Antiurolithiatic activity of ethanolic extract of Eleusine indica Linn. (Poaceae) leaves in ethylene glycol-induced urolithiasis in male Sprague Dawley rats

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ABSTRACT: Urolithiasis is the process of forming stones in the kidney or urinary tract. This study aimed to determine the antiurolithiatic potential of Eleusine indica ethanolic extract. The animals were randomly divided into five groups with five animals each. Ethylene glycol (0.75% w/v) was used for inducing urolithiasis, and Cystone® (750mg/kg) was used as the standard herbal supplement. The ethylene glycol-induced rats were treated with ethanolic leaf extract at doses 200 and 400mg/kg BW for 14 days starting from the 15th until the 28th day of the experiment. The effects of 200 and 400mg/kg of E. indica ethanolic extract were compared with Cystone® as the standard, and distilled water as the control. Data obtained from the experiment was analyzed using one-way ANOVA followed by Tukey-HSD for multiple comparison between groups. Mann Whitney U-test was used in analyzing the renal scoring, using SPSS version 21 with 95% level of confidence. The results revealed that E. indica crude extract produced significant ($p<0.05$) effects in the urinary pH and microscopic examination of calcium oxalate crystals on the 21st day, and serum BUN on the 28th day. While no significant effects were seen in the urine calcium and creatinine, serum creatinine, and kidney histopathological examination. It can be concluded that E. indica possesses no significant antiurolithiatic activity.

Keywords: Antiurolithiatic, Urolithiasis, Eleusine indica Linn.

INTRODUCTION

Urinary stone diseases have been affecting humans since antiquity and still afflict people up to this day. At present, urinary

stones or urolithiasis affects 10-12% of the population in industrialized countries, and the peak incidence seems to be within the ages of 20-40 years (Chen, 2012). Urolithiasis is classified as one of the most painful (Ghori, Alam, Abrar, Khan, & Fatima, 2015) and third most common disorder of the urinary tract, others being frequently occurring urinary tract infections and benign prostatic hyperplasia as reported by Jain & Argal (2013). A stone forming belt has been stretched across Saudi Arabia, UAE, Myanmar, Thailand, Indonesia and the

Philippines (Ganesamoni & Singh, 2012). According to "Statistics by Country for Kidney Stones" (2015), Philippines has an extrapolated incidence of 317, 065 in 86, 241, 697 estimated population in 1996. The statistics mentioned is only an extrapolation of various prevalence or incidence rates against the populations of a particular country or region.

According to the National Kidney Foundation (2016), urinary system is a filtration network of the body as it carries out the different purposes in maintaining homeostasis. It includes metabolic waste excretion, electrolyte balance and regulation of blood pressure. Functionally, kidneys are the major part of the system. Each kidney is comprised of around a million units of nephron, which is responsible for filtration. Nephron, if damaged, may lose its capability to excrete toxins and minerals. At times, waste products may precipitate to crystals and accumulate to form stone or calculi. These stone aggregations are termed nephrolithiasis when found in the kidney; however, formation of these crystals anywhere in the urinary tract is called urolithiasis.

Urinary stone is a common disorder of the urinary tract and recurrent stone formation is often encountered. A stone is a hard object formed from chemicals in the urine containing calcium oxalate (CaC_2O_4) as one of its primary constituent, but other components such as magnesium ammonium phosphate type 2 and 3 can also be found. Approximately 80% of the analyzed kidney stones are composed of calcium oxalate and calcium phosphate, and 10% struvite which are produced during bacterial infection (Shamina & Jishamol, 2014). The calculi are formed when the urine is supersaturated with salt and minerals such as calcium oxalate, struvite (ammonium magnesium phosphate), uric acid and cystine. The crystals attract other elements and fuse together to create an even bigger solid that continues to grow unless passed out of the body with the urine. They vary considerably in size from small 'gravel-like' stones to large staghorn calculi. The calculi may stay in the position where they

are formed, or migrate down the urinary tract, producing symptoms such as severe pain on either side of your lower back, more vague pain or stomach ache that does not go away, blood in the urine, nausea or vomiting, fever and chills, and urine that smells bad or looks cloudy (Henderson, 2015). As a general rule, the larger the stone, the more noticeable the symptoms are (National Kidney Foundation, 2016). Secondary risks such as urinary tract infection may arise due to urolithiasis. Stones that cause obstruction to the flow of urine set up an environment of urine stasis and bacterial growth. The irritation caused by the stones results in infections leading to pyelonephritis, cystitis and urethritis (Grant, 2012).

In spite of tremendous advances in the field of medicine, there is no truly satisfactory drug for the treatment of renal calculi. Most patients still have to undergo surgery to get rid of this painful disease. Medical management of urolithiasis has been employed such as extracorporeal shock wave lithotripsy, percutaneous nephrolithotomy, ureteroscopy and open surgery. Although they cause side effects such as hemorrhage, hypertension, tubular necrosis, subsequent fibrosis of the kidney and also increase in stone recurrence (Patel, Vyas, Joshi & Gandhi, 2016). Phytotherapy is often sought to overcome the undesirable effects of the abovementioned treatments (Ghori et al., 2015; Henderson, 2015; National Institute of Diabetes and Digestive and Kidney Disease, 2016; National Kidney Foundation, 2016; Yadav, Jain, Alok, Mahor, Bhar & Jaiswal, 2011). In the traditional systems of medicine, most of the remedies were taken from plants and since then, there has been a growing demand for plant-based medicines (Jain & Argal, 2013; Shamina & Jishamol, 2014). Several plant extracts have been used to treat calculi with promising effect in prevention and treatment (Jagannath, Chikkannasetty, Govindadas, & Devasankaraiah, 2012). Included in this vast biodiversity is Eleusine indica Linn. commonly known as goose grass or paragis.

