wistar rats, the reference values for serum uric acid is between 0.20-0.91 mg/dL (12-54 µmol.L-1), creatinine between 0.35-0.54 mg/dL (31.0-48.0 µmol.L-1) and urea between 24.02-55.86 mg/dL (4.0-9.30 mmol.L-1). Taking into account these reference values, in the urolithiasis control (Group II), the renal damage was evidenced by the elevation of serum creatinine and uric acid levels. The levels of serum uric acid in the experimental groups were also found to be lower than in the control group II. These high values in the urolithiasis control animals (group II) may be due to the obstruction of the outflow of urine by stones in the urinary system.

The administration of EG + AC in drinking water increased the levels of uric acid, urea and creatinine when compared with group I (healthy control). In the case of the group that received Cystone®, the increase was significantly lower than in the groups treated with the extract at doses of 100 and 200 mg/kg, obtaining the best results at a dose of 400 mg/kg. In urolithiasis, the glomerular filtration rate decreases due to the obstruction to the outflow of urine by stones in the urinary system and due to the damage to renal parenchyma. Therefore, the

waste products, particularly nitrogenous substances such as urea, creatinine, and uric acid, are accumulated in the blood (Selvam et al., 2001; Bashir and Gilani, 2009). The decrease in the serum levels of these is due to the antiurolithiatic effect of the *Boldoa* aqueous extract.

Renal dysfunction decreases the ability to purify creatinine (Fig.7), so the level of creatinine increases in the blood. The normalization of this parameter in animals treated with the aqueous *Boldoa* extract, compared to hyperoxaluric animals, shows that this species protects the deterioration of renal function by minimizing tubular damage and crystal deposition (Vargas et al., 1999).

The histopathological study of the kidneys of the rats without treatment (group I) showed a normal appearance with normal glomeruli, proximal and distal convoluted tubules without inflammatory changes, normal blood vessels and without deposits of calcium oxalate (Fig. 8A). Microscopic examination using polarized light of urolithiatic kidney sections showed intratubular and interstitial crystal deposits in Group II rats (Fig. 8B). However, rats treated with aqueous extract of *B. purpurascens* had far less kidney calcification (Fig. 8D, E and F). Histopathological examination of the healthy control (Group I) showed normal size tubules with single epithelial lining along the margin. In the urolithiasis control rats, there was a marked dilatation of the tubules and total degeneration of the epithelial lining with infiltration of the inflammatory cells into the interstitial space because of the crystals. Animals treated with Cystone® as well as those treated with aqueous *Boldoa* extract at all doses appeared normal in the tubular epithelial cells and glomeruli, with the presence of few crystals, as well as the appearance of mild edema and dilation(Christina et al., 2006).

Hyperoxaluria is one of the major risk factor in the pathogenesis of kidney stone formation, as it cause oxidative stress and damages the renal epithelial cells thereby providing a nidus for crystals attachment and ultimately cause crystal aggregation retention and deposition in the kidney (Byer and Khan, 2005). Therefore, decrease in oxalate may explain its decrease in oxidative stress and renal crystal deposition. It not uncommon that plant extracts may interfere with oxalate metabolism in animal model of urolithiasis. For example, the extract of extract of *Aerva lananta* decrease the oxalate excretion, in ethylene glycol fed rats, by decreasing the formation of oxalate synthesizing enzymes like glycolic acid oxidase (GAO) in liver and lactate dehydrogenase (LDH) in liver and kidney (Soundararajan et al., 2006), similar results were found in the extract of *Tribulus terrestris* (Sangeeta et al., 1994).

Several *in vivo* and *in vitro* studies have demonstrated that hyperoxaluria, a major risk factor for calcium oxalate nephrolithiasis, results in greater production of superoxide and hydroxyl free radicals, leading to antioxidant imbalance, cell membrane rapture and cell death (Santhosh et al.,2003) which leads to CaOx crystal adherence and retention in renal tubules (Thamilselvan et al., 2003). Thus, it can be speculated that decrease in oxalate might be useful in hyperoxaluric kidney stone formers and that the inhibitory effect of the plant extract on CaOx crystal deposition in renal tubules is possibly caused by its antioxidant activity.

Not published studies demonstrated the antioxidant effects of extracts from *Boldoa purpurascens*. On the other hand, it has been reported that the extract of the plant has anti-inflammatory (González et al., 2018) and antimicrobial (Machín et al., 2008) activities, which can complement its beneficial effect since infection and inflammation are associated with the process of urolithiasis.

6. Conclusions

Findings of the present study clearly demonstrate the antiurolithiatic potential of the *B. purpurascens* leaf extract against CaOx urolithiasis *in vitro*. The extract showed prominent inhibition of all phases of CaOx stone formation (nucleation, growth and aggregation), and favored the formation of more amenable COD crystals. In addition, the administration of the extract decreased the development of urolithiasis in a rat model of urolithiasis.

Conflict of interest

No conflict of interest is declared.

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