Evaluation of Safety, Antioxidant Effects and Antiurolithiatic Potentials of Selected Ethiopian Medicinal Plant Extracts

Tilahun Alelign Wassie

Addis Ababa University

Addis Ababa, Ethiopia

June 2021

ADDIS ABABA UNIVERSITY
COLLEGE OF NATURAL AND COMPUTATIONAL SCIENCE

Evaluation of Safety, Antioxidant Effects and Antiurolithiatic Potentials of Selected Ethiopian Medicinal Plant Extracts

By

Tilahun Alelign Wassie

A Thesis Presented to the School of Graduate Studies of the Addis Ababa University in Partial Fulfillment of the Requirements for the PhD Degree in Biology (Biomedical Sciences)

Approved by Examining Board:

1. Prof. Beyene Petros (Advisor) _____
2. Dr. Asfaw Debella (Advisor) _____
3. Dr. Endalkachew Nibret (External Examiner) _____
4. Prof. Eyasu Makonnen (Internal Examiner) _____
5. Dr. Adey Feleke (Chair) _____

Addis Ababa University
Department Of Microbial Cellular Molecular Biology
College Of Natural Sciences

Evaluation of Safety, Antioxidant Effects and Antiurolithiatic Potentials of Selected Ethiopian Medicinal Plant Extracts
Tilahun Alelign Wassie, PhD Dissertation
Addis Ababa University, 2021

Abstract

Urolithiasis is a public health problem with limited treatment options. In the Ethiopian folk medicine, there is a claim that medicinal plants can treat urolithiasis. The present study investigated the safety, antioxidant activities and antiurolithiatic efficacies of *Achyrathes aspera*, *Rumex abyssinicus*, *Satureja punctata*, *Chenopodium murale*, *Aloe pulcherrima*, *Chenopodium ambrosioides*, *Inula confertiflora*, *Gomphocarpus fruticosus* and *Commiphora myrrha* extracts. Primarily, thirteen years retrospective clinical records from St. Paul's Tertiary Referral Hospital were retrieved and analyzed to estimate the overall prevalence of urolithiasis in Ethiopia. In addition, plant extracts were tested for acute and sub-acute toxicity in female Wistar rats. The *in vitro* DPPH (2,2-diphenyl-1-picrylhydrazyl) and calcium oxalate (CaOx) assays were measured using UV-Vis-NIR spectrophotometery. Also, urolithiasis was induced in male Wistar rats by feeding ethylene glycolated (0.75%) water for 28 days. Preventive and curative studies were based on oral exposure of rats to 200 mg/kg extracts for 14 and 28 days, respectively. Urine samples were collected on 13^{th} and 27^{th} day before sacrificing the rats, whereas the liver and kidneys collected after sacrificing on days 14 and 28. Blood samples were collected from retro-orbital sinus under the anaesthetic condition. The bioactive constituents of *G. fruticosus* extracts were characterized by GC-MS analysis. Among 32,370 surgically treated patients, referred from all over the country, 2.3% (757) urolithiatic cases were recorded. Trend analysis revealed an increasing prevalence of urinary stones. Acute toxicity tests resulted in body weight loss for *C. murale, R. abyssinicus, C. ambrosioides* and *I. confertiflora* extracts at dose 2000 mg/kg. Moreover, *C. murale* ($p<0.001$) extracts showed significant reduction in platelet count.

Histopathological studies for *R. abyssinicus* extract indicated mild liver injuries in sub-acute toxicity test. The DPPH scavenging capacities of *S. punctata, G. fruticosus,* and *A. pulcherrima* aqueous extracts were 92.3%, 81.6%, and 72.3%, respectively compared to ascorbic acid, the standard therapy in use, which showed 87.6%, 94.5%, and 92.3% at concentrations of 0.20 mg/ml, 0.78 mg/ml and 3.13 mg/ml, respectively. The IC_{50} antioxidant value of *S. punctata* aqueous extract was 0.01 ±0.003 mg/ml, compared to ascorbic acid (0.03 ±0.007 mg/ml). The *in vitro* inhibition of CaOx nucleation at 3200 μg/ml aqueous extracts of *A. pulcherrima* (22.5%), *S. punctata* (26%) and *G. fruticosus* (37.6%) was observed in 30 minutes incubation; whereas their potency of aggregation inhibition at 200 μg/ml was 32.9%, 3.8% and 33.3%, respectively. Likewise, the nucleation inhibition of *G. fruticosus* EtOAc fraction was 56.9%, and its fraction II aggregation inhibition was 58.9% at 3200 μg/ml. Furthermore, *in vivo* studies also confirmed that *S. punctata* aqueous extract had CaOx deposition inhibitory effects in the preventive study ($p<0.001$), whereas *G. fruticosus* aqueous and EtOAc fraction ($p<0.01$) reduced CaOx deposition in curative study. In the urine, serum and kidney homogenates, stone forming constituents were lowered by *G. fruticosus* EtOAc fraction ($p<0.05$). GC-MS analysis of *G. fruticosus* fraction II revealed 29 compounds, of which di-isooctyl phthalate, n-hexadecanoic acid, isoborneol acrylate, and benzoic acid were the major constituents. In general, the *in vitro* and *in vivo* findings were found to be nearly complementary in demonstrating CaOx preventive and therapeutic potencies of *S. punctata* and *G. fruticosus* extracts, respectively. However, further investigation may be required to assess the efficacy of *G. fruticosus* major compounds and *S. punctata* fractions against urolithiasis.

Key words: Antioxidants, GC-MS analysis, *In vitro*, *In vivo*, Medicinal plants, Toxicity, Urolithiasis

Acknowledgements

First of all, I would like to express my deepest gratitude to my supervisor, Prof. Beyene Petros, for offering me his great expertise, guidance, and encouragement from the beginning to the completion of this thesis work. Indeed, he helped me to build up my writing capabilities, which enabled me to write this dissertation. I would also like to extend my gratitude to my co-supervisor, Dr. Asfaw Debella for his appreciable feedback in facilitating the research on animal studies. Without their valuable help, the dream of completing this highly demanding work would not have come to reality.

I express my thanks to Dr. Adey Feleke, Head, Department of MCMB, College of Natural Sciences, Addis Ababa University (AAU) for her encouragement and immense financial support. Also, I thank Dr. Gurja Belay, former Head of MCMB, for his administrative help during the period of my academic study. My heartfelt gratitude extends to Haji Sheh Ali Adem, who was a key informant of a medicinal plant, which I used in the study, and Prof. Zemede Asfaw and Dr. Ermias Lulekal, who helped me in plant species identification. Furthermore, I thank very much Prof. Ariaya Hymete for his insightful feedback and suggestions. I would also like to appreciate the generous support given to me by Mr. Solomon Genet in purchasing a drug-Cystone from Mumbai, India. My especial thanks also go to Dr. Abraham Asefa and Dr. Kidist Bobosha, from the Armauer Hansen Research Institute (AHRI), who helped me to access kits purchased from Bio Vision PLC, USA through AHRI's procurement office. I would also thank Dr. Mekonnen, Mr. Melaku, and Mr. Jibreal from Chemistry Department, AAU who allowed me to use lab equipment, and help with techniques. I also thank Dr. Dawit Solomon from St. Paul's Hospital for his contribution in histopathological studies; Mr. Yosef Tolcha from Ethiopian Public Health

Institution (EPHI), Mrs. Sinknesh Wolde (AHRI), and Mrs. Mekdes Tesfaye (St. Paul's Hospital) for their technical assistance in lab techniques.

I would like to thank Eng. Mesfin Engdawok for his help in lab equipments calibration and maintenance during my study. I appreciate Mrs. Yewubdar (EPHI) and Emahoy (AAU) for keeping clean the houses of the experimental animals. I am also indebted to Mrs. Kidist Terefe, an executive secretary of the Department of MCMB, for her facilitation made during my PhD study. Moreover, I am grateful to all individuals (friends, colleagues and classmates) whose names are not mentioned here for helping me to develop this thesis.

I would like to thank Addis Ababa University (AAU) for its financial support via an adaptive research grant, Debre Berhan University for sponsoring me to attend my PhD studies, St. Paulos hospital surgical staff for giving space during the retrospective study, Traditional and Modern Medicine Research Directorate of EPHI, and AHRI for their support in lab facilities.

Finally, I would like to express my heartfelt gratitude to my wife Mrs. Zinayitu Tafere for helping in plant sample collection, the responsibility of taking care of our lovely kids as well as offering me great encouragement to accomplish my PhD studies. I thank my kids Ruhama and Natnael for bestowing pure love on me whenever I miss them. Lastly, I thank my late father, whom this thesis is dedicated to. Above all, I thank Almighty God for being with me in all the ups and downs, and blessing me to be where I am today.

Table of Contents

List of Figures

List of Tables

List of Appendices

List of Acronyms/Abbreviations

ALP - Alkaline Phosphatase
ALT - Alanine Aminotransferase
ANOVA - Analysis of Variance
Aq - Aqueous extract
AST - Aspartate aminotransferase
b.w.- body weight
CaOx - Calcium oxalate
CaP - Calcium phosphate
CD -Collecting duct
Chl - Chloroform
CHPD - Calcium hydrogen phosphate dihydrate
COD - Calcium oxalate dihydrate/ weddellite
COM - Calcium oxalate monohydrate/ whewellite
DPPH - 2, 2-diphenyl-1-picrylhydrazyl
DT - distal tubule
EDTA-Ethylene diamine tetra acetic acid
EG - Ethylene glycol
ESWL - Extracorporeal shock wave lithotripsy
EtOH - Ethanol
EtOAc- Ethyl acetate
HK-2- human kidney- proximal tubule epithelial cell line
IR - Infrared
LLC-PK1 cells- Lilly Laboratories Cell - Pig Kidney Epithelial Cells
MDCK cells - Madin-Darby Canine kidney epithelial cells
mg/kg- milligram per kilogram
OECD-Organization for Economic Cooperation and Development
Ox - Oxalate
p38 MAPK-p38 mitogen-activated protein kinases
PCNL - Percutaneous nephrolithotomy
PET - Petroleum ether
ppm - Parts per million
PT- Proximal tubule
RBC - Red blood cells
ROS - Reactive oxygen species
rpm - Revolution per minute
SD - Standard Deviation
STD - Standard
THP - Tamm-Horsfall protein
TM -Traditional medicine
URS - Ureteroscopy
WBC - White Blood Cell
WHO - World Health Organization

1. Introduction

1.1. Herbal Medicine

The utilization of medicinal plants is as old as the history of mankind and gave birth to pharmacology, which was derived from human experience with herbal drugs (Dog, 2004). Over the last few decades, there has been a renaissance of interest in medicinal plants, which are culturally acceptable as an alternative treatment for many human diseases. Globally, about 25% of drugs prescribed are developed from plants (Rates, 2001). Herbal medicine is an alternative natural remedy in primary health care in developing countries (Ankur *et al.*, 2010; WHO, 2011). In Africa, it was estimated that 70-80% patients were treated through the help of traditional medicine (TM) (Diallo *et al.*, 1996). In Ethiopia, it has been estimated that 70% of humans and 90% of livestock depend on TM (Bekele, 2007).

Plants synthesize a variety of metabolites, and some of which may be beneficial or potentially toxic to mankind (Kale *et al.*, 2019). Also, it has been true that drugs may be therapeutic at one dose and toxic at another (Sharif *et al.*, 2015), and drugs synthesized from plants continue to be an important source of modern medicine (Newman and Cragg, 2016). In order to ensure safety, there must be a toxicity study of plants claimed to be beneficial to human and animal diseases treatment. Therefore, considering the safety issues of medicinal plants would be necessary before deciding to use them in therapy (Moreira *et al.*, 2018).

1.2. Safety of herbal medicine

Safety is a relative term best evaluated in terms of the expected benefit weighed against the likelihood that the substance will cause harm or adverse reactions. In fact, undesirable effects are the common characteristics of known pharmacological products (Bejar *et al.*,

2004). The *in vitro* and *in vivo* studies provide important information about the safety of medicinal plants, although they have limitations in accurately predicting the physiological effects in humans (Dog, 2004). Traditional practitioners would recognize short term exposure, although they cannot detect long-term exposure effects, which may damage organs such as liver and kidneys (Arsad *et al.,* 2013). Hence, in modern drug development, about one-third of the drug candidates have been reported to require high cost toxicity studies (Guengerich, 2011).

Toxicokinetic and toxicodynamic factors can determine the toxicity of xenobiotics (foreign matter) like herbal extracts. The selective uptake or accumulation of a particular xenobiotic in a specific cell or tissue can lead to toxicity. Alternatively, the inhibition of the normal exports of potentially toxic metabolites from a cell to the outside may cause toxicity (Boelsterli, 2003). Furthermore, the dynamic interaction of toxicants with biological targets such as cells and the activation of cellular receptors can also lead to toxicity (Boelsterli, 2003; Saad *et al.,* 2006).

Acute, sub-acute and chronic toxicity tests are routinely performed during the investigation of natural products or drugs with therapeutic potentials. In acute toxicity test, which is the first step, a single dose of test material is used once to determine the adverse effect of substances within 14 days of administration (Rhiouani *et al.,* 2008; Bhardwaj and Gupta, 2012). The median lethal dose (LD_{50}) for a particular toxic substance is the amount that results in the death of half (i.e. 50%) of the test animals (usually rats or mice). The most frequently determined LD_{50} is through oral route administrations (Gadanya *et al.*, 2011). In sub-acute toxicity test, drugs are daily administered (Bhardwaj and Gupta, 2012), usually from three weeks to three months in rodents (rats), dogs and monkeys (Gandhare *et al.*,

2013; Singh *et al.,* 2014). The starting dose could be that the most likely to cause mortality and other toxic effects in the dosed animals (OECD, 2001). Furthermore, the previous studies administered extracts at dose 2000 mg/kg (Jaganathan *et al.,* 2012; Ogbuehi *et al.,* 2015), and 5000 mg/kg dose for 28 days to determine sub-acute toxicity (Unuofin *et al.,* 2018).

The liver and kidneys are the primary organs affected by metabolic reactions caused by toxicants (Dybing *et al.,* 2002; Saad *et al.,* 2006) and are useful in predicting toxicity effects of phytotherapeutic products or drugs (Bello *et al.,* 2016). The liver is the main target for toxic compounds because of its prior exposure to foreign substances absorbed in the intestine before it reaches the blood circulation (Rhiouani *et al.,* 2008; Samuel *et al.,* 2012). Although toxins may harm the liver, these are detoxified (toxic substances removed) by it (Ravikumar and Gnanadesigan, 2012). In experimental animals, the liver function test would allow to understand the toxic effects, which can be extrapolated for safety if used in humans.

Blood parameters are relevant indicators of potential health hazards and have a higher predictive value for toxicity (Arsad *et al.,* 2013; Ghadirkhomi *et al.,* 2016). The values of calculated red blood cell indices, that is, the mean corpuscular volume (MCV) and the mean corpuscular hemoglobin concentration (MCHC) are used for diagnosis of anemia (Amna *et al.,* 2013). Hematological indices such as packed cell volume (PCV) and hemoglobin (HB) are associated with the total population of red blood cells (RBCs). MCV reflects the size of red blood cells, whereas the mean corpuscular hemoglobin (MCH) and MCHC are used mathematically to define the concentration of hemoglobin (Mahmoud, 2013). The packed cell volume (hematocrit) represents the percentage of RBCs of whole blood volume, which

is clinically used to determine anemia (Wintrobe and Greer, 2009). These biomarkers would help to determine toxicity in relation to dose and time-response (WHO, 1993).