Eleusine indica is a species of grass that belongs to the family Poaceae. E indica is commonly referred to as goose grass. In the Philippines, it is also known as “paragis” or “palagtiki”. It is a typical weedy species of the tropics and sub-tropics, flourishing in cultivated and other disturbed situations on a wide range of soil types. It is also a widely distributed weed along riverbanks, roads, and settled areas throughout the Philippines. It is a tufted annual grass, prostrate and spreading, or erect to about 10 cm to 1 m in height, depending on density of vegetation but not usually rooting

at the nodes. Leaves are flat to V-shaped, up to 8 mm wide, 15 cm long and emanate to a longer, acute, boat-shaped tip. They are glabrous and usually quite bright, fresh green in color (Rojas-Sandoval & Acevedo-Rodríguez, 2014).

Figure 1. Eleusine indica, close up, (paragis) a stubborn grass along roadsides and everywhere else (Tau'olunga, 2007)

E. indica is used for various purposes, ranging from animal feed to its use for making fiber. The plant's short, creeping rhizome is regarded as the most valuable fodder plant and extensively used to make hay (Okokon, Odomena, Imabong, Obot & Udobang, 2010). Its seeds, when cooked, are sometimes used as a famine food. Aside from that, the stems are used to make mats, baskets, and are suitable for paper manufacture. The plant is also used in agroforestry for stabilizing sandy soil (Fern, 2016).

Traditionally, the plant has been the component of basic remedy in Vietnamese traditional medicine. The whole plant is said to be depurative, diuretic, febrifuge and laxative, and hence is used for the treatment of influenza, hypertension, oliguria and urine retention (Al-Zubairi, Abdul, Abdelwahab, Peng, Mohan, & Elhassan, 2009). According to Quattrocchi (2016), the plant is used in Ayurveda and Siddha by uprooting the whole plant followed by washing and is chewed for the treatment of diarrhea and dysentery. It is also reported to be boiled for treatment of sprains and fevers. A number of studies have been conducted with regards to the medicinal uses of E. indica. It was reported that extracts of the plant possess antibacterial, antioxidant, antiplasmodial, antidiabetic, antimicrobial and anthelminthic activities (Al-Zubairi et al., 2009; Okokon et al., 2010; Morah & Otuk, 2015).

Furthermore, these studies reported the presence of various secondary metabolites in E. indica extracts that produce the mentioned medicinal properties. In a study conducted by Okokon et al. (2010), ethanolic extract showed the presence of alkaloids, terpenes, flavonoids, tannins, anthraquinones,

saponins and cardiac glycosides. These phytochemical constituents present in the extract of E. indica, have general mechanisms of action that that is relatable to antiurolithiatic activity.

Flavonoids have the ability to disintegrate mucoproteins as shown in the study conducted by Ghori et al., (2015) on Desmostachya bipinnata ethanolic extract. Mucoproteins have significant affinity for calcium oxalate surface and promote the growth of crystals and cement them. Flavonoids might have reduced calcium and oxalate deposition by pre-coating calcium oxalate crystals and disintegrating the mucoproteins. They also minimize reactive oxygen species (ROS) by free radical scavenging activity and prevented further generation by metal chelating property.

Aside from flavonoids, another component that has actions against urolithiasis is alkaloid. In a study, alkaloid extract of Phyllanthus amarus relaxes smooth muscles specific to the urinary and biliary tract which eases the excretion of kidney and bladder stones (Eweka & Enogieru, 2011).

In addition, saponin derivatives appear to be a component of medicinal herbs with antiurolithiasis claims (Patel et al., 2016). Saranya & Geetha (2014) reported that the saponins in Beta vulgaris leaf and root aqueous extracts appear to inhibit mineral phase formation of calcium phosphate and growth of calcium oxalate monohydrate crystals. Saponins also disintegrate mucoproteins thereby decreasing calcium oxalate adhesion to renal epithelial cells (Kishore, Moosavi & Varma, 2013).

Aside from phytochemical screenings of the extracts of E. indica, a study conducted by Okokon et al. (2010) performed acute toxicity test on the ethanolic leaf extracts of E. indica. The median lethal dose (LD50) of the extract was estimated using the method of Lorke. This involved the administration of different doses of the extract to groups of three mice each. The animals were observed for manifestation of physical signs of toxicity such as writhing, decreased motor activity, decreased body/limb tone, decreased respiration and death. Results of the toxicity test showed that E. indica (1-5g/kg) produced physical signs of toxicity in the animals depending on the dose given ranging from writhing, decreased respiration and death 24 hours after administration of the extract. All the animals administered with 3.5g/kg and above doses of the extract died. The LD50 of the extract was calculated to be 3.24g/kg.

In addition, studies of plants related to E. indica were conducted which could contribute to its potential as an antiurolithiatic agent. In the statistical analysis of Ahmed, Hasan & Mahmood (2016), Poaceae has been shown to be one of the most cited families used traditionally for treating kidney stones with a total of 16 plants which can provide opportunities for future research and development of new natural antiurolithiatic compounds. Two of the species of plants belonging to the family Poaceae with studies for their antiurolithiatic properties are Saccharum spontaneum and Desmostachya bipinnata. S. spontaneum, also known as Kasa or wild sugarcane, is a traditional herb with excellent medicinal value. The study conducted by Sathya and Kokilavani (2013) reported the decrease in the levels of lipid peroxidation in homogenates of liver and kidney when compared to urolithiatic rats which might be an indication of recovery due to the administration of ethanolic root extract of the plant which possesses free radical scavenging activity and antiurolithiatic property.

Another plant that has protective effect against oxalate-induced lipid peroxidation is Desmostachya bipinnata, commonly known as halfa grass. Based on the plant's phytochemical screening, it contains flavonoids which act by disintegrating the mucoproteins, thereby preventing calcium and oxalate deposition and excretion (Ghori et al., 2015).

In a number of antiurolithiatic studies such as that of D. bipinnata, ethylene glycol is used to induce urolithiasis in rats. Rats are commonly used to study the pathogenesis of human CaC2O4 stone disease as the metabolism is regarded as almost similar in rats and humans. Male rats are used instead of female ones because estrogen inhibits the formation of calcium oxalate crystals by lowering urinary calcium and calcium oxalate saturation (Heller, Sakhaee, Moe, & Pak, 2002; Patel et al., 2016). Ingestion of ethylene glycol has been found to be a reliable inducer of oxalate lithiasis in rats. It is converted to endogenous oxalic acid by the liver enzyme glycolate oxidase and adenylyl cyclase which induces urinary acidification thus favoring adhesion and retention of CaC2O4 particles within the renal tubules (Ghori et al., 2015). Acting against the formation of calculi is Cystone®, which is the standard herbal supplement commonly used in antiurolithiatic studies. Cystone® is a product of Himalaya Drug Company which is a natural choice for urinary calculi and UTI. Cystone® has anti-lithiatic and lithotriptic properties. It prevents supersaturation of lithogenic substances and inhibits

calculogenesis by reducing stone forming constituents thereby causing their expulsion by micropulverization. It causes disintegration of the calculi and the crystals by acting on the mucin, which binds the particles together (Sam, 2012).

Currently, medical management of urolithiasis is either costly or with side effects. However, the search for antiurolithiatic drugs from natural sources has assumed greater importance (Shamina & Jishamol, 2014). A number of studies reported the medicinal values of E. indica extracts although none focused on its potential antiurolithiatic activity. In this regard, this study aims to evaluate the antiurolithiatic potential of ethanolic extract of E. indica leaves in ethylene glycol-induced urolithiasis in male Sprague Dawley rats through assessment of urine and serum tests, and kidney histopathology. This study also aims to determine the antiurolithiatic activity of E. indica ethanolic leaf extracts at 200mg/kg and 400mg/kg BW in ethylene glycol-induced urolithiatic male Sprague Dawley rats afterwards, results were compared to the standard herbal supplement Cystone®. Since calcium oxalate is the most common component of urinary calculi, the researchers also evaluated the plant extract's activity against calcium oxalate-containing urinary stones. If this study is proven to be significantly effective, this would lead to the advancement in the use of indigenous herbal medicines for urolithiasis. This study may eventually lead to a new and accessible solution to the high cost medical management, and bring cure and treatment to urolithiasis that will alleviate the burden of urinary calculi among Filipinos and even in other races. This study may also help other researchers who aim to confer more knowledge about the medicinal usefulness of E. indica in treating urolithiasis.

This research hypothesizes that the ethanolic extracts of E. indica can significantly reduce urinary stones in ethylene glycol-induced Sprague Dawley rats and that the effect can be comparable to that of Cystone®. It is also expected that the antiurolithiatic effects of E. indica in ethylene glycol-induced Sprague Dawley rats will be dose dependent.

This research is confined in determining the antiurolithiatic activity of ethanolic extract of E. indica leaves against ethylene glycol-induced urolithiatic male Sprague Dawley rats. The study did not perform the identification, isolation, and quantification of specific phytochemical constituents of E. indica leaves. Also, this study did not conduct the determination of bacterial contamination in the 24-hour urine samples. After thorough search in relation to

other studies conducted, no scientific study regarding the antiurolithiatic effect of E. indica has been conducted in the Philippines.

MATERIALS AND METHODS

Collection and authentication of plant material

Mature leaves of Eleusine indica (Linn.) Gaernt. were collected at Barangay Rizal, Lipa, Batangas. The plant was verified by John Rey C. Callado, Museum Researcher II – Botany Division from the National Museum located at Padre Burgos Avenue, Ermita, Manila.

Reagents and materials

Analytical grade ethanol was purchased from RTC Laboratory Services & Supply while tween 80, concentrated hydrochloric acid and ethylene glycol were purchased from Belman Laboratories. The standard herbal supplement, Cystone® (Didymocarpus pedicellata, Saxifraga ligulata, Rubia cordifolia, Cyperus scariosus, Achyranthes aspera, Onosma bracteatum, Vernonia cinerea, Shilajeet (Purified), and Hajrul yahood bhasma), was purchased from Himalaya Drug Company.