Furthermore, the considerations of platelet indices such as mean platelet volume (MPV), platelet distribution width (PDW) and a platelet large cell ratio (P-LCR) are also used to screen toxicity (Hawkins, 1972; Kaito *et al.,* 2005; Elsewefy *et al.,* 2014). The *in vitro* platelet toxicity assays can be used to evaluate the predictive values of hypersensitivity reactions against drugs (Elzagallaai *et al.,* 2013). Increasing or decreasing platelet counts result in clotting or bleeding abnormalities, respectively. Therefore, the change in platelet function may be induced by drugs, and is important to consider it for toxicity screening (Hawkins, 1972). There are limited scientific evidences regarding safety profiles, although the wider use of the traditional medicine including antiurolithiatic plants in developing countries (Frassetto and Kohlstadt, 2011; Arsad *et al.,* 2013).

Although the mode of actions of most phytotoxins is unclear (Agarwal *et al.,* 2003), it has been suggested that some act on microtubules (Iwasaki, 1998), interfere with nutrient absorption (Leonard and Takayuki, 2009), and exert hypersensitivity (Guengerich, 2011). On the other hand, some polyphenols and flavonoids could be pro-oxidants (chemicals that induce oxidative stress by generating reactive oxygen species or inhibiting antioxidant systems (Halliwell, 2007). It has been also suggested that toxicity may be due to the introduction of soil contaminants such as heavy metals, aflatoxins and pathogenic microbes during the preparation of plant extracts (Parshuram and Sateesh, 2019).

1.3. Antioxidant systems

The formation of free radical (highly reactive oxygen species) through the activity of the respiratory chain in the mitochondria is part of the cell's normal metabolic process, including detoxification process and immune defenses (Elzbieta *et al.,* 2005). Oxidative stress can be initiated if the generation of free radicals exceeds the body's ability to protect the effect of antioxidants. The excessive generation of free radicals such as nitric oxide and peroxynitrite (unstable structural isomer of nitrate) (Polterat, 1997), superoxide anions, hydrogen peroxides and hydroxyl radicals are capable of inducing oxidative damage to the body (Larkins, 1999; Elzbieta *et al.,* 2005). Radicals cause damage to various cellular macromolecules such as DNA, proteins and lipids, which trigger various human chronic diseases (Polterat, 1997; Lobo *et al.,* 2010). However, the proper physiological functions of the body could be maintained by endogenous antioxidant systems (Rahman, 2007), and by ingestion of exogenous antioxidants (Lobo *et al.,* 2010)**.** Therefore, adequate antioxidant defense within and outside cells is important to offer protection against oxidative damage (Sun, 1990).

In aerobic organisms, the defense system against free radicals is provided by scavengers, which act as anti-oxidants. Human cells have an array of protecting mechanisms by preventing the production of free radicals through enzymatic and non-enzymatic antioxidants. The first antioxidant defense may involve enzymes such as superoxide dismutase, catalase and glutathione peroxidase, whereas the non-enzymatic mechanisms include nutrients and minerals (Niki *et al.,* 1994; Aggarwal *et al*., 2005). The glutathione detoxifies hydroxyl radicals (Ighodaro and Akinloye, 2018). The activities of antioxidant enzymes are reduced due to defects in the antioxidant defense system (Mulay *et al.,* 2013),

which can be restored by the antioxidant constituents of medicinal plants leading to protection from renal cell injury (Dinnimath and Jalalpure, 2018). The non-enzymatic antioxidants can be classified as water-soluble or lipid-soluble depending on whether they act primarily in the aqueous phase or in the lipophilic region of the cell membrane. The hydrophilic antioxidants include vitamin C and certain polyphenol flavonoid groups; whereas the lipophilic antioxidants include ubiquinols, retinoids, carotenoids, apocynin and procyanidins (Middleton *et al*., 2000). Ascorbic acid (vitamin C) is a naturally occurring antioxidant found in medicinal plants, vegetables, fruits and whole grains (Sharma and Bhat, 2009).

The antioxidant properties of herbal extracts may protect the body from the deleterious effects of free radicals. Antioxidants block the effects of reactive oxygen species, and reduce the risk of disease. The herbal extracts may exert their antioxidant effects by (i) decreasing the local O_2 concentrations; (ii) removing catalytic metal ions; and (iii) removing reactive oxygen species such as oxygen free radicals and H_2O_2; (iv) scavenging the initiating radicals such as hydroxyl ions; (v) breaking the chain of an initiated sequences; and (vi) quenching oxygen (rearrangement of electrons that produce oxygen rapidly) (Gutteridge, 1994).

1.3.1. DPPH antioxidant activity

An organic compound, 2,2-diphenyl-1-picrylhydrazyl (DPPH) is a dark-red colored crystalline powder composed of free-radical molecules (Sanchez-Mareno, 2002). The DPPH assay is a widely used model to evaluate antioxidants because of its ease of use and reproducibility (Hu *et al*., 2004; Sharma and Bhat, 2009). In the DPPH assay, the initial electron transfer occurs very quickly and the subsequent hydrogen transfer takes place more slowly, and depends on the hydrogen-bond accepting solvents. The DPPH free radical

scavenging effects of antioxidants is due to their hydrogen atom-donating ability (Bortolomeazzi *et al.,* 2007; Fagali *et al*., 2008). In antioxidant assays, a strong hydrogen-bond accepting solvents such as methanol and ethanol are usually used (Foti *et al.,* 2004). The electron transfer assays measure the reducing ability of the substrate (antioxidant), whereas the hydrogen transfer assays measure hydrogen donating ability of the substrate. The previous studies reported that assays that measure hydrogen transfer would be preferable to assays that measure electron transfer (MacDonald-Wicks *et al.,* 2006; Alam *et al.,* 2012).

Absorbance can be measured after the reaction of antioxidants with DPPH, which are dissolved in methanol or ethanol (Sanchez-Mareno, 2002). The DPPH radical has a maximum ultraviolet-visible (UV-Vis) Spectrophotometry absorption in the range between 515 nm and 519 nm (Brand-Williams *et al.,* 1995; Sanchez-Mareno, 2002). The reduction in the absorbance of the DPPH radicals in the solution revealed the action of antioxidant agents (Fagali *et al*., 2008; Rao *et al.,* 2009). Therefore, the ability of the plant extracts to scavenge the DPPH free radicals could contribute to the inhibition of the inflammatory processes (Kaushik *et al.,* 2017). The polyphenol of plant extracts is an excellent antioxidant, which acts by hydrogen donation of its phenolic hydroxyl group to stop free radical chain reactions (John and Shahidi, 2010). On the other hand, the synthetic antioxidants such as butylated hydroxytoluene and butylated hydroxyanisole have been reported to be dangerous for human health (Lobo *et al.,* 2010). Therefore, searching an effective, non-toxic natural antioxidant could be an effective treatment option to manage renal oxidative stress.

1.4. Overview of Urolithiasis

Urolithiasis refers to the formation of stone(s) in the urinary tract (Romero *et al.,* 2010), which is a disease affecting people worldwide (Moe, 2006; Noshad *et al.,* 2014). Stones are mainly lodged in the kidneys (Giannossi and Summa, 2012; Atodariya *et al.,* 2013). The stone diseases present in the kidneys are termed as nephrolithiasis (renal calculi or kidney stones), while in the ureter is described as ureterolithiasis (ureteral calculi), and in the bladder as cystolithiasis (bladder calculi) (Havagiray *et al.,* 2010; Borisov and Dzeranov, 2011). Urolithiasis is a systemic disorder associated with metabolic syndrome (Sakhaee, 2008) of increasing risk of chronic kidney diseases (Rule *et al.,* 2009; Sigurjonsdottir *et al.,* 2015), end-stage renal failure (El-Zoghby *et al.,* 2012; Mikawlrawng *et al.,* 2014), cardiovascular diseases (Worcester and Coe, 2008; Rule *et al.,* 2010), *Diabetes mellitus*, hypertension and obesity (Stamatelou *et al.,* 2003; Taylor *et al.,* 2005; Uyeturk *et al.,* 2014). Urolithiasis (nephrolithiasis) is responsible for 2 to 3% of end-stage renal failure (Courbebaisse *et al.,* 2017).

There is evidence that shows mankind has been afflicted with urinary stones for millennia, dating back to 4000 B.C.(Lopez and Hoppe, 2008). The prevalence of kidney stone diseases is increasing worldwide (Knoll, 2010) with increasing cost of treatment (Durkee and Balcom, 2006). Furthermore, its recurrence remains to be a serious problem in human health (Mikawlrawng *et al.,* 2014), the prevention requires a better understanding of the mechanisms involved in stone formation (Khan *et al.,* 2016). Thus, treating renal stone requires time and money; therefore, it results in adverse consequences on the quality of life in particular and on the nation's economy in general (Baheti and Kadam, 2013b).

The clinical symptoms of kidney stone relate to their location (kidneys, ureter, or urinary bladder) (Kumar *et al.,* 2012a). Thus, the common signs and symptoms of stone diseases are renal colic (intense cramping pain) generally associated with nausea and vomiting, flank pain, hematuria, obstructive uropathy, urinary tract infections, blockage of urine flow, and hydronephrosis (dilation of the kidneys) (Murugan and Satishkumar, 2001; Teichman and Joel, 2004).

1.5. Epidemiology of Kidney Stones

Urolithiasis affects the population of the world in the range of 2% to 20%, at some stage in their lifetime (Buchholz *et al.*, 2003; Indridason *et al.*, 2006; Chauhan *et al.*, 2008). It occurs in all ages, sexes and races (Al-Eisa *et al.,* 2002; Romero *et al.,* 2010), but it is more prevalent among men than women within the age of 20 to 49 years (Edvardsson *et al*., 2013). Calcium oxalate crystallization is of great interest in medicine because it is the main constituent among the majority of kidney stones (Kavanagh *et al.*, 2000; Grases *et al.*, 2002).

Kidney stone prevalence may also be influenced by exposure to the sun, which enhances vitamin D production, leading to an increase in 25-hydroxy vitamin D (Penniston *et al*., 2009). Vitamin D metabolites could increase calciuria and promote urinary stone formation. Also, the steroid hormone 1,25-dihydroxy-cholecalciferol stimulates the intestinal absorptions of calcium by genomic (receptor mediated) and non-genomic (transcaltachia mediated) mechanisms (Norman, 1990). These mechanisms involve the synthesis of a calcium transport protein, which shuttles calcium from the brush border across the basolateral sides of the mucosal cells (Heaney, 2008).

In the past decades, various studies have reported that urolithiasis has been increasing globally. An increasing trend in stone disease is believed to be associated with changes in lifestyle, such as dietary habits, lack of physical activities (Robertson *et al*., 1979; Singh and Sailo, 2013; Sofia *et. al.*, 2016) and global warming (Romero *et al.*, 2010). If patients do not apply metaphylaxis, the relapsing rate of secondary stone formations is estimated to be 10 - 23% per year, 50% in 5-10 years, and 75% in 20 years in the patients (Moe, 2006). However, the lifetime recurrence rate is higher in males, although the incidence of nephrolithiasis is increasing among females (Afsar *et al.*, 2016). In the United States, kidney stone affects 1 in 11 people (Scales *et al*., 2012), and it is estimated that 600,000 people suffer from urinary stones per year (Joseph *et al*., 2005). In Germany, there has been an increase in the prevalence of urolithiasis (Hesse *et al.*, 2003), and it has been reported that kidney stones are frequently formed in men than in women between 20 and 49 years old in Oman, Batinah North Governorate (Al-Risi *et al.*, 2014).

1.6. Kidney Stone Disease in Ethiopia

According to the report of the Ethiopian Ministry of Health (FMoH, 2013), the renal diseases accounted for 1.2 to 6% of the adult hospital admissions. In Ethiopia, deaths due to kidney diseases were 12,038 (1.47%) of the total deaths in the hospitals (Kore *et al..*, 2018). In Ethiopia, urinary tract problems were the 8th leading cause of morbidity in the general population, which affected 931,348 persons. Similarly, urinary tract infections were reported as the 5th (558,551 cases) cause of morbidity among females (FMoH, 2013). Among the top ten causes of morbidity in the general population, urinary tract infections were ranked 7th with 1,471,078 cases (4.26%) (FMoH, 2015). However, reports on the prevalence of urolithiasis are scarce in Ethiopia.

1.7. Etiology of Kidney Stones

The causes of kidney stones are multi-factorial including epidemiological, biochemical and genetic factors (Sayer, 2008; Alaya *et al.*, 2012). This includes intrinsic factors (age, sex and heredity) and extrinsic factors (geography, climate, diet, mineral composition and water intake) (Moe, 2006). A kidney stone is formed due to an imbalance between crystal promoters and inhibitors in the urine (Finlayson, 1974) (Figure 1).

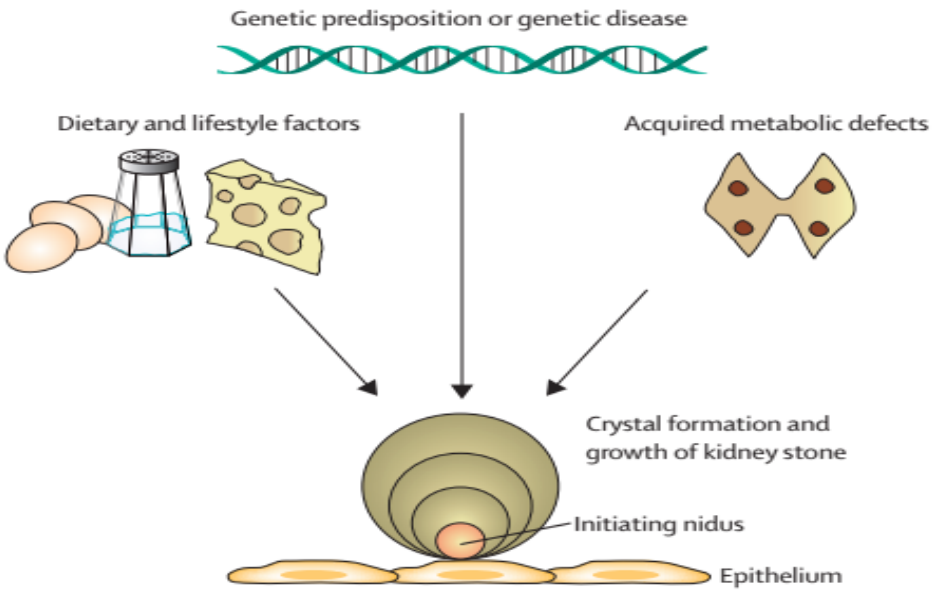


Figure 1. Pathogenesis of kidney stones. Adopted from Moe (2006).