Preparation of Extract

E. indica leaves were air dried for two weeks, and powdered using a blender. The 174g powdered leaves were macerated in a percolator in approximately 1L of 95% ethanol for 72 hours and was repeated twice for 24 hours each. The liquid filtrate was concentrated in a rotary evaporator at 40ºC at 80 rpm. The resulting liquid was then placed in a water bath until a semisolid mass was obtained. The extract was stored in a refrigerator until further used in the experiment (Okokon et al., 2010). The percentage yield of ethanolic extract of E. indica leaves was calculated using the formula,

$$\text{Percentage yield of extract (\%) w/w} = \frac{WEE - WED}{WTS} \times 100$$

Where: WEE – weight of evaporating dish and extract
WED – weight of empty evaporating dish
WTS – weight of sample

Animals and Diet

Twenty-five male Sprague Dawley rats (2 months old) with weights ranging from 100g-170g were purchased from the Department of Pharmacology and Toxicology, College of Medicine – University of the Philippines Manila. They were acclimatized for one week prior to the start of the research in an apartment conducive to the experiment in an improvised cage patterned after a standard polypropylene cage, and a 12h light and 12h dark cycle. The animals were given standard pellets and distilled water ad libitum. The experiment was conducted in accordance to the Amended Animal Welfare Act (R.A 8485 or R.A 10631).

Renal calculi induction

Animals were randomly divided into five groups with five rats each. Group I served as the control group. Groups II-V received ethylene glycol (0.75% w/v) that was incorporated in the animals' drinking water for 28 days.

Experimental protocol

The ethanolic extract of E. indica and the standard herbal supplement, Cystone®, were insoluble in distilled water hence; they were suspended in 3% w/v tween 80 prior to administration to the experimental animals (Kumar R., Kumar T., Kamboj V. & Chander H., 2012). The concentrations given were prepared in 1mL/100g BW (Kishore et al., 2013).

Group I: Control group was treated with normal diet for 28 days
Group II: Urolithic control was given 0.75% w/v ethylene glycol incorporated in their drinking water for 28 days
Group III: Standard group. Urolithic rats + Cystone® (750mg/kg BW suspended in 3% w/v tween 80), treated once daily PO from 15th to 28th day
Group IV: Urolithic rats + ethanolic extract of E. indica leaves, treated with 200mg/kg BW once daily PO from 15th to 28th day
Group V: Urolithic control + ethanolic extract of E. indica leaves, treated with 400mg/kg BW once daily PO from 15th to 28th day

Collection and analysis of urine

The male Sprague Dawley rats were kept in improvised metabolic cages separately to obtain 24-hour urine samples.

Collection of urine samples was done on the 0th, 7th, 14th, 21st and 28th day of calculi induction treatment to determine the urinary pH. The samples were also subjected to microscopic examination under high power field for the quantity of calcium oxalate crystals since it is the most common component of urinary calculi. On the 28th day, urinary volume was determined, and analyzed for calcium and creatinine levels. Animals were given free access to drinking water during the urine collection. A drop of concentrated hydrochloric acid was added to each urine sample collected (Kishore et al., 2013).

Serum analysis

On the 28th day, the rats were anesthetized using Zoletil® IM and blood was collected through intracardiac route. The blood samples were analyzed for creatinine and BUN levels (Kishore et al., 2013). The collection of blood was done under the supervision of a veterinarian.

Histopathological Examination

With the help of a veterinarian, the kidneys of the animals were removed by dissection and placed in 10% formalin. The kidneys were examined for necrosis (Kishore et al., 2013). Tissue samples were prepared and analyzed by the veterinarian. A renal scoring was provided in accordance with FDA (Blank et al., 2009). Necrosis was measured and graded as 0 to 5 where, grade 0 showed normal histology; grade 1, showing degeneration only without necrosis; grade 2, necrosis involving <25%; grade 3, necrosis affecting >25% but <50%; grade 4, necrosis involving >50% but <75%; and grade 5, necrosis affecting >75% of the field.

Statistical analysis

The results were expressed as the mean ± standard error of the mean, and analyzed using Statistical Package for the Social Sciences version 21 and one-way ANOVA to determine statistically significant differences between the groups followed by Tukey-HSD. Mann Whitney U-test was used in analyzing the renal scoring. Significance level was tested at 95%.

RESULTS

Plant extraction

The extract obtained from the ethanol-macerated E. indica leaves had a syrupy consistency and was dark green in color with an herb-like smell. The percentage yield of ethanolic extract of E.

indica leaves was 11.58% w/w. It was calculated using the formula,

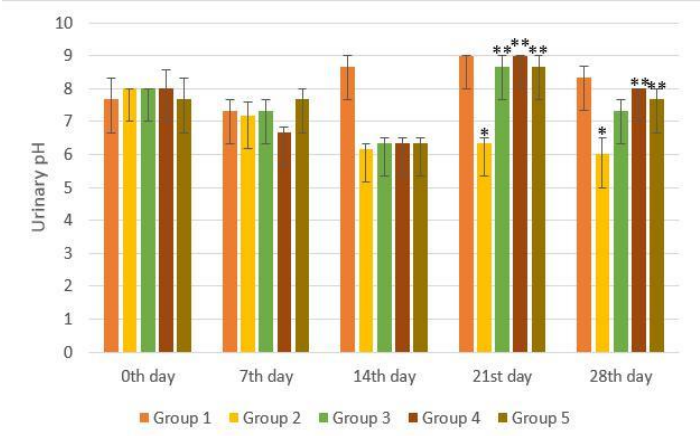
$$\text{Percentage yield of extract (\%) w/w} = \frac{88.1934\text{g} - 68.0423\text{g}}{174\text{g}} \times 100$$

Where: 88.1934g – weight of evaporating dish and extract
68.0423g – weight of empty evaporating dish
174g – weight of powdered plant

Urine Analysis

Urinary pH

Administration of EG (0.75% w/v) in the disease control group for 28 days caused significantly ($p<0.05$) decreased pH compared to the normal group starting from the 14th day. Standard herbal supplement Cystone® (750mg/kg) caused significant ($p<0.05$) increase in urinary pH when compared to EG alone treated group. Treatment with E. indica ethanolic extract 200 and 400mg/kg caused significant ($p<0.05$) increase in urinary pH when compared to EG alone treated group (Figure 2).



Legend: Data are expressed as mean ± SEM (n=3). *($p<0.05$) vs normal group, ** ($p<0.05$) vs disease control
Group 1 – Normal; Group 2 – Disease control;
Group 3 – Standard; Group 4 – Extract treated 200mg/kg;
Group 5 – Extract treated 400mg/kg

Figure 2. Change in urinary pH of rats for 28 days' observation period

Microscopic Examination of Urine

Administration of EG (0.75% w/v) in the disease control group for 28 days caused significantly ($p<0.05$) increased calcium oxalate crystals compared to the normal group starting from the 21st day. Standard herbal supplement Cystone® (750mg/kg) caused significant ($p<0.05$) decrease in calcium oxalate crystals when compared to EG alone treated group. Treatment with E. indica ethanolic extract 200 and 400mg/kg caused significant ($p<0.05$) decrease in calcium oxalate crystals when compared to EG alone treated group (Figure 3).

Urinary Volume

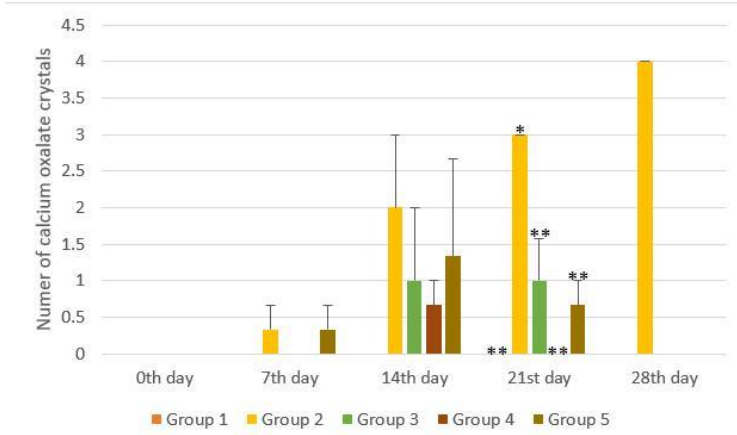
The increase in urine volume seen in the ethanolic extract-treated group at 200mg/kg was significant ($p<0.05$) when compared to the normal control group while the group treated with 400mg/kg showed no significant difference (Figure 4).

Estimation of Calcium and Creatinine

The data analysis showed an increase in calcium and creatinine urinary levels in the disease control group (Group II) as compared to the normal control group (Group I) although there are decreases in the urinary levels of calcium and creatinine in extract-treated groups (Groups IV & V), these differences are not considered significant in comparison (Figures 5 & 6).

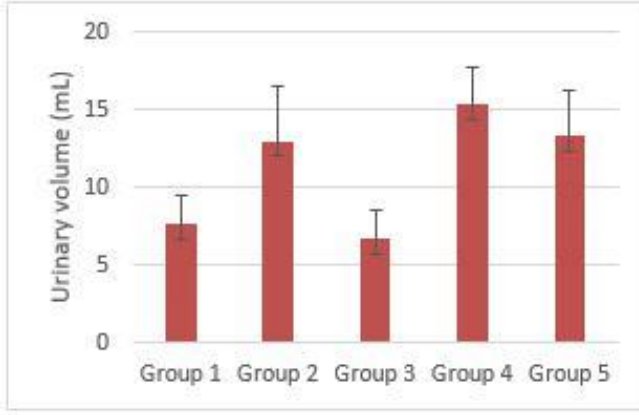
Serum Analysis

The serum creatinine and BUN levels showed increased concentrations on the ethylene glycol-induced rats (Group II) as compared to the normal control group (Group I), Cystone®-treated group (Group III) and extract-treated groups (Groups IV & V). The reduction in the serum creatinine levels of Groups IV and V showed no significant difference while the reduction in the serum BUN levels produced by the ethanolic extract-treated groups at 200 and 400 mg/kg presented significant ($p<0.05$) difference when compared to the disease control group (Group II) (Figures 7 & 8).



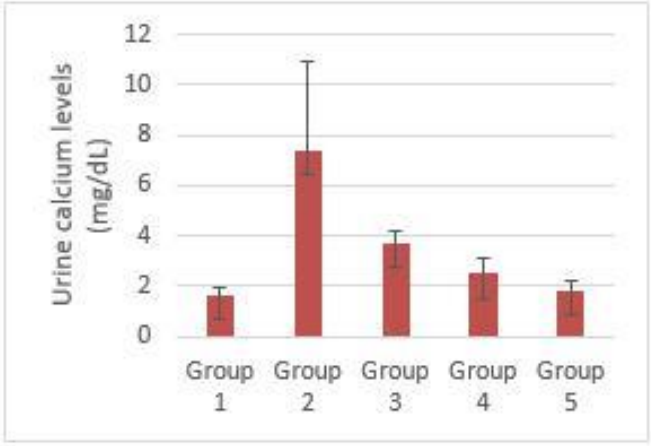
Legend: Data are expressed as mean ± SEM (n=3). *(p<0.05) vs. normal control group, **(p<0.05) vs. disease control
group. Microscopic Crystal scoring: 0 = 0; 1 = 0-2; 2 = 2-5; 3 = 5-20; 4 = >20.
Group 1 – Normal; Group 2 – Disease control;
Group 3 – Standard; Group 4 – Extract treated 200mg/kg;
Group 5 – Extract treated 400mg/kg

Figure 3. Change in urine calcium oxalate crystals during the 28 days' observation period



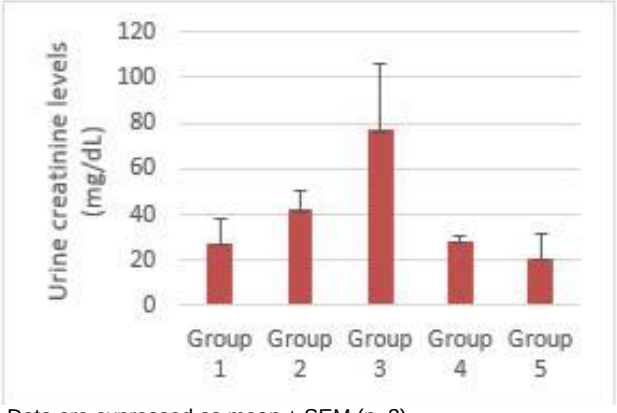
Legend: Data are expressed as mean ± SEM (n=3)
Group 1 – Normal; Group 2 – Disease control;
Group 3 – Standard; Group 4 – Extract treated 200mg/kg;
Group 5 – Extract treated 400mg/kg

Figure 4. Descriptive Statistics on the Effect of Ethanolic extract of Eleusine indica on Urinary Volume



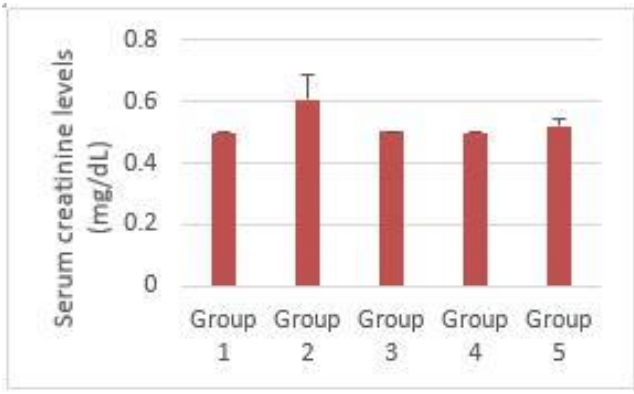
Legend: Data are expressed as mean ± SEM (n=3)
Group 1 – Normal; Group 2 – Disease control;
Group 3 – Standard; Group 4 – Extract treated 200mg/kg;
Group 5 – Extract treated 400mg/kg

Figure 5. Descriptive Statistics Calcium Urinary Levels



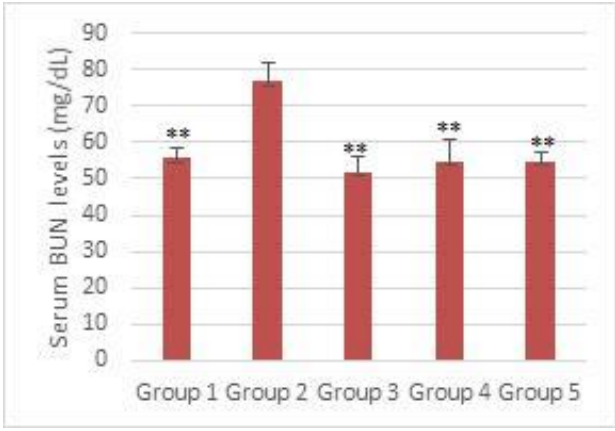
Legend: Data are expressed as mean ± SEM (n=3)
Group 1 – Normal; Group 2 – Disease control;
Group 3 – Standard; Group 4 – Extract treated 200mg/kg;
Group 5 – Extract treated 400mg/kg

Figure 6. Descriptive Statistics Creatinine Urinary Levels



Legend: Data are expressed as mean ± SEM (n=3)
Group 1 – Normal; Group 2 – Disease control;
Group 3 – Standard; Group 4 – Extract treated 200mg/kg;
Group 5 – Extract treated 400mg/kg

Figure 7. Descriptive Statistics Serum Creatinine



Legend: Data are expressed as mean ± SEM (n=3)
Group 1 – Normal; Group 2 – Disease control;
Group 3 – Standard; Group 4 – Extract treated 200mg/kg;
Group 5 – Extract treated 400mg/kg

Figure 8. Descriptive Statistics Serum BUN

Histopathological Examination

Renal Medulla

The histopathological study of the kidney sections of the normal group showed mild tubular cell degeneration while moderate cell degeneration and necrosis were observed in the kidney samples of Group II. The renal medulla of Groups III and

IV were normal, and there were no intratubular casts, inflammation and parenchymal mineralization seen. Renal medulla samples of Group V were also normal, and no intratubular casts, inflammation and parenchymal mineralization were seen although there was mild tubular degeneration observed.

Renal Cortex

Normal group (Group I) showed mild tubular cell degeneration and infiltration with small, darkly stained round cells, possibly lymphocytes, around the glomeruli and degenerative tubules. There was mild inflammation, intratubular casts and parenchymal mineralization seen in the renal cortex of the ethylene glycol-induced urolithiatic group (Group II). The group treated with Cystone® (Group III) showed mild tubular cell degeneration and tubular dilatation. The renal cortex of Group IV is normal but intratubular casts and mild glomerulopathy were seen, although both are rare. Group V's renal cortex is also normal but with tubular dilatation and mild tubular degeneration as well.

A scoring for the kidney histopathological examinations, in accordance with FDA, was also provided by the veterinarian although the differences in the renal scoring were considered to be insignificant at $p<0.05$ (Figure 9).

DISCUSSION

In the study conducted, 25 male Sprague Dawley rats were used for renal calculi induction as the metabolism is regarded almost as similar in rats and humans. Male rats were used instead of female ones because estrogen inhibits the formation of calcium oxalate crystals in female rats (Patel et al., 2016). Urinary supersaturation of various stone-forming elements is commonly considered being one the causative factors in stone formation. Previous studies reported that after the 28 days' period of ethylene glycol induction in the drinking water of laboratory animals significantly cause renal stone formation consisting mainly of calcium oxalate by increasing the urinary concentration of oxalate (Ghelani, Chapala, & Jadav, 2016). As reported in the study of Ghori et al. (2015), ingestion of ethylene glycol has been found to be a reliable inducer of oxalate lithiasis in rats as it is converted by the liver enzyme glycolate oxidase into endogenous oxalic acid which binds to calcium to form CaC2O4 crystals.

As mentioned in previous studies, the type of stone formed can be predicted from urinary pH. Since crystalluria is pH-dependent, a urinary pH of 5.0-6.5 promotes mostly calcium

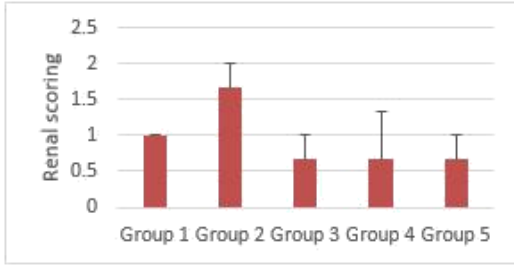
oxalate type of crystals (Ghori et al., 2015). From this research, the ethanolic extract of E. indica was able to restore the urinary pH (7.67-8.0) indicating the ability to reduce calcium oxalate crystals evident in the decline of crystals in the microscopic examination of the urine although this decrease was not found to be significant.

Traditionally E. indica has been used as a diuretic. The diuretic action is important in increasing the amount of fluid going through the kidneys to flush out the deposits. The increase in urinary volume reduces the saturation of the salts, thus preventing the precipitation of crystals at physiologic pH (Kishore et al., 2013). Only the group which received 200mg/kg BW ethanolic extract of E. indica showed significant ($p<0.05$) increase in urine volume thus exhibiting non-dose dependent effects. It was reported earlier that administration of ethylene glycol causes urolithiasis due to an increase in the concentration of calcium and creatinine which leads to nucleation and precipitation of calcium oxalate from urine (Sethiya, Brahmbhat, Chauhan, & Mishra, 2016). There was no significant decrease in the supersaturation of lithogenic ions such as calcium and creatinine in the extract-treated groups (Groups IV and V) as compared to the disease control group (Group II), although Cystone® did not produce a remarkable decrease in the urinary creatinine as well.

In urolithiasis, the glomerular filtration rate decreases due to the obstruction to the urine outflow caused by stone accumulation in the urinary system. Because of this, nitrogenous waste products like creatinine and BUN gets accumulated into the blood (Jarald, Kushwah, Edwin, Asghar, & Patni, 2011). In calculi-induced rats (Group II), the elevated serum levels of creatinine and BUN indicate marked renal damage. Although upon administration of E. indica ethanolic extract only the serum BUN was significantly ($p<0.05$) reduced while the extract produced no promising decrease in the serum creatinine level.

At present, it seems clear that renal epithelial cell injuries play a crucial role in renal calculi development, and in fact the lithogenic effect caused by ethylene glycol (EG) can be mainly attributed to the oxidative damage caused by the high level of oxalate generation (Khan, Shinge, & Naikwade, 2010). The histopathological examination of the renal medulla and renal cortex revealed necrosis which can be defined as cell injury which is an evident sign of exposure to CaC_2O_4 crystals. Even though there are noticeable differences between the normal group (Group I), disease control group (Group II), Cystone®-treated group

(Group III) and ethanolic extract-treated groups (Groups IV & V) in the histopathological examination, the renal scoring provided by the veterinarian showed that these visible differences were still considered to be insignificant thus demonstrating the inefficiency of the extract to reduce renal damage.

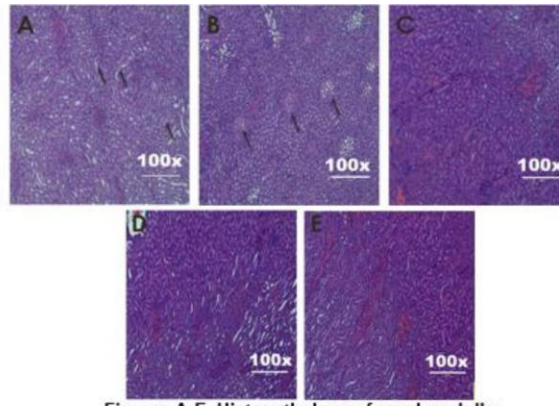


Legend: Data are expressed as mean ± SEM (n=3) FDA Renal Scoring 0 = normal histology; 1 = degeneration only without necrosis; 2 = <25%; 3 = >25% but <50%; 4 = >50% but <75%; 5 = >75% Group 1 – Normal; Group 2 – Disease control; Group 3 – Standard; Group 4 – Extract treated 200mg/kg; Group 5 – Extract treated 400mg/kg

Figure 9. Descriptive Statistics of Renal Scoring

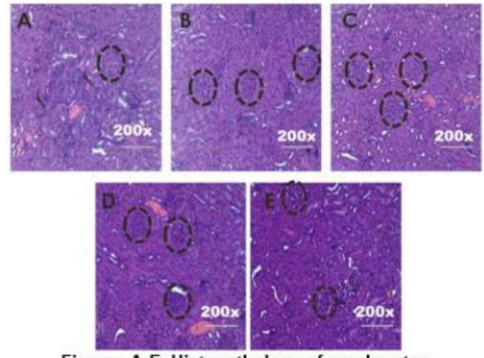
SUMMARY

In our study, although the ethanolic extract was able to produce a significant ($p<0.05$) increase in the urinary volume at 200mg/kg, it was not enough to produce a significant decrease in the CaC2O4 crystals and in the supersaturation of lithogenic ions such as calcium and creatinine. Therefore, administration of ethanolic extracts was not sufficient to cure the renal impairment caused by these calculogenic substances despite the significant decrease in serum BUN levels. In some parameters measured, ethanolic extract showed effects comparable to Cystone®.



Figures A-E. Histopathology of renal medulla

Group I (A: Normal Control Group) - Mild tubular cell degeneration (arrows);
Group II (B: Disease Control Group) - Moderate cell degeneration and necrosis (arrows);
Group III (C: Standard herbal supplement Cystone-treated Group at 750mg/kg BW p.o.) - Normal; No intratubular casts, inflammation and parenchymal mineralization were seen;
Group IV (D: Extract-treated Group at 200mg/kg BW p.o.) - Normal; No intratubular casts, inflammation and parenchymal mineralization were seen; and
Group V (E: Extract-treated Group at 400mg/kg BW p.o.) - Normal; Mild tubular degeneration; No intratubular casts, inflammation and parenchymal mineralization were seen.



Figures A-E. Histopathology of renal cortex

Group I (A: Normal Control Group) - Mild tubular cell degeneration; infiltration with small, darkly stained round cells, possibly lymphocytes (arrows) around glomeruli and degenerative tubules;
Group II (B: Disease Control Group) - Mild inflammation was seen. Intratubular casts and parenchymal mineralization were seen;
Group III (C: Standard herbal supplement Cystone-treated Group at 750mg/kg BW p.o.) - Mild tubular cell degeneration; Tubular dilatation;
Group IV (D: Extract-treated Group at 200mg/kg BW p.o.) - Normal; Intratubular cast was seen, although rare. Mild glomerulopathy, although rare; and
Group V (E: Extract-treated Group at 400mg/kg BW p.o.) - Normal; Tubular dilatation, mild tubular degeneration

*Glomeruli- dashed circles

CONCLUSION

Eleusine indica crude extract was found to be inefficient to work as an antiurolithiatic agent despite some significant values

based on the parameters measured. There was no significant decrease in the calculogenic substances to treat the renal impairment formed. Even though some of the parameters measured, such as urinary pH, urine volume at 200mg/kg and serum BUN, were found to be significant, those results alone are insufficient to support its antiurolithiatic activity against ethylene glycol-induced urolithiasis in male Sprague Dawley rats. Also, it was found that the relationship of the dose of the extract to the activity does not demonstrate a dose-dependent relationship. From this research, it can be concluded that there is a lack of promising antiurolithiatic potential due to inability of E. indica extract to suffice all the parameters necessary to assert its antiurolithiatic activity.

RECOMMENDATION

It is recommended to the succeeding researchers to investigate the possible use of the ethanolic extract as a urine alkalinizer since the urinary pH parameter was shown to be significant ($p<0.05$). Also, further research is recommended to determine the specific mechanism of action of Eleusine indica in lowering serum BUN levels.

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