The risk factors for kidney stone formation could be excessive intake of animal proteins (Knoll, 2010), eating food too rich in calories and table salt, deficiencies in fiber and alkali, lack of physical exercise (Straub and Hautmann, 2005), metabolic disorders such as hypercalciuria and hyperoxaluria in the urine (Dursun *et al.*, 2015); hypercystinuria (Dal-Moro *et al.*, 2005); hypercalcemic disorders (hyperparathyroidism) (Giannossi and Summa, 2012); excessive excretion of calcium, oxalate, and uric acid, low levels of citrate and magnesium, and inadequate water intake (Heilberg and Schor, 2006).

Furthermore, the following are also incriminated recurrent urinary tract bacterial infections, which alkalinate the urinary pH by urease enzymes such as *Proteus mirabilis* (Dursun *et al.*, 2015); abnormal genetic disorders on the autosome (Taylor *et al.,* 2005); kidney anatomical abnormalities (Johri *et al.*, 2010); hypertension and obesity (Obligado and Goldfarb, 2008); climate change (global warming); occupation (Giannossi and Summa, 2012); inflammatory bowel diseases (Taylor *et al*., 2005); the absence of intestinal oxalate degrading bacteria such as *Oxalobacter formigenes* (Kwak *et al.,* 2003) and lithogenic drugs such as indinavir (Dursun *et al*., 2015).

In addition, hyperoxaluria caused by intestinal absorption of excess oxalic acid is the major risk factor of kidney stone diseases (Khan *et al.,* 1992; Menon and Koul, 1992). It has been also postulated that an increased dietary protein intake, alters renal excretion and increases hepatic oxalate production (Menon and Koul, 1992).

1.8. Types of Kidney Stones

Stones differ in size, shape and chemical composition (Chhiber *et al*., 2014). Based on variations in mineral composition and pathogenesis, kidney stones are commonly classified into five types (Barbasa *et al*., 2002).

1.8.1. Calcium Stone

Calcium stones are dominant stone types in the kidneys with a frequency of 60-85% (Khan, 1997; Parmar, 2004; Coe *et al.,* 2005; Moe, 2006). Calcium stones account for pure calcium oxalate (CaOx) (50%), calcium phosphate (CaP, termed as apatite) (5%), and a mixture of CaOx and CaP (45%) **(**Chaudhary *et al*., 2010). Calcium hydrogen phosphate or hydroxyapatite is the main constituent of calcium stones (Coe *et al*., 1992; Skolarikos *et al*., 2015). Calcium oxalate exists in nature in three different hydrated forms. Calcium oxalate

monohydrate (COM, $CaC_2O_4 \cdot H_2O$; termed as whewellite), calcium oxalate dihydrate (COD, $CaC_2O_4 \cdot 2H_2O$, weddellite), and calcium oxalate trihydrate (COT, $CaC_2O_4 \cdot 3H_2O$, Caoxite) (Ouyang *et al.*, 2006). The main disease forms are hypercalciuria, primary hyperparathyroidism, and low urinary citrate level (Parmar, 2004; Moe, 2006).

Among the different forms of calcium oxalate, COM is thermodynamically the most stable type followed by COT and COD (tetragonal), respectively. COM and COD are the major constituents of most kidney stones (accounts greater than 60%), but COT is not often found in the urine. It is believed that COT act as the precursor of COD and COM crystal formation (Deganello, 1986; Ouyang *et al.*, 2006). COM is commonly observed in clinical stones than COD, because it is the most thermodynamically stable (Basavaraj *et al*., 2007) and with sharp edges that can physically scratch or injure the renal epithelial cells. In addition, the COM structure has a large oxalate-rich face, which has been found to be harmful to the renal epithelial cells (Hackett *et al*.,1995; Wiessner *et al.*, 2001). The other two hydrate structures have rounder and smoother edges. Mostly, urinary pH of 5.0 to 6.5 promotes CaOx stones (Kishore *et al.*, 2013), whereas CaP stones occur when the pH is greater than 7.5. The recurrence of calcium stones is higher than other stone types (Kumar *et al*., 2012a).

1.8.2. Struvite Stone

Struvite stones are a mixture of magnesium, ammonium and phosphate (Parmar, 2004; Moe, 2006). They occur to the extent of 10-15% and are also known as infectious stones or triple phosphate stones since they are common among patients with chronic urinary tract infections producing urease, the most common pathogen being *Proteus mirabilis* and the less common pathogens consists of *Klebsiella pneumonia*, *Pseudomonas aeruginosa* and

Enterobacter species (Coe *et al*., 2005; Giannossi and Summa, 2012). Urease is necessary to split/cleave urea to ammonia and CO_2, making the urine more alkaline, which elevates pH (typically greater than 7). Phosphate precipitates on to the insoluble ammonium products and yields a large staghorn stone (Griffith, 1978). Thus, women are more likely to develop this type of stone than males due to their biological differences for infections (Dursun *et al.*, 2015).

1.8.3. Uric acid Stone

Uric acid stone accounts for approximately 3 to 10% of all stone types (Coe *et al.*, 2005; Giannossi and Summa, 2012), and is also known as urate, and its composition is uric acid anhydrous/dihydrate (Parmar, 2004; Moe, 2006). The main causes are animal protein diets such as meat and fish, which contain high purines resulting in hyperuricosuria, low urine volume, and acidic urinary pH (less than 5.05) that exacerbate uric acid stone formation (Ngo and Assimos, 2007; Kumar *et al.*, 2012a). The most important cause of uric acid is idiopathic and is more common in men than in women (Dursun *et al.*, 2015).

1.8.4. Cystine Stone

This stone comprises less than 2% of all stone types. Cystine stones are genetic disorder in the transport of amino acids (cystine), which results from excess cystinuria excretions in the urine (Coe *et al.*, 2005; Giannossi and Summa, 2012). It is an autosomal recessive defect of rBAT gene on chromosome 2 (Ahmed *et al.*, 2006), resulting in impaired renal tubular absorption of cystine (or leaking cystine into the urine) (Kumar *et al.*, 2012a). People who are homozygous for cystinuria excretes more than 600 millimole insoluble cystine per day (Barbasa *et al.*, 2002). Urinary cystine detection is the only clinical manifestation of cystine stones (Ahmed *et al.*, 2006).

1.8.5. Drug Induced Stones

Lithogenic drugs such as guaifenesin, triamterene, atazanavir and sulfa drugs could induce stones, which account for 1% of all stone types (Giannossi and Summa, 2012). For instance, people who take the protease inhibitors such as indinavir sulphate, a drug used to treat HIV infection and other drugs are at risk of developing kidney stones (Barbasa *et al*., 2002). The metabolites of drugs may deposit to form a nidus or added on preexisting renal calculi. On the other hand, the metabolic actions of drugs may interfere with calcium oxalate or purine metabolisms (Dursun *et al*., 2015).

1.9. Kidney Stone Compositions

Kidney stones are composed of organic and inorganic components (Khan, 1995a). The chemical composition of kidney stones depends on variations in urine chemical compositions. Approximately 98% weight of a kidney stone is crystalline inorganic content, 90% of which is made of CaOx mixed with calcium phosphates (Deganello, 1986). The organic matrix (non-crystalline content) of urinary stones consists of macromolecules such as glycosaminoglycans, lipids, carbohydrates, and proteins, which promote or inhibit kidney stone development. The stone matrix comprises mainly proteins (64%), non-amino sugars (9.6%), hexosamine as Glucosamine (5%), water (10%) and inorganic ash (10.4%). The matrix acts as a template, which contains about 8.6% of phospholipids and participates in the assembly of stones. The phospholipids of the cell membrane, as part of the organic matrix, promotes the formation of CaOx and CaP stones (Aggarwal *et al.*, 2013). Albumin is the major component of the matrix of all stone types (Khan and Kok, 2004). Brushite stones are hard phosphate minerals with an increasing incidence rate, and a quarter of calcium phosphate (CaP) former patients contain brushite stones (Krambeck *et al*., 2010a).

In the urinary tract, CaP may be present in the form of hydroxyapatite, carbonate apatite, or brushite (calcium monohydrogen phosphate dihydrate, $CaHPO_4 \cdot 2H_2O$). Brushite is resistant to shock wave and ultrasonic lithotripsy treatment (Krambeck *et al*., 2010b).

1.10. Kidney Stone Formation

The pathogenesis of kidney stones (biomineralization process) is a complex biochemical event, which remains incompletely understood (Aggarwal *et al.*, 2013). Renal stone formation is a biological process that involves various physicochemical changes. In supersaturated urine, solutes precipitate in the urine, leading to nucleation and then, crystal concretions (Basavaraj *et al*., 2007). Crystallization is the first phase when the concentrations of two ions exceed their saturation point in the solution (Parmar, 2004). Supersaturation is a main driving force, which occurs when the concentration of ionic species in the solution exceeds the saturation point.

The transformation of liquids to a solid phase is influenced by pH and specific concentrations of excess substances. The level of urinary saturation with respect to stone-forming constituents like calcium, phosphorus, uric acid, oxalate, cystine, and low urine volume are risk factors for crystallization (Malhotra, 2008; Giannossi and Summa, 2012) and stone formation. The crystallization process depends on the thermodynamics (the rate of nucleation) and kinetics (rate of crystal growth) of a supersaturated solution (Kok *et al.*, 1990).

It is also known that stone formation depends on the level of imbalance between urinary inhibitors and promoters of crystallization. All stones share similar events with respect to the mineral phase of stone formation. But, the sequence of events leading to stone formation differs depending on the type of stone and urine chemistry. For instance, crystallization of

calcium-based stones (calcium oxalate or calcium phosphate) occurs in supersaturated urine if it is with low concentrations of inhibitors. Uric acid interferes with the solubility of CaOx and promotes its formation and the crystallization process is prevented by inhibitors in the urine (Giannossi and Summa, 2012). The sequence of events that trigger stone formation includes nucleation, growth, aggregation, and retention of crystals within the kidneys (Chhiber *et al.,* 2014; Cunningham *et al.,* 2016).

1.10.1. Nucleation

The first step in kidney stone development begins with nuclei formation (termed as nidus) from supersaturated urine retained in the kidneys (Khan and Kok, 2004; Kumar *et al.,* 2012a). In a supersaturated liquid, free atoms, ions, or molecules start forming microscopic clusters that precipitate when the bulk free energy of the cluster is less than that of the liquid. That is, for nucleation to occur, the system has to overcome the Gibbs free energy barrier (Khan, 1995a; Christmas, 2001). Once a nidus has been achieved (or if it is anchored), the overall free energy is decreased by adding new crystal components to its surface (Bawari *et al.,* 2018). That is, less energy is required to precipitate more crystal layers, and the total free energy of the cluster is increased by the surface energy (Khan *et al*., 1996). Nucleation starts in supersaturated solutions if crystal promoters exceed that of inhibitors (Basavaraj *et al*., 2007). In other words, the charged ions of calcium and oxalate combine to form calcium oxalate crystals, which become insoluble crystal (Basavaraj *et al.,* 2007). Nucleation may be formed in the kidney through free particle or fixed particle mechanisms (Basavaraj *et al*., 2007; Evan, 2010).

A crystal can occur by homogeneous nucleation when local supersaturation allows the spontaneous organization of the atoms into the appropriate lattice. Heterogeneous nucleation

is more likely to occur within complex mixtures of molecules used to form the initial crystal lattices (Cerini *et al.,* 1999). The presence of cellular debris could induce nucleation by changing from homogeneous to heterogeneous systems. Renal tubular cell injury promotes CaOx crystallization by providing substances for heterogeneous nucleation (Fasano and Khan, 2001). Existing epithelial cells, urinary casts, RBCs, and other crystals in urine can act as nucleating centers in the process of nuclei formation (heterogeneous nucleation) (Aggarwal *et al.,* 2013). The kidney walls are protected by an anti-adherent glycosaminoglycan layer, in which nucleation can only occur at certain damaged areas or at interstitial sites (Evan *et al.*, 2003; Ratkalkar and Kleinman, 2011).

The organic matrix such as mucopolysaccharides act as binding agents for heterogeneous nucleation and aggregation (Ahmed *et al.,* 2016). In addition, nanobacteria potentiate stone formation by forming apatite structures serving as crystallization center for stone formation (Kajander and Ciftcioglu, 1998). On the other hand, oxalate degrading bacteria, such as *Oxalobacter formigenes* prevent CaOx stone formation (Xu *et al.,* 2013). Thus, treatment targeting nucleation intervention is one of the best approach to control kidney stone formation.

1.10.2. Crystal growth

Crystal growth is the major step of stone formation following nucleation. The crystals in the urine have to stick together to become a hard mass of stones. Crystal growth is determined by aggregations of preformed crystals or secondary nucleation's on the matrix-coated surfaces. It is normal that calcium oxalate will nucleate crystals inside kidneys due to supersaturation of calcium and oxalate in the urine. However, in non-stone forming patients, calcium oxalate crystals are small in size (less than 2 μm) and are excreted out of the

nephrons without causing damage to the epithelial cells (Ratkalkar and Kleinman, 2011). On the other hand, crystal growth may be enhanced by changes in supersaturation, pH, and defects in crystal structure. Stone growth is a slow process and requires a longer time to obstruct the renal tubules (Basavaraj *et al*., 2007). In the organic matrix, both Tamm-Horsfall proteins and Osteopontin are promoters of CaOx stone formation (Knoll, 2010). Lipids of cellular membranes are basically believed to be involved in nucleation of crystals (Khan *et al*., 1996).

1.10.3. Crystal aggregation

Crystal aggregation is considered to be the most critical step in kidney stone formation. In the process, the small hard mass of crystals in a solution stick together to form a larger stone (Aggarwal *et al.*, 2013). Large stone formation is initiated by crystals retained in the kidneys that cannot pass through the nephrons. If the attractive forces between particle interactions (crystal-crystal interactions**)** dominate the overall forces in the solution, crystal aggregation occurs (Khan, 1995b; Christmas, 2001), which is determined by a balance of aggregating and disaggregating forces. The smaller inter-particle distances, increase the attractive forces and favour particle aggregations (Basavaraj *et al.*, 2007). All models of CaOx urolithiasis have shown that crystal aggregations involve the crystal retention within the kidneys (Aggarwal *et al.*, 2013).

1.10.4. Crystal cell interaction

Crystal-cell interaction involves the attachment of crystals with the epithelial cells of renal tubules (Schepers *et al*., 2002; Aggarwal *et al*., 2013). Renal stone formation requires persistent retention of crystals in the kidneys after crystallization process is completed (Lieske *et al.*, 2000; Wiessner *et al*., 2001). Urothelium is generally thought to be resistant

to crystal adherence; however, chemical or mechanical damage to the urothelial may promote crystal binding and aggregation (Gnessin *et al.,* 2010). Two hypotheses have been forwarded for retention of urinary stones, which are either found freely (free particle hypothesis), or attached to the renal papillae (fixed particle hypothesis) (Kok and Khan, 1994).

According to the free particle hypothesis¸ nucleation occurs in the tubular lumen, and crystals rapidly aggregate and grow large enough to get stuck within the tubular lumen (Gnessin *et al.,* 2010). According to the fixed particle hypothesis, it has been proposed that crystals adhered to a fixed point such as renal epithelial cells or Randall's plaque (Evan *et al.,* 2006; Evan *et al.,* 2015). That is, the beginning of calcium phosphate (CaP) deposition in the interstitium is nuclei formation. The formation of CaP in the basement membrane of the loops of Henle, the inner medullary collecting ducts, and the ducts of Bellini serves as an attachment site for stone development. Idiopathic stone formers develop CaOx attached to fixed sites of interstitial plaque. Stones of the distal tubular acidosis attach to the plugs protruding from dilated ducts of Bellini, whereas cystinuria stones do not attach to the renal plagues and found freely (Evan, 2010). Calcium phosphate, uric acid, or cystine crystals formed in the renal tubules plug at the terminal collecting ducts. When mineralization reaches the renal papillary surface, plaque ruptures the surface, exposing CaP crystals to the pelvic urine. Following this, urinary macromolecules deposited over CaP crystals and promotes CaOx depositions (Khan *et al.,* 2016).

Calcium oxalate crystals aggregate in the renal tubules due to an increased retention force between crystals and injured tubular epithelial cells, which promote crystallization (Verkoelen *et al.,* 2000). Experimental findings demonstrated that stone calcification is

triggered by reactive oxygen species and the development of oxidative stress (Khan, 2014), which are thought to be one of the factors involved in renal cell injury (Khan *et al.*, 2002). Although most stones are found in the renal pelvis, stone forming process actually starts in the nephrons, and crystals frequently get precipitated in the renal tubular fluids of stone-forming as individuals (Kok and Khan, 1994). Due to exposure to high oxalate concentrations or sharp calcium oxalate monohydrate (COM) crystals, tubular cells could be injured(Tsujihata, 2008; Courbebaisse *et al.*, 2017).

The interaction of COM crystals with a surface of renal epithelial cells is a critical initiating event in nephrolithiasis (Verkoelen *et al.*, 2000). The concentration of oxalate is higher in the renal collecting ducts (~0.1-0.5 mM) than in the proximal tubules (Wiessner *et al.*, 2001). Crystal-cell interactions result in the movement of the crystals from the basolateral side of the cells to the basement membrane (Courbebaisse *et al.*, 2017). Then, crystals could be taken into the cells and anchored to the basement membrane of the kidneys. However, most of the crystals attached to epithelial cells are thought to be digested by macrophages and/or lysosomes inside cells and then discharged with urine (Tsujihata, 2008). Following renal cellular degradations, numerous membrane vesicles produced, which are nucleators of calcium crystals as supported by *in vitro* and *in vivo* studies (Aggarwal *et al.*, 2013). The injured cells release substances like renal prothrombin fragment-1, or other anionic proteins, which induce COM crystal agglomerations (Moryama *et al.*, 2005).

Injured cells potentiate to invert the cell membrane, which is anionic to the urinary environment and acts as the site of crystal adherence. COM crystals have a stronger affinity of attachment towards the inverted anionic membrane (Khan *et al.*, 2002), than calcium oxalate dihydrate (COD) crystals (Barros *et al.*, 2003). A study reported that COM crystals

deposited in Madin-Darby canine kidney (MDCK) epithelial cells than proximal tubular epithelial cells of pig kidney (LLC-PK1) cells (Rabinovich *et al.,* 2006). The addition of CaOx crystals onto MDCK cell lines showed an increase in the release of lysosomal enzymes, prostaglandin E2, and cytosolic enzymes (Rashed *et al.*, 2004). This may be due to the presence of a binding molecule such as hyaluronan on Madin-Darby canine kidney epithelial cells for COM crystal attachment (Verkoelen *et al.,* 2000). Although the detailed mechanisms of crystal-cell interactions remain unexplored, treatment targeting this step could be an option.

COM crystals rapidly adhere to microvilli on the renal tubular cell surface, and subsequently internalized (endocytosized or engulfed). Crystals may be endocytosed by cells are transported to the interstitium. It has been suggested that injured cells develop a nidus which promotes the retention of particles on the renal papillary surface (Fasano and Khan, 2001). The polyanion molecules present in tubular fluid (urine) such as glycosaminoglycans, glycoproteins, and citrate may coat crystals and inhibits the binding of COM crystals to the cell membrane (Aggarwal *et al.,* 2013). The *in vitro* (Asselman *et al*., 2003; Singhto *et al.,* 2010) and the *in vivo* (Yamaguchi *et al.,* 2005; Hirose *et al*., 2010) studies have demonstrated that CaOx crystals are toxic to renal epithelial cells which produce injury and renal cell death. Similarly, hypercalciuria produces cellular injury, and reactive oxygen species induced lipid peroxidation, which can stimulate the CaOx deposition (Xi *et al.,* 2015).

Exposure to higher levels of oxalate or CaOx crystals induce epithelial cell injury, which is a predisposing factor for the subsequent stone formation (Khan and Thamilselvan, 1999; Khan, 2004). CaOx crystal depositions in the kidney up regulate the expression and

synthesis of macromolecules that can promote inflammation (Hackett *et al.,* 1994). A study on animal models also revealed that administration of high concentrations of CaOx crystals or oxalate ions appears to be toxic, causing renal tubular cell damage (Aggarwal *et al.,* 2013). It has been suggested that oxalate increases the availability of free radicals by inhibiting enzymes responsible for their degradation. For instance, reactive oxygen species can damage the mitochondrial membrane and reduces its transmembrane potential. These events are known features of early process in the apoptotic pathway (Chaturvedi *et al*., 2002). Among individuals with hyperoxaluria, the renal tubular cells are injured and crystals become attached to them (Tsujihata, 2008). Modulators targeting the steps from super-saturation to crystal retention may be a potential means to block stone formation (Aggarwal *et al.,* 2013).

1.10.5. Randall's Plaques

Randall's plaques appear to be the precursor of urinary stone formation, although it is not clear whether it involves in all stone types or not (Ratkalkar and Kleinman, 2011). Initially, CaP crystals along with organic matrix are deposited at the basement membranes of the loops of henle or collecting ducts producing membrane vesicles at the basal side, which leads to plaque formation (Khan, 2014). Most of CaOx stones are attached to renal papillae at sites of Randall's plaque (Evan, 2010), and are located in the interstitial basement membrane of the loop of Henle (Evan *et al.,* 2003; Evan *et al.,* 2006), extended into the interstitial spaces to the urothelium (Knoll, 2010). Calcium phosphate (apatite) and purine crystal compositions were identified within the plaques (Daudon *et al.,* 2015). Evidences suggest that primary interstitial apatite crystal formation leads to CaOx stone formation (Knoll, 2010). Due to renal cell injury, plaque is exposed to supersaturated urine. In

supersaturated urine, the crystals adhere to the urothelium, which enhance subsequent stone growth (Dawson and Thomson, 2012).

The epithelial cell damage (degradation) products promote heterogeneous nucleation and crystal adherence to renal cells. The Randall plaque calcification is triggered by oxidative stress. Cells may express molecules at distal and collecting tubules, which act as crystal binding sites such as Phosphatidylserine, CD44, Osteopontin, and hyaluronan (Yuen *et al.*, 2010; Chhiber *et al.*, 2014). Thus, the apatite crystal deposits could act as nidus for CaOx stone formation by attaching to matrix molecules (Knoll, 2010; Khan, 2014). However, the driving forces and the matrix molecules involved in plaque formation remain elusive. A summary of the various steps involved in stone formation is illustrated in Figure 2.

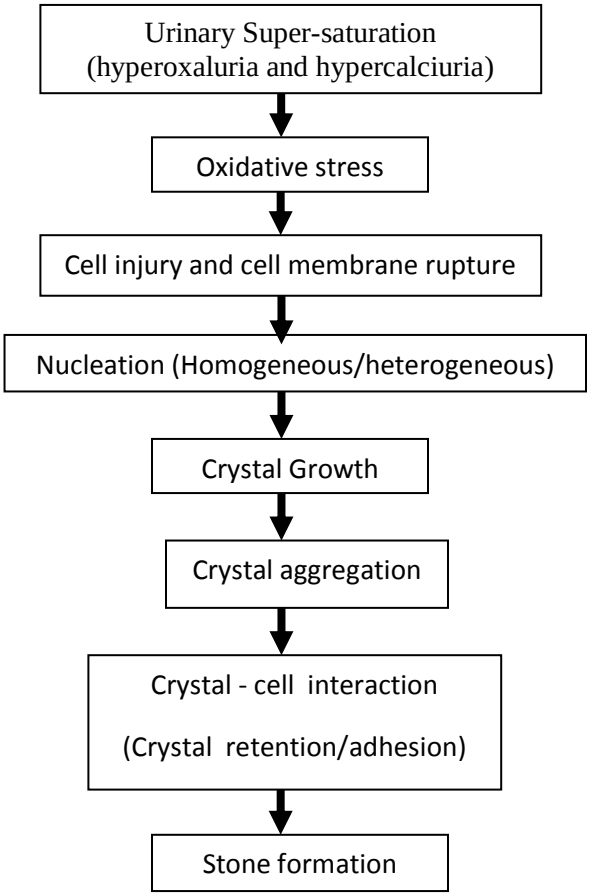


Figure 2. **Schematic representation of various events of kidney stone formation**.

1.10.6. Promoters and Inhibitors of Stone Formation

The process of stone formation is influenced by several promoters and inhibitors, as well as some morphoanatomic, dietary and environmental factors. Naturally, urine contains a stone inhibitor and promoter chemicals. Trace elements in the urine, such as fluoride, iron, iodine, manganese, molybdenum, nickel, selenium, silicone, germanium, vanadium, copper, zinc, chromium, and lithium are found to initiate the process of crystallization. These elements act as a nucleus for the formation of the stone, or influence the external morphology of growing crystals (Słojewski, 2011). On the other hand, crystal formation inhibitors in the urine include small organic anions (such as citrate), small inorganic anions (pyrophosphates), multivalent metallic cations (magnesium) (Ratkalkar and Kleinman, 2011; Aggarwal *et al*., 2013). Stone formation inhibitors may act either directly by interacting with crystals or indirectly by influencing the urinary environment (Khan and Kok, 2004). When inhibitory compounds adsorb onto the surface of the crystal, they inhibit nucleation, crystal growth, aggregation or crystal-cell adherence (Aggarwal *et al.,* 2013). Inhibitors decrease the initiation of supersaturation, nucleation, crystal growth and rate of aggregation (Basavaraj *et al.,* 2007). However, inhibitors do not work equally for everyone and hence some persons form stones while others do not.

Promoters are substances, which facilitate stone formation (Ratkalkar and Kleinman, 2011), such as oxalate, calcium, sodium and cystine (Basavaraj *et al.,* 2007). The genetic defects (dysfunction) of renal epithelial cells influence the urinary constituents, leading to stone formation (Khan and Canales, 2009). Among recurrent stone formers, urinary oxalate excretion was higher, whereas citrate excretion was lower (Cakıroglu *et al.,* 2016). A study also indicated that oxalate can facilitate chloride, sodium and water re-absorption in the

proximal tubules and activate multiple signaling pathways in renal epithelial cells (Marengo and Romani, 2008). Generally, an imbalance between urinary stone inhibitors and promoters has been suggested to be the cause of stone formation (Basavaraj *et al.*, 2007). The location of stones may vary as indicated in Figure 3.

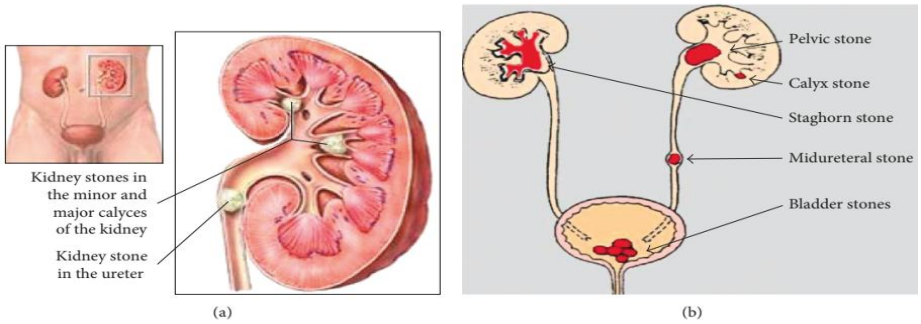


Figure 3. **Kidney stone locations in the urinary system.** (**a**) Adopted from Zahid *et al.* (2013); and (**b**) Adopted from Evan (2010).

1.11. Prevention of Urolithiasis

Prevention of urolithiasis depends on addressing the causes of stone formation, dietary issues, and medical therapy. Regardless of the underlying etiologies and drug treatments, kidney stone diseased patients should be instructed to increase water/fluid intake, which reduces urinary saturation and dilutes the promoters of CaOx crystallization by enhancing urine output at least 2 liters per day (Trinchieri, 2013; Prezioso *et al.*, 2015).

The dietary recommendations for hyperoxaluric patients is low intake of oxalate, and to increase the intake of dietary calcium (Xu *et al.*, 2013). In contrast, some studies advise restriction of dairy products and foods with high calcium content for those who are calcium stone formers (Tiselius, 2000). However, it is also reported that a reduced intake of calcium leads to an increased intestinal absorption of oxalate, which in turn facilitates stone formation. Therefore, calcium supplements may reduce oxalate absorption because calcium binds with dietary oxalate in the gut lumen. On the other hand, vitamin C intake should be

limited because of conversions of ascorbic acid to oxalate under *in vivo* conditions, which results in stone formation(Park and Pearle, 2007). Therefore, dietary intake needs adjustment based on the individual's metabolic abnormalities.

A high sodium chloride intake boosts the risk of stone formation (Trinchieri, 2013) by reducing renal tubular calcium absorption and increasing urinary calcium (Park and Pearle, 2007; Prezioso *et al.,* 2015). Lowering animal protein intake is encouraged (Prezioso *et al.,* 2015) because of its high content of sulfur-containing amino acids, which increases acid load (Trinchieri, 2013). Thus, high protein intake reduces urine pH and the level of citrate, which enhances urinary calcium excretion via bone reabsorption. Therefore, if a person is with very high acidic urine, it is recommended to eat less meat, and avoid food with vitamin D (NIH, 2005). However, increasing intake of fruits and vegetables rich in potassium are recommended (Heilberg and Schor, 2006).

In the prevention of calcium oxalate, cystine and uric acid stones, urine should be alkalinized by eating a diet high in fruits and vegetables, taking supplements (prescription citrate) or drinking alkaline mineral water. In uric acid stone formers, gout needs to be controlled and in the case of cystine stone formers, the intake of sodium and proteins need to be restricted. In the prevention of calcium phosphate and struvite stones, urine should be acidified. For struvite stones, acidifying the urine is the single most important step (Frassetto and Kohlstadt, 2011). To prevent primary or secondary episodes of kidney stone formation, the proper management of dietary intervention is required, which is a low cost public health initiative compared to its treatment strategy (Samal *et al.*, 2011). The current treatment modalities are not effective to prevent urolithiasis.

1.12. Treatment of Urolithiasis

1.12.1. Diagnosis

Diagnosis of urinary stone starts with a physical examination, patients medical history, dietary data, complete blood cell count, routine urine analysis, and serum creatinine measurement (Tchey *et al.,* 2011). Furthermore, ultrasound and X-ray or computerized tomography (CT) scan, can be utilized. Some stones may not be visualized in X-ray, therefore, Ultrasonography is preferred as it detects all types of kidney stones (Frassetto and Kohlstadt, 2011). The choice of intervention depends on various factors like metabolic abnormalities (Malhotra, 2008), stone composition analysis (Giannossi and Summa, 2012), size of calculi, degree of obstruction, the status of kidney function, location of stone(s) and its density, and associated infections. The size of stones less than 5 mm in diameter has a high chance of being passed out of the body without any problem, while those of 5 to7 mm in diameter have a 50% chance of removal, and those over 7 mm in diameter always required urological interventions (Giannossi and Summa, 2012). However, the rate of stone passage with urine is highly variable, and depends on its size and location.

1.12.2. Surgical Treatments

The various approaches used to manage stone diseases comprise surgical treatments such as extracorporeal shock wave lithotripsy (ESWL), ureteroscopy (URS) (Knoll, 2007; Butterweck and Khan, 2009), Percutaneous Nephrolithotomy (PCNL) and open surgery (Butterweck and Khan, 2009). Open surgery is performed to remove larger stones, which block the flow out of urine, damage kidney tissues and causes bleeding (Cunningham *et al.*, 2016), and it is an invasive treatment option with major complications. However, more than 50% of kidney stone patients face the problem of kidney stone recurrence and have to

repeat the surgery again and again. Therefore, alternative methods such as extracorporeal shock wave lithotripsy (ESWL) was developed in order to manage kidney stone diseases.

ESWL uses sound waves to break stones into small pieces, and it excretes them out safely. The shock wave impulse is created outside the body in a therapeutic dose, and projected through the body tissues, until it hits stones to break down into sand-like particles that can easily pass out with patient's urine (Silberstein *et al.,* 2008). However, treatment of calcium oxalate monohydrate and cystine stones using ESWL is less effective than treatment of calcium oxalate dihydrate and uric acid stones (Srisubat *et al.,* 2009). In addition, it does not prevent the recurrence of kidney stones since the remaining stone residual fragments in the kidneys could be sites for new stone formation (Bhaskar and Shelke, 2012). Therefore, patients have to go a repeated or long term ESWL therapy that causes undesirable effects such as hypertension, acute renal failure (Butterweck and Khan, 2009), acute renal hemorrhage (Bhaskar and Shelke, 2012), surrounding tissues and abdominal organs damage (Gecit *et al.,* 2014; Hassan and Zietlow, 2002), tubular necrosis, acute renal injury (Bhaskar and Shelke, 2012) and *Diabetes mellitus* (Krambeck *et al.,* 2006).

1.12.3. Drug Therapy

There are several measures usually taken to manage urolithiasis (Sasikala *et al.,* 2013), and the treatment includes anti-inflammatory drugs, calcium antagonists, α-blockers (Masarani and Dinneen, 2007), and antibiotics (Rivers *et al*., 2000; Atmani *et al*., 2003). Although progress has been made in the last decades to improve therapy, the current pharmacological agents are not effective and safe (Heilberg and Schor, 2006), with low success rate, and infertility as well as stone recurrences on long term use (Mattle and Hess, 2005; Samal *et al.,* 2011).

Treatment of urolithiasis focuses on preventing stone recurrences and dissolving existing stones. Several oral supplements have been given to prevent stone recurrences. Among these, citrate and magnesium salts ($MgCl_2$) are known inhibitors of kidney stone formation for they competitively bind with free oxalate ions forming water soluble complexes (Hammarsten, 1929).

1.12.3.1. Potassium citrate

Potassium citrate is an alkali allopathic agent used to treat kidney stones, and renal tubular acidosis (Coe *et al.,* 2005; Zerwekh *et al.,* 2007). It is salt of citric acid, white, odorless and hygroscopic crystalline powder with a saline taste. It was approved in the United States by Food and Drug Administration in 1985 to treat a wide variety of disorders that cause stone formation (Spivacow *et al*., 2010). Potassium citrate (K-Cit) has been the mainstay of medical intervention for more than 25 years (Robinson *et al.,* 2009). It is commonly used with thiazide diuretics in the management of recurrent hypercalciuric nephrolithiasis (Odvina *et al.,* 2003). Potassium citrate is a low molecular weight inhibitor of crystallization acting through raising urinary citrate and pH levels (alkalinization), reducing calcium concentrations by complexing calcium, and increasing the solubility (dissociation) of uric acid. Citrate reduces saturation of calcium phosphate and calcium oxalate and inhibits CaOx crystallization and enlargement (Spivacow *et al*., 2010).

However, the most commonly used drugs like thiazides (diuretics) and alkali-citrates (stone recurrences inhibitors) are less efficacious (Pak, 1989). Potassium citrate (2.5 g/kg) is widely used as oral supplement in the treatment of CaOx urolithiasis (Serhat and Kupeli, 2006; Siddiqui *et al.,* 2018), but some patients remain stone formers (Mattle and Hess, 2005). The long-term use of potassium citrate (14 years) decreased stone formation by 93%

(Robinson *et al.,* 2009). Despite the wider use of citrate therapy for prevention and treatment of CaOx stones, the evidence to support its clinical efficacy remains uncertain (Phillips *et al.,* 2015). Therefore, there is a need to look for alternative therapies for urolithiasis.

1.12.3.2. Cystone

Cystone is a marketed polyherbal formulation approved by Ministry of Health and Family Welfare of India's Drug Regulatory Authority (Jayaramaiah *et al.,* 2013) since 1943. It has been developed for treatment of urolithiasis and urinary tract infections (UTI), and is manufactured and sold virtually worldwide by Himalaya healthcare. In the United States, this product is known as uricare (Erickson *et al.,* 2011). It's treatment efficacy against urolithiasis was observed to be 80% with negligible adverse effects in clinical studies (Karamakar and Patki, 2008). However, in another study, cystone showed a complete cure for 54.4% urolithiatic patients, where expulsion of the bigger-sized renal stones ranging from 7 to 12 mm was observed (Palaniyamma and Jeyaraman, 2017). In contrast, the study did not support the reported efficacy of cystone for CaOx stone treatment following 6 to 52 weeks administration, which rather increased stone formations (Erickson *et al.,* 2011).

A cystone tablet comprises the following plant extracts in definite proportions: *Didymocarpus pedicellata* R. Br. (Stem) (130mg); *Saxifraga ligulata* (whole plant) syn. *Bergenia ligulata*/ciliate (98 mg); *Onosma bracteatum* Wall. (roots) (32 mg); *Cyperus scariosus* R. Br. (Rhizome) (32 mg); *Achyranthes aspera* L. (roots) (32 mg); *Rubia cordifolia* L. (whole plant) (32 mg); *Vernonia cinerea* (whole plant) (32 mg); *Asphaltum punjabianum* (a kind of mineral resin that exudates from the cracks of the rocks) (purified) (26 mg),; *Lapis judaicus* (seed) (32 mg) (Karamakar and Patki, 2008; Erickson *et al.,* 2011;

Dulanjali and Srikaran, 2020), and *Cyanthillium cinereum* (L.) H.Rob. (whole plant) (weight is not stated) (Kumaran and Patki, 2011).

Cystone prevents stone forming constituents in the urine like oxalic acid, and calcium by minimizing supersaturation, through micropulverization, antimicrobial and anti-inflammatory activities with symptomatic relief of ureteric colic, burning and micturition. Similarly, studies have also reported that cystone disintegrates calculi due to its lithotriptic effects on mucin, which binds particles together in a calculus, have diuretic properties (increased urine volume) and relaxes the smooth musculature of the urinary tract (antispasmodic effect), which averts supersaturation of stone forming agents in urine, thereby inhibiting the crystallization process and kidney stone formation (Satish *et al.,* 2010; Rathod *et al.,* 2013a). Furthermore, other herbal formulations used to treat urolithiasis are Calcury, Chandraprabha bati, Trinapanchamool, Rencare Capsule, Patherina tablet, Ber Patthar Bhasma and Chander Prabha Vati (Deo *et al*., 2011).

A number of the *in vitro* and the *in vivo* models of urolithiasis have been developed to investigate pathogenesis of CaOx nephrolithiasis and to develop therapeutic agent and protocols to test their efficacy. Although no single model is thought to be perfect, valuable information can be obtained using a combination of the *in vitro* and the *in vivo* experimental models (Khan, 2018).

1.13. *In vitro* Models for Urolithiatic Studies

1.13.1. Calcium Oxalate Crystallization Assay

Crystal nucleation inhibitors are among possible therapeutic agents to prevent recurrent stones (Agarwal and Varma, 2013; Rathod *et al.*, 2013b). The plant extracts, which inhibit crystal nucleation and dissolutions of existing stones could be evaluated by measuring

turbidity changes of CaOx solutions under the *in vitro* assays (Hess *et al.,* 1995; Phatak and Hendre, 2015; Vennila and Mariyal, 2015). Accordingly, the rate of homogenous nucleation and aggregation of CaOx crystals can be produced by mixing metastable solutions (artificial urine**)** of calcium chloride ($CaCl_2$) and sodium oxalate ($Na_2C_2O_4$) (Saha and Verma, 2013; Phatak and Hendre, 2015).

In the previous studies, nucleation assay was prepared by mixing 5 mmol/l calcium chloride and 7.5 mmol/l sodium oxalate solutions in 0.05 mol/l Tris-HCl and 0.15 mol/l NaCl buffering conditions (pH 6.5) (Bawari *et al*., 2018). The rationale for the selection of $CaCl_2$ and $Na_2C_2O_4$ solutions is their swift solubility and capability of forming CaOx crystals rapidly (Sujatha *et al*., 2015). It is easier to create COM crystals than COD crystals approximately at pH 6 under the *in vitro* studies (Riese *et al.,* 1988; Wiessner *et al*., 2003).

The mixtures of calcium chloride and sodium oxalate comprising test agents could be incubated at 37ºC, and absorbance can be measured at 620 nm (wave length) using UV/Vis Spectrophotometer (Kalpana *et al.,* 2013; Phatak and Hendre, 2015; Bawari *et al.,* 2018). The time required to measure the absorbance of the solution varies among different researchers such as after 10 minutes (Khare *et al*., 2014); from 20 to 30 minutes (Hess *et al.*, 1995) and after 30 minutes (Bensatal and Ouahrani, 2008). The light absorbance increases as a mixture nucleates crystals. The growth of calcium oxalate crystals is formed by the following reaction:

$$CaCl_2 + Na_2C_2O_4 \rightarrow CaC_2O_4 + 2NaCl$$

The percent inhibition of nucleation by test extracts is calculated as: Percent inhibition = [(C-S) /C] × 100, Where C is the turbidity slope without plant extract; S is the turbidity

slope in the presence of the plant extract (inhibitor) (Aggarwal *et al.,* 2010; Saranya and Geetha, 2014). Calcium oxalate crystal formation in the presence of the extract was compared with that of the control (absence of the extract) and the reference drugs (potassium citrate and cystone). The *in vitro* data cannot be simply extrapolated to infer results of more complex *in vivo* systems, but this study gives an insight into the activity related to the efficacy of testing compounds or extracts (Bawari *et al.,* 2018).

1.14. *In vivo* Model for Urolithiatic Studies

1.14.1. Rat Model of Urolithiasis

Male rats are frequently used to induce CaOx nephrolithiasis due to similarity of their urinary system with humans (Khan *et al*., 2001; Vyas *et al.,* 2011), although mouse model possesses greater genomic closeness (Tzou *et al.,* 2016). The previous studies reported that there was a higher amount of stone deposited in the adult male rats compared to female rats (Prasad *et al.,* 1993; Khan, 2004), because of the inhibitory effects of estrogen against CaOx formation (Patel *et al.,* 2016a). Furthermore, the oxalate metabolism in rats is considered to be almost identical to humans (Khan and Hackett, 1985).

CaOx stones in hyperoxaluria rats and humans are usually located on renal papillary surfaces, which consists of organic matrix and CaOx crystals (Yamaguchi *et al.,* 2005). CaOx nephrolithiasis is formed in humans is identical to rats (Kumar *et al.,* 1991; Khan, 1997; Hennequin *et al*., 1998). Even though the kidneys of rats and humans have inherited differences, the adult rat kidneys are smaller, weighing 0.75-1.2 g, measuring 1.6×1×0.9 cm, unipapillary, while human's kidney weighs approximately 170 g, measuring 11×6×2 cm and multi-papillary. Despite these gross differences, the cortex to medulla ratio (2:1) of rat

kidneys is similar to humans (Khan, 2013a). Therefore, the rat model of urolithiasis is well established and relatively economical.

1.14.2. Urolithiasis Induction in Rats

Urolithiasis induction in the rats have relied on exogenous administration of lithogenic agents including sodium oxalate, glycolic acid, hydroxy-L-proline (HLP), and ethylene glycol (EG) (Tzou *et al.*, 2016). The delivery of these agents in the rat ranges from drinking water modification, enriched chow, gavage instillation, intraperitoneal injection, and even subcutaneous implantation of oxalate-containing osmotic mini-pumps (Khan, 2013a). A summary of rats hyperoxaluria induction (Tzou *et al.*, 2016) is indicated in Table 1.

Table 1. Rat models related to hypercalciuria and hyperoxaluria.

Types of approach	Lithogenic agents, reference	Diet/administration	Effects
Exogenous induction	Sodium oxalate	Intraperitoneal injection of 10 mg/kg sodium oxalate	Prompt CaOx crystal deposits; Crystal aggregation in the ducts of Bellini
	Glycolic acid	Free drinking water with powdered 3% glycolic acid	Hyperoxaluria; Hypocitraturia; CaOx crystal deposits
	Hydroxy-L-proline (HLP)	Intraperitoneal injection of 2.5 kg/kg HLP; Mixed in chow of 5% HLP; Hyperoxaluria; CaOx crystal deposit	Hyperoxaluria; CaOx crystal deposits; Less toxic compared to other agents
	Ethylene Glycol (EG)	0.75% EG in water with/without ammonium chloride, vitamin D, calcium chloride; Hyperoxaluria; CaOx crystalluri	Hyperoxaluria; CaOx crystalluria; CaOx crystal deposits; Renal toxicity

Dietary manipulation	Potassium oxalate supplement; Magnesium (Mg) deficiency; Vitamin B6 (pyridoxine) deficiency	5% level of potassium oxalate; Dietary Mg deprivation; Dietary intentional deficiency of pyridoxine	CaOx crystal deposit; Increase of CaP crystal deposits; Hyperoxaluria; Hypocitraturia; CaOx crystal deposits
Surgery	Intestinal Resection	Resection of distal 40-45 cm of the terminal ileum; Combination diet of high oxalate/low calcium/high lipid fat	Hyperoxaluria; Hypocitraturia; CaOx, CaP, CaCO3 crystal deposits
	Gastric Bypass Surgery	Roux-en-Y gastric bypass; 40% fat, and 1.5% sodium oxalate diet	Hyperoxaluria; CaOx crystal deposits

Ethylene glycol (EG) can be used alone (Khan *et al*., 2002), or in combination with ammonium chloride (NH_4Cl) in drinking water or vitamin D3, which induces hypercalcemia and hypercalciuria to induce urolithiasis in rats (de-Bruijn *et al.,*1994; de-Water *et al.,* 1996). These methods are economical and easily administered via drinking water. Thus, CaOx crystal deposits in the kidneys can be observed in a short period of time. The use of EG is an effective method for inducing calcium oxalate crystals in rats (Lee *et al*., 1991; Santhoshi *et al.,* 2015).

In experimental rats, the main component of the stone formed was COD when EG alone administered, whereas COM crystals were predominantly formed and COD will be present in trace amounts when NH_4Cl combined with EG. On the other hand, it was not clear why COM crystals were made with low urinary pH (Riese *et al.,* 1988; Wiessner *et al.,* 2003). In cell culture, the degree of attachments of COM and COD crystals to the renal epithelial cells depends on pH and urinary proteins (Wesson *et al.,* 1998; Wiessner *et al.,* 2003). Evidences from the previous studies indicated that 14 days administration of EG (1% v/v) to young

male albino rats produced CaOx crystals (Gilhotra and Christina, 2011). Similarly, 15 days administration of EG (0.75%) with 0.5% to 2% of ammonium chloride (w/v) in drinking water leads to CaOx crystallization (Fouada *et al.,* 2006; Khan *et al.,* 2010). Addition of NH_4Cl (1%) induces urinary acidification by lowering pH, and promotes stone formation by favoring the retention of CaOx crystals within the renal tubules. However, it leads to weight loss and ultimately death if given for more than 5 days (Vanachayangkul *et al.,* 2011; Kishore *et al*., 2013; Siddiqui *et al*., 2018).

EG is widely used as an automobile antifreeze agent (Saha and Verma, 2012). The basic mechanism behind EG induced calculi is hypercalciuria and hyperoxaluria (Karadi *et al.,* 2006). The previous studies reported that EG induced hyperoxaluria is a major risk factor for CaC_2O_4 nephrolithiasis and it is a widely acceptable model for urolithiatic studies (Atmani *et al*., 2004; Santhoshi *et al.,* 2015). However, this model has been criticized due to the side effects of the metabolic acidosis induced by EG consumption (Green *et al.,* 2005). That is, EG causes injury and inflammations of many organs, including the renal epithelium and producing metabolic acidosis if used at a concentration of more than 7.5% (Khan *et al*., 2006; Khan, 2018).

EG is rapidly absorbed from the gastrointestinal tract and metabolized in the liver. In rats, EG is broken down into four organic acids: glycoaldehyde, glycolic acid, glyoxylic acid and oxalic acid (Leth and Gregersen, 2005). The oxalate (oxalic acid) is a metabolic breakdown end product of the Krebs cycle, which is the major constituent of the most renal stones. First, EG is metabolized by the liver enzyme (alcohol dehydrogenase) to glycolaldehyde, then to glycolic acid (GA) (via aldehyde dehydrogenase). GA is a toxic metabolite that accumulates in the human plasma (Hess *et al.,* 2004). Subsequently, GA is oxidized or

metabolized into glyoxylic acid and further oxidized into endogenous oxalic acid (oxalate) by glycolate oxidase in the liver. Thus, leading to chronic hyperoxaluria, which binds with calcium to form CaC_2O_4 crystals, and precipitates in the kidneys (Fan *et al.*, 1999; Katzung and Trevor, 2004). Furthermore, some pathways in humans also involve the conversion of glycolaldehyde to glyoxal, glyoxylate to glycine (and its conjugate hippurate in the presence of benzoic acid), and formic acid (Gabow *et al.*, 1986). This deposit produces renal mitochondrial toxicity, similar to the clinical renal calculi (McMartin and Wallace, 2005) (Figure 4).

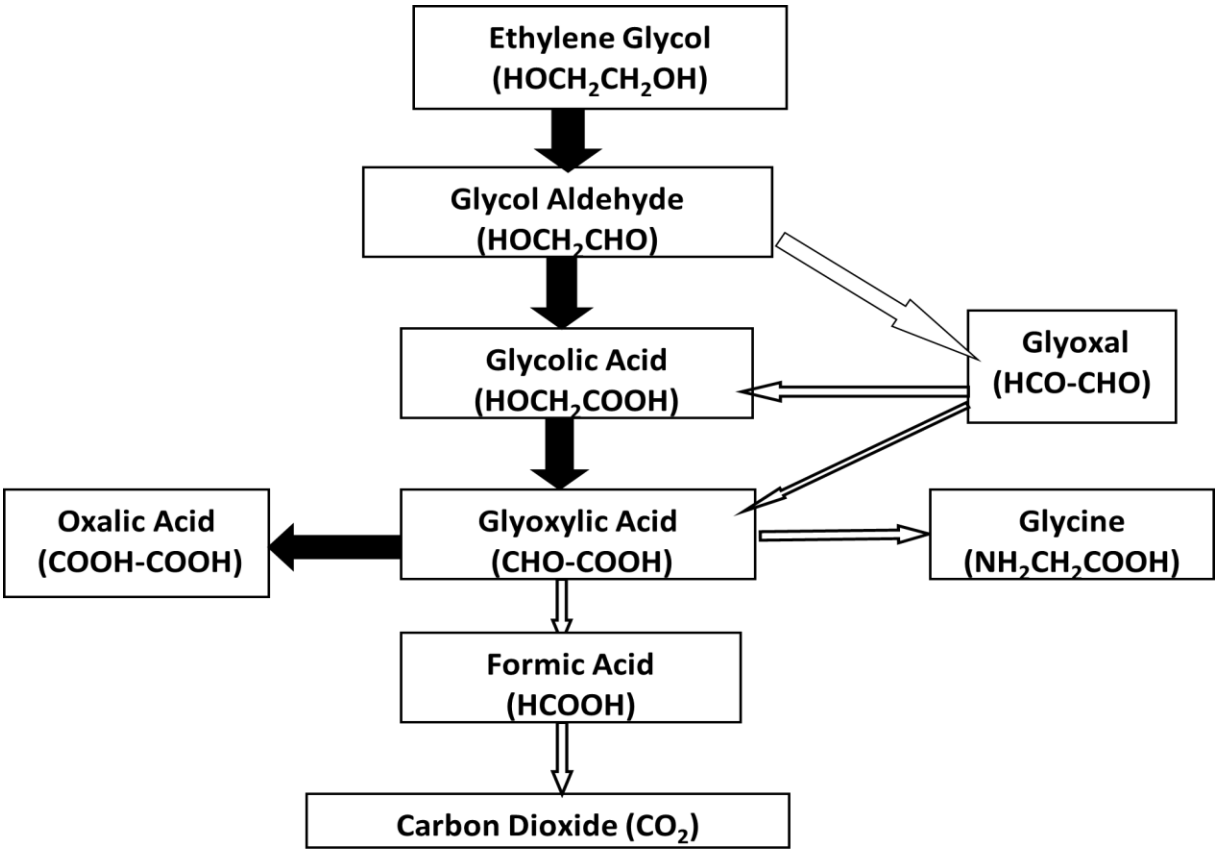


Figure 4. Metabolism of Ethylene glycol. Bold arrows indicate the major pathways (Hess *et al*., 2004).

1.15. Medicinal Plants with Anti-urolithiatic Effects

There are several phytoconstituents responsible for antiurolithiatic activities such as saponins, tannins, flavonoids and phenols **(**Bawari *et al.,* 2018). The phytoconstituents may exert renal stone preventive or curative effects through multiple mechanism, including diuretic activity, anticalcifying activity, crystallization inhibitions (nucleation, aggregations and growth); breaking mucin the bindings of calculi lattices, which is lithotriptic activity and regulating oxalate metabolisms. Moreover, phytochemicals may regulate the crystalloid-colloid imbalance and improve renal function, improve the renal tissue antioxidant status and cell membrane integrity. It also prevents kidney stone recurrences, and relieve symptoms like burning micturition and haematuria (which could show analgesic and anti-inflammatory activities) (Habermann, 2006; Yadav *et al.,* 2011).

The effect of herbal extracts may be through modifying the absorption, distribution, metabolism, and/or elimination of drugs (Bejar *et al.,* 2004). Herbal remedies such as kidney beans, watermelon, horsetail tea, lemon juice, olive oil, apple cider vinegar, and barley are used to treat kidney stones (Deo *et al*., 2011). Furthermore, some medicinal plants studied so far to manage urolithiasis are listed in Table 2.

Table 2. Medicinal plants possessing anti-urolithiatic effects.

No.	Plants name (Family)	Parts used	Study Model	Results/Effects	References
1	*Jasminum auriculatum* (Oleaceae)	Leaves	Male rats	Curative and preventive	Chanchal *et al.*, 2016
		Flowers	Male rats	Curative and preventive	Bahuguna *et al.*, 2009b
2	*Scoparia dulcis L.* (Scrophulariaceae)	Roots, shoots	In vitro	Nucleation and aggregation inhibitions	Binu and Vijayakumari, 2013
3	*Achyranthes indica* (Amaranthaceae)	Leaves	In vitro	Inhibitions of nucleation and crystal growth	Pareta *et al.*, 2011a
4	*Sesbania grandiflora* (Fabaceae)	Leaves	Male rats	Antiurolithiatic effect	Doddola *et al.*, 2008
5	*Mimusops elengi* (Sapotaceae)	Bark	Male rats	Curative and preventive	Ashok *et al.*, 2010
6	*Herniaria hirsuta L.* (Carryophyllaceae)	Leaves	In vitro	Aggregation inhibitions	Atmani and Khan, 2000
		Leaves	Male rats	Prevents CaOx formation and depositions	Atmani *et al.*, 2004; Begun and Knoll, 2004
7	*Hibiscus sabdariffa* linn. (Malvaceae)	Leaves	Male rats	Curative effect	Kalyan *et al.*, 2009
8	*Raphanus sativus* (Brassicaceae)	Leaves	Male rats	Prevent stones formation	Ch *et al.*, 2017
9	*Moringa oleifera* Lam. (Moringaceae)	Root-wood (barks removed)	Male rats	Curative and preventive	Karadi *et al*., 2006; Deo *et al.*, 2011
10	*Hygrophila spinosa* (Acanthaceae)	Whole plant	Male rats	Curative and preventive	Sathish *et al.*, 2010
11	*Rotula aquatica* (Boraginaceae)	Roots	Male rats	Prevent stone growth	Gilhotra and Christina, 2011
12	*Sesbania grandiflora* (Leguminosae)	Leaves	Male rats	Prevent CaOx stones	Doddola *et al*., 2008
13	*Phyllanthus niruri* L**.** (Euphorbiaceae)	Whole plant	Male rats*	Inhibits crystal growth	Freitas *et al.*, 2002
14	*Portulaca oleracea* Linn. (Portulacaceae)	Aerial parts	Male rats	Protects urolithiasis	Kishore *et al.*, 2013
15	*Rubia cordifolia* (Rubiaceae)	Roots	Male rats	Protects urolithiasis	Divakar *et al.*, 2010
16	*Ocimum gratissimum* L. (Lamiaceae)	Leaves	*In vitro*	Inhibit CaOx formation	Agarwal and Varma, 2014b
17	*Terminalia arjuna* (Combretaceae)	Bark	*In vitro*	Inhibits calcium phosphate and CaOx crystals	Chaudhary *et al.*, 2010

18	*Foeniculum vulgare* Mill. (Apiaceae)	Seeds	Male rats	Inhibit stone formation	Ibrahim and El-Khateeb, 2013
19	*Chenopodium album* (Chenopodiaceae)	Leaves	Male rats	Stone inhibitory and dissolution effect	Sikarwar *et al.*, 2017.
20	*Achyranthus aspera L.* (Amaranthaceae)]	Leaves	*In vitro*	Inhibit CaOx nucleation	Agarwal and Varma, 2014a
21	*Boerhavia diffusa* L. (Nyctaginaceae)	Whole parts	*In vitro*	Dissolves CaOx	Yasir and Waqar, 2011
		Roots	Male rats	Inhibits CaOx stones	Pareta *et al.*, 2011b

Note: Male rats = Adult male albino Wistar rats (EG induced); Male rats*= CaOx seeds placed in the bladder of rats.

1.16. Description of Medicinal Plants Used in the Present Study

1.16.1. Achyranthes aspera

A. aspera L. (Family: Amaranthaceae) is a perennial herb sometimes with a woody base, stems 0.2-2m long, stiffly erect to prostrate, straggling or scrambling, simple to considerably branched, stems and branches subglabrous to densely tomentose. Leaves vary in shape and size, from elliptic to almost round, covering varying from subglabrous through subglabrous above and densely covered with appressed grey hairs below to densely tomentose on both surfaces. Inflorescences with young dense flowers, elongating as the flowers mature up to 40 cm long (Edwards *et al.,* 2000). It grows throughout the tropics and warmer parts of the world in nearly all types of habitat such as along roadsides in acacia woodland, shaded banks near rivers, grassland, rocky hills, slopes in gorges, montane forests, on termite mounds, as a weed of cultivation, 350-2300 m. It is an indigenous plant to Ethiopia, and found in most regions of the country. It is a flowering plant all year round, and honeybees collect pollen and nectar from the flowers (Fichtl and Adi, 1994; Edwards *et al.,* 2000).

The traditional use of *A. aspera* leaves and roots include the treatment of cough, colic, debility, dropsy, dog bite, asthma, bleeding, gynaecological disorders and arthritis, and as antifertility (spermicidal), laxative, ecbolic, abortifacients, antihelminthic, aphrodisiac,

antiviral, anti-plasmodic, antihypertensive, anticoagulant, diuretic and anti-tumor agents (Uniyal *et al.,* 2006). It is also used to treat a scorpion bite, gonorrhea, obstetric disorders, *Diabetes mellitus*, fever and dysentery, and to prevent hypersensitivity reactions of the skin (Okon *et al.,* 2015). Nephroprotective, antiparasitic, hypoglycaemic, analgesic, antipyretic, and purgative activities of *A. aspera* were also reported (Reddy and Kamble, 2014).

1.16.2. Rumex abyssinicus

R. abyssinicus Jacq. (Family: Polygonaceae) is a perennial herb, up to 3 m tall with thick fleshy rhizomes. Stems are erect, stout, grooved, pale green or reddish, ochreae funnel-shaped, and brownish. Leaves are petiole as long as blade, thin, hastate, basal leaves up to 25 x 20 cm with palmate venation, and stem leaves are much smaller. Inflorescence is a very large and richly branched panicle, up to 40 cm long.

Flowers are in clusters on slender pedicels, up to 5 mm long. It is a common weed found in the field and plantations, 1200-3300 m, in many parts of Ethiopia. The tender shoots are edible, and it is an indigenous plant to Ethiopia, and the rhizomes are used to refine butter and give it a rich yellow colour. They are also used medicinally and their extracts are drunk to control mild forms of diabetes (Edwards *et al.,* 2000).

In Ethiopian traditional medicine, the rhizomes of *R. abyssinicus* is claimed to be used for the treatment of gonorrhea, lung TB, leprosy, fever, liver disease, hypertension, hemorrhoids, scabies, antiemetic, aphrodisiac, cough, rabies, vermifuge, rheumatism and migraine. It was also used as a diuretic, anti-microbial, anti-inflammatory and analgesic activities. Root powder paste with lime juice applied for *Tinea nigra* and *T. versicolor* (Getie *et al.,* 2003; Mekonnen *et al.,* 2010).

1.16.3. Satureja punctata

S. punctata (Benth.) Briq. (Family: Lamiaceae) is a woody herb up to 100 cm high. Flowers are purple, sometimes red or violet, arranged in axillary clusters. Leaves are sessile or shortly petiolate: blade often leathery, circular to ovate or narrowly lanceolate, margin entire, often thickened in a white line, revolute. Inflorescence with many cymes. Cymes are mostly many flowered, pedunculate. The stems are strongly lemon-scented when crushed, and they are flowering plants throughout the year, mostly after the rains, and honeybees collect pollen and nectar from them. It is also an indigenous plant to Ethiopia. They inhabit a stony slopes and rocks, Acacia-savanna, Erica-Myrica shrub, often on limestone, 600-3840 m, and are found in many regions of Ethiopia, Eritrea, Djibouti and other parts of Africa (Fichtl and Adi, 1994; Hedberg *et al.*, 2006).

In Ethiopian traditional medicine, the aerial parts of *S. punctata* was used to treat headache, stop menstruation, relieve stomach pain (Abate, 1989), relieve muscle pain, treat stomach and intestinal disorders such as cramps, nausea, indigestion and diarrhea, and as tonic and carminative agent (Momtaz and Abdollahi, 2010; Tepe and Cilkiz, 2015). Moreover, the essential oils of *Satureja* species are used in various industrial applications as flavoring agent, medicine and perfumes (Teklu *et al.,* 1998). The leaves are traditionally used against fever and common cold. The plant is also used to improve the quality of milk (Hedberg *et al.*, 2006)

1.16.4. Chenopodium murale

C. murale L. (Family: Chenopodiaceae) is a herb up to 90 cm high, usually highly branched, and rarely dense. Leaves are variable, commonly rhombic-ovate, rarely narrower, tip acute, base cuneate into winged petiole of 1.6-2.5 cm long, margins with 3-15 course

irregular, usually sharp teeth in each side. It is indigenous to Ethiopia and found in different areas with altitudes ranging from 1300 to 2900 m above sea level. It is a cosmopolitan weed found along poorly drained sites (Edwards *et al.,* 2000**)**.

C. murale leaves are traditionally used as diuretic, mild purgative, anthelmintic, tranquilizer, laxative and tonic for liver (Andrews, 2009). They also have antibacterial, antifungal, insecticidal, cytotoxic, anthelmintic, anti-diaphoretic, stomachic, antispasmodic, emmenagogue, anti-asthmatic, abortifacients, migraine, digestive problems, sterility, hair loss, anxiolytic, antidepressant and anti-hypertensive effects (Ahmad *et al.,* 2003), and to treat jaundice (Jan *et al.,* 2009).

1.16.5. Chenopodium ambrosioides

C. ambrosioides L. (Family: Chenopodiaceae) is a strongly aromatic herb up to 1.2 m high. It is also known as American worm-seed, ant-weed, Mexican tea, worm-seed (English), and synonyms to *Chenopodium anthelminiticum*. It was introduced to Ethiopia, and grow as a weed of cultivation and in the areas of wet sites with altitudes ranging from 950 to 2500 m. It originated from America, and spread throughout the tropics and sub-tropics. It is found in most Ethiopian regions (Edwards *et al.,* 2000). This herb is used in folk medicine as anti-parasitic, anti-inflammatory and antibiotic, whose efficacies have been scientifically proven. It is also used as a vermifuge to expel roundworms and hookworms. The essential oil of *C. ambrosioides* is known to impede the growth of yeast species, dermatophytes and other filamentous fungi, and its hexane extract is known to inhibit the growth of filamentous fungi (Sousa *et al.,* 2012).

1.16.6. Aloe pulcherrima

A. pulcherrima Gilbert and Sebsebe (Family: Aloaceae) is a prostrate and pendent shrub, mostly un-branched, and its stem to 1 meter long and 8 cm thick, sometimes dichotomously branched at apex within leaf rosette, especially when cultivated. Leaves 35-50 in dense rosette, pale blue-green, slightly glaucous with fine but distinct longitudinal lines, and especially in dry season, red margin, mariginal teeth almost absent up to 3 per 10 cm. Its habitat is along a steep basalt slopes or cliffs with sparse cover of ever-green bush land from 2480 to 2700 m. It is endemic to Ethiopia, and found in Shewa only as indicated in Flora of Ethiopia (Edwards *et al.,* 1997). In Ethiopian traditional medicine, the gel/latex is used to treat various infectious diseases like malaria and for wound healing (Demissew *et al.,* 2011; Abdissa *et al.,* 2017).

1.16.7. Inula confertiflora

I. confertiflora A. Rich. (Family: Asteraceae) is an erect hairy annual herb growing to 2.5 m high. The stem is much branched and leaves are densely formed towards the apex of the branches. It grows in habitats of margins and clearings in Juniperus-podocarpus forest, rocky places, ranging from 2400 to 3730 m above sea level. It is endemic to Ethiopia and found in Shewa, Wollo, Arsi, Bale and Hararge, and is not known elsewhere (Hedberg *et al.,* 2004). In rural areas of Ethiopia, the roots of the plant are dried and smoked to be used as a fumigant during childbirth, and suspension of leaves pounded in water is applied to diseased eyes of cattle. The roots are used against leprosy (Hedberg *et al.,* 2004).

1.16.8. Gomphocarpus fruticosus

G. fruticosus (L.) (Family: Asclepiadaceae) is an erect, sparsely branched perennial shrub that can grow up to 2 m tall. It is a short lived herb from non-tuberous tap roots; milky latex.

The calyx is mostly tinged with purple, yellow or greenish yellow corolla. The stem is 0.5-1.5 m tall erect, much branched above the base, woody below, densely spreading pubescent, and the leaves are linear or linear-lanceolate. It mostly found in disturbed areas and in roadside gravel from 1200 to 2000 m (Hedberg *et al*., 2003). This plant has quite a number of vernacular names that include narrow-leaved cotton plant, cotton bush, swan plant, bristle-fruited silk-weed and Moby-Dick. *G. fruticosus* (Syn. *Asclepias fruticosa*) is a species of milkweed (Goyder and Nicholas, 2001). This plant is used by the traditional herbalists in Ethiopia for treatment of urolithiasis as mentioned by a key informant.

Cardiac glycosides are an important class of naturally occurring compounds in this plant (Roberts *et al.,* 2015). *G. physocarpus* is widely used in traditional medicine in South Africa. The roots are used to treat stomach ache. The leaves are dried and ground into powder to be taken as snuff for headaches. The milky latex is used to treat warts. Seeds are blown away from the pods as a charm to placate the ancestors. The stems are used for fiber. Fresh stems and leaves stuffed into mole holes are said to be an effective deterrent. This plant is poisonous if ingested and has caused death to sheep feed large amounts. The inflated fruits last well in the vase, when dried, and can be used in fresh and dried floral arrangements. Wash your hands after handling the cut stems and dispose of all clippings (Notten, 2010).

In Africa, *G. fruticosus* is traditionally used to treat malaria, diabetes, asthma, bronchitis, cardiac palpitations, diuretic and treatment for anthrax in cattle (Burkill, 1985). It is used to treat tumors, skin diseases, scabies and itching in Arabian Peninsula (Mothana *et al.,* 2014). Several cardenolides were identified in *G. fruticosus* plants of different geographical origins. The cardiac glycosides extracted from *G. fruticosus* have been used for treatment of heart

failure. Pregnane glycosides isolated from different Asclepidaceae plants showed various biological activities including cytotoxicity (Wang *et al.,* 2008; Liu *et al.*,2014); antidyslipidemic and antioxidant activities (Sethi *et al.,* 2013); anti-trypanosomal (Gurib-Fakim and Mahomoodally, 2013); chondroprotective (Sanyacharernkul *et al.,* 2009); and anti-obesity effects (Abdel-Mogib and Raghib, 2013; Elsebai and Mohamed, 2015). The identification of a new pregnane glycoside, cardenolides and triterpenoids from *G. fruticosus* extract warrants more pharmacological studies (Marzouk *et al.,* 2016).

1.16.9. Commiphora myrrha

C. myrrha (Nees) Engl. (Family: Burseraceae) is a tree up to 4 m high. It is indigenous to Ethiopia. It is found in *Acacia - Commiphora* woodlands and bush-lands on sandy to loamy soil at altitudes of 250-1300 m, in Afar, Bale, Hararge and Sidamo, Kenya, Somalia, and Arabia. The gum-resin of this species is the raw material of the renowned myrrh. Some authors use the name *C. myrrha* for the Arabian plant and use the name *Commiphora molmol* for the African. The two names are clearly synonymous. The gum is obtained from *Commiphora* by bark incising which results in gum exudation. Myrrh is an aromatic oleo-gum-resin of pale yellow color, changing to dark red upon hardening. Myrrh is also collected from a number of other species in Ethiopia, mainly for local use (Hedberg and Edwards, 1989).

In Ethiopian traditional medicine, myrrh is used by women who fumigate their body in health care. The essential oil is used in different parts of the world for asthma, bronchitis, for mature and wrinkled skin, gargle for mouth ulcers and thrush and cleanser for the womb. The six main compounds of myrrh are furanoeudesma-1,3-diene, lindestrene, furanodiene,

2-methoxyfuranodiene, 2-acetoxyfuranodiene and isofuranogermacrene (Syn. Curzerene) (Hedberg and Edwards, 1989).

Therefore, many patients rely on phytotherapy for the primary health care in Ethiopia (Yineger and Yewhalaw, 2007; Giday and Teklehaymanot, 2013), and it remains the mainstay of treatment (Serhat and Kupeli, 2006). This is because the pharmaceutical products are expensive, inaccessible and ineffective, and the 'folk' pharmacopoeia provides apparently effective remedies for many diseases.

1.17. Rationale of the Present Study

In many developing countries, including Ethiopia, most patients rely on medicinal plants as an alternative therapy for many diseases including urolithiasis. Medicinal plants are affordable, accessible, and effective; they are usually compatible with the human body and hence cause less side effects (Bensatal and Ouahrani, 2008) compared to the conventional drugs (Ankur *et al*., 2010). Despite the wider use of folk medicine in developing countries, scientific evidences regarding safety and antioxidant profiles are limited (Frassetto and Kohlstadt, 2011; Arsad *et al.*, 2013). The same is true for modern drugs, which may have therapeutic effect at one dose and toxic at another (Sharif *et al*., 2015).

Urolithiasis is a common urinary tract problem worldwide, which may lead to end stage kidney failure. Although studies reported that the incidence of urolithiasis is quite high globally, there is no scientific information available on the prevalence of urolithiasis in Ethiopia. This imposes a significant impact on quality of life, and the nation's economy (Baheti and Kadam, 2013b). The pathogenesis of kidney stones remain incompletely understood (Aggarwal *et al.*, 2013) and its treatment remains a challenge, although there are

modern treatment modalities including ESWL (Singh and Sailo, 2013). Urolithiasis is caused by an imbalance between stone promoters and inhibitors in the kidneys. The main challenge of drug development for urolithiasis may be the presence of various chemical compositions of stones, and multifactorial nature (Butterweck and Khan, 2009). Despite considerable improvements in medical intervention such as the utility of extracorporeal shock wave lithotripsy (ESWL), there is no satisfactory drug to treat renal calculi (Mikawlrawng *et al.,* 2014). ESWL treatment is associated with acute renal injury due to traumatic effect of the shock wave, and infections after treatment (Tiwari *et al.,* 2012). It also results in incomplete stone clearance (Mittal *et al.,* 2012), and is unable to prevent new stone formations (Coe *et al.,* 2005), or stone recurrences (often up to 60%) (Atmani and khan, 2000; Yasir and Waqar, 2011). After the first episode of a stone, the recurrence rate is more than 50% within 10-years (Ljunghall and Danielson, 1984; Trinchieri *et al.,* 1999). Therefore, kidney stone recurrence is a major risk, which is considered the un-met clinical challenge in the area of urology (Sujatha *et al.,* 2015). Stone forming patients are prone to its recurrences after the first surgical therapy (Abraham and Smith, 1984). Even though the chemical composition of a stone influences the choice of intervention (Channa *et al.,* 2007), open surgery remains to be the mainstay of treatment (Hounnasso *et al.,* 2015).

In Ethiopia, various medicinal plants have been claimed to cure various ailments including urinary tract stones. In the present study, *Achyranthes aspera* L., *Rumex abyssinicus* Jacq., *Satureja punctata* (Benth.) Briq., *Chenopodium murale* L., *Aloe pulcherrima*-Gilbert and Sebsebe, *Chenopodium ambrosioides* L., *Inula confertiflora, Gomphocarpus fruticosus* (L.) Ait.f., and *Commiphora myrrha* (Nees) Engl. were selected to examine their effects on experimentally induced urolithiasis. A thorough literature survey was carried out to

ascertain that none of the selected medicinal plant parts or combinations have been studied so far for anti-urolithiatic activities. Furthermore, there is also insufficient scientific evidence reported on these plants for toxicity and antioxidant effects. Therefore, the present study has been intended to verify the scientific basis for the (i) safety, (ii) antioxidant effects and (iii) anti-urolithiatic activities of the selected medicinal plants extracts.

Research Hypothesis: The extracts of selected medicinal plants may be potentially safe, would have a significant antioxidant effect and could potentially be sources of effective bioactive compounds for treatment of urolithiasis.

1.18. Objectives of the Study

1.18.1. General Objective

■ The general objective of the study was to assess trends in the prevalence of urolithiasis in Ethiopia, to investigate the toxicity of solvent extracts, the antioxidant activity and the antiurolithiatic efficacies of selected medicinal plant extracts in Ethiopia

1.18.2. Specific Objectives

The specific objectives were to:

☞ Determine the trend in the prevalence of urolithiasis at the St. Paulos Referral Hospital during the last 13 years (2005 to 2017);

☞ Screen for phytochemical constituents of medicinal plant extracts;

☞ Evaluate the acute and sub-acute toxicity profiles of medicinal plants extract in Wistar albino female rats;

☞ Assess the *in vitro* antioxidant activities of medicinal plant crude extracts traditionally used to treat kidney stones;

☞ Determine *in vitro* inhibition of medicinal plants extracts against calcium oxalate nucleation and aggregation events;

☞ Evaluate the preventive efficacy of medicinal plants extracts in experimentally induced nephrolithiatic Wistar male rats;

☞ Evaluate the curative efficacy of medicinal plants extracts in experimentally induced nephrolithiatic Wistar male rats, and

☞ Describe the chemical constituents of the bioactive fractions of a plant with promising anti-urolithiatic activity under *in vitro* study.

2. Materials and Methods

2.1. The Prevalence of Urolithiasis in Ethiopia

The thirteen years' retrospective data were extracted from medical records (surgical theatre registry books and electronic databases) between September 2005 and 2017 from St. Paul's Hospital, Millennium Medical College (SPHMC), Addis Ababa, Ethiopia. The SPHMC is one of the tertiary hospitals in Ethiopia, receiving patients referred from all over the country. Electronic medical records were available only for the last 2 years in this hospital (2015 to 2017).

The study population was patients who were admitted to the SPHMC during the study period. Among all patients who underwent open surgery, those identified as stone cases were enrolled in the study. The data retrieved include the age, sex, anatomical positions of stones in the urinary system, co-morbidities or conditions associated with stones, and the history of stone recurrences. The patient records with incomplete information and that did not appear readable were excluded from the study.

2.2. Description of the Study Sites

The actual places of plant specimen collection were Addis Ababa ("Arat Kilo" Campus, the former Science Faculty, located at altitude 2447 m (latitude: $9^0$2'8.41"N; Longitude: $38^0$45'58.69"E); Sheger Park, near Sheraton Hotel with altitude 2427 m (latitude: $9^0$2'6.97"E; Longitude:$38^0$45'55.07"N); Entoto forest area, altitude 2819 m (latitude: $9^0$4'58.07"E; Longitude: $38^0$45'43.51"N), and Bole Bulbula (altitude: 2334 m). From North Shewa Zone, around Debre Berhan from Keyit (altitude: 2787 m), and Saria (altitude:2,840 m), and Ankober District, near to Minilik Palace (altitude: 2,465 m, located at 9° 22’ 0”- 9°

45’ 0” N and 039° 40’ 0”- 039° 53’ 0” E), which is 42 km East of Debre Berhan town as recorded by Lulekal *et al.* (2014). The flow chart of the study is indicated in Figure 5.

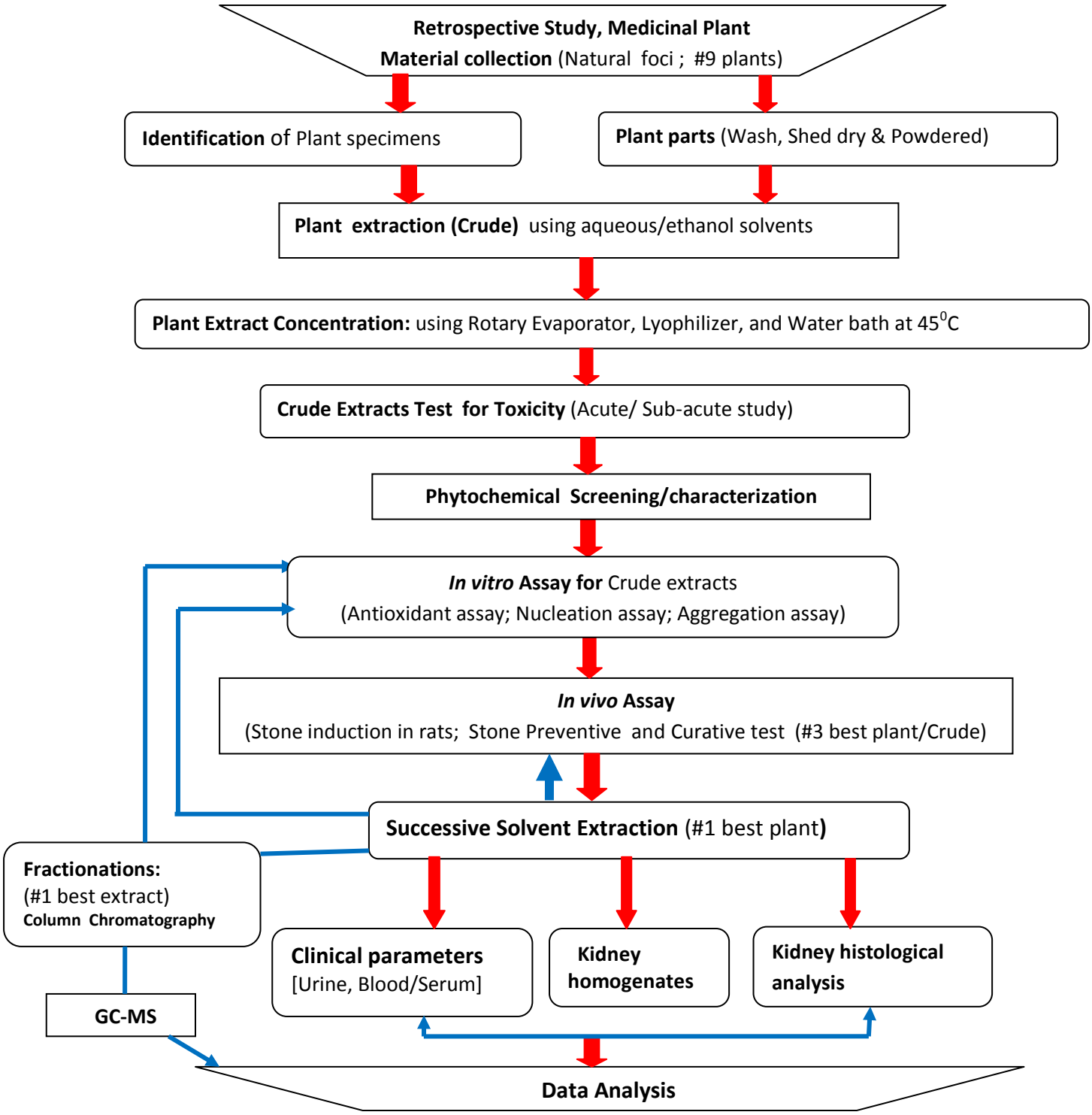


Figure 5. Schematic representation of conceptual framework of the study.

2.3. Medicinal Plants Investigated in the Present Study

All plant materials, except *Commiphora myrrha* were collected during their flowering or fruiting seasons. *Achyranthes aspera* L. (Amaranthaceae) (Abate, 1989; Aggarwal *et al.*, 2010; Belay, 2014); *Satureja punctata* (Benth.) Briq. (Lamiaceae) (Abate, 1989; Belay, 2014); *Inula confertiflora* A.Rich. (Compositae) (Abate, 1989; Belay, 2014); *Rumex abyssinicus* Jacq.(Polygonaceae)(Belay, 2014); *Chenopodium murale* L. (Chenopodiaceae), *Aloe pulcherrima*-Gilbert and Sebsebe (Aloaceae), *Chenopodium ambrosioides* L. (Chenopodiaceae), *Commiphora myrrha* (Nees) Engl. (Burseraceae) (Belay, 2014), and *Gomphocarpus fruticosus* (L.) Ait.f (Asclepiadaceae) (the key informant) were claimed to have urolithiasis treatment. The plant specimens were submitted to the National Herbarium, Department of Plant Biology and Biodiversity Management, Addis Ababa University (AAU) for taxonomic authentication and the corresponding collection number was given. The specimens were deposited in the National Herbarium of AAU for future references (Table 3**;** Appendix 1).

Table 3. Medicinal plants collected with reported anti-urolithiatic effect in the present study.

Serial No.	Scientific name (Voucher /accession number)	Local name (Amharic)	Parts Used	Collection Site and Time
1	*Achyranthes aspera* L. (TA231)	Telenji	Leaves, Roots	"Arat Kilo" campus of the Science Faculty, AAU, October 2017
2	*Rumex abyssinicus* Jacq. (TA232	Mekimeko	Rhizomes	"Arat Kilo" campus of the Science Faculty, AAU, and near to Sheraton Hotel, Addis Ababa, October 2017
3	*Satureja punctata* (Benth.) Briq. (TA233)	Yelomi-eshet	Aerial parts	Entoto, around Addis Ababa, November 2018
4	*Chenopodium murale* L. (TA234)	Amedmado	Leaves	Keyit, Debre Berhan, April 2017
5	*Aloe pulcherrima* -Gilbert & Sebsebe (TA235)	Sete-Eret	Gel	Ankober, North Shewa, December 2017
6	*Chenopodium ambrosioides* L. (TA236)	Gimy/Gundan-abir	Leaves	Ankober, North Shewa, November 2017
7	*Inula confertiflora* A.Rich. (TA237)	Tikur woinagift	Leaves	"Saria", around Wonkishet Mariam Church", North Shewa, December 2017.
8	*Gomphocarpus fruticosus* (L.) Ait.f (TA238)	Tifriena	Leaves	Bole Bulbula around "93 Mazoria" Addis Ababa, October 2018
9	*Commiphora myrrha* resins (Nees) Engl. (TA239)	Kerbie	Resins	Purchased from "Merkato" local market of Addis Ababa, December 2017

2.4. Preparation of Crude Extracts

The leaves, inflorescences and roots of *A. aspera*, the rhizomes of *R. abyssinicus*, the aerial parts of *S. punctata*, and the leaves of *C. murale*, *C. ambrosioides, I. confertiflora,* and *G. fruticosus*, and the resins of *C. myrrha* were washed thoroughly with tap water to remove contaminants and dried in shed at room temperature from 2 to 3 weeks in the Biomedical Sciences laboratory, AAU. The gel of *A. pulcherrima* was collected by spilling-out its fresh leaves with the knife. The dried plant parts, and resins were finely powdered

using a kitchen grinder (Mortar and Pestle, sized about 9 inches in diameter). The powder was put through a sieve of 3 mesh sizes so as to filter the gross solid matter.

The extracts were prepared using a procedure similar to that often used by traditional healers or patients, with some minor modifications. The plant powder was soaked in distilled water and hydro-ethanol (70%), which was placed on a shaker for 72 hours. The mixture was filtered through cotton/gauze, then through Whatman filter paper number 1 (pore size: 11 μm) to remove fine solid plant particles or insoluble constituents. The entire extracts were concentrated to dryness using a Rotary evaporator by removing ethanol (EtOH) at 45°C under reduced pressure. The distilled water extracts were concentrated using Lyophilizer. Then, the semi-solid concentrates poured into a glass petri-plates and allowed to completely dry in water bath adjusted to 45°C. The final dried extracts were collected and stored in labeled sterile bottles covered with tightly fitted stopper and kept in freezer at -20^0C until used in the experiments.

In addition, the percentage yield of plants crude extracts were calculated using the formula: Yield (%) = Weight of the dry extract (residual) divided by the weight of dried plant material (initially dissolved in solvent) × 100 as described by Gahlot *et al.* (2018).

2.5. Preparations of Successive Solvent Fractions

After conducting the *in vitro* anti-urolithiatic screening test for nine (9) plant crude extracts, one very promising plant crude extract was further partitioned by solvent-solvent extraction. This successive extraction with organic solvents was based on the bioactive guided findings of the aqueous water crude extracts. Therefore, the mother extract (the aqueous extract) was partitioned with solvents of increasing polarity from non-polar to polar

solvents ensuring that a wider polarity range of compounds could be extracted. Successive extraction was done sequentially beginning with petroleum ether, followed by chloroform, ethyl acetate, butanol and water, and the solvents were removed and concentrated under Rotary vacuum evaporator (Figure 6)**.** The distilled water extract was lyophilized under freeze dryer to obtain the powdered residues. The dried extracts were capped tightly with stoppers and stored in glass bottles, and kept in a refrigerator at -20^{0}C until used in the experiment.

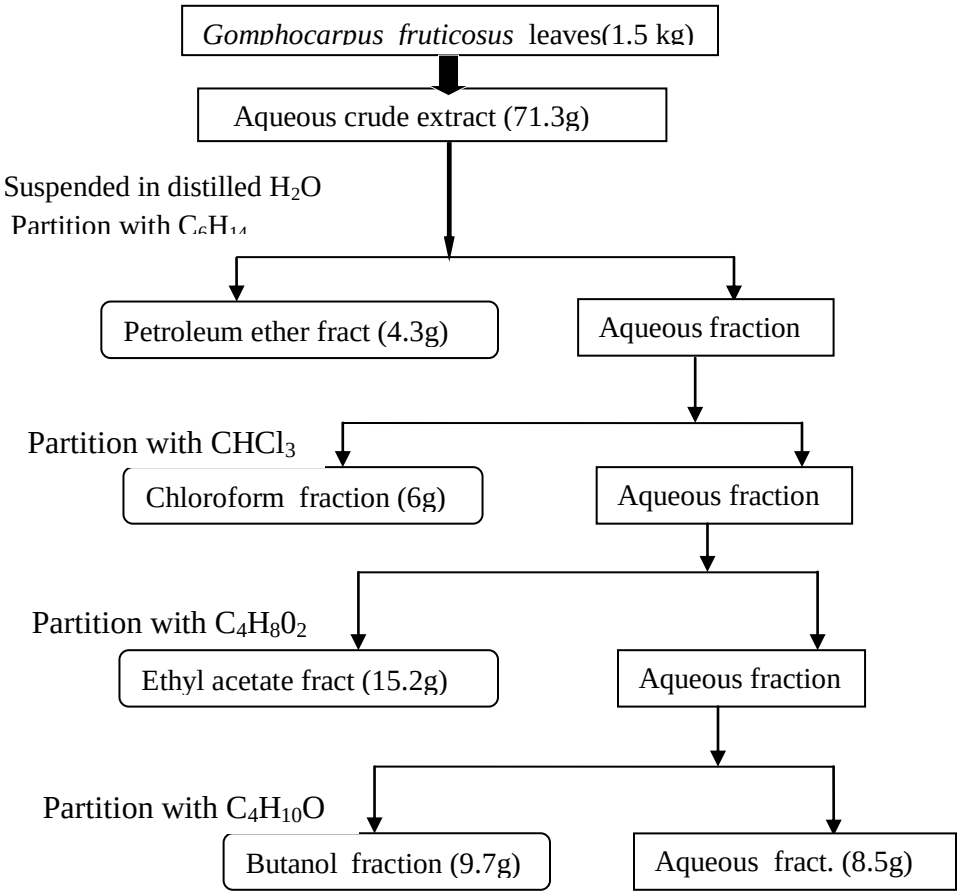


Figure 6. Successive solvent extraction of *G. fruticosus* leaves.

2.6. Phytochemical Screening

A preliminary phytochemical screening of the selected medicinal plants of the aqueous crude extracts was carried out for qualitative estimation of phytoconstituents. The presence

of secondary metabolites (phenol, flavonoids, tannins, steroids, terpenoids, alkaloids, saponin, resins, and glycosides) were analyzed using phytochemical methods as described by Yadav and Agarwala (2011), Hossain *et al.* (2013) and Keo *et al.* (2017) (Appendix 4).

2.7. Chemicals and Reagents

The chemicals and reagents/ kits used were analytical grade purchased from various sources. The procurement of petroleum ether, chloroform, ethyl acetate, butanol and ethanol were from Wisteam PLC (Addis Ababa, Ethiopia), potassium dihydrogen phosphate (anhydrous), sodium phosphate (dibasic anhydrous extra pure), calcium chloride monohydrate ($CaCl_2.H_2O$), sodium oxalate ($Na_2C_2O_4$), Tris-HCl buffer ($C_4H_{11}NO_3$), sodium chloride (NaCl), DPPH (2,2-diphenyl-1-picrylhydrazyl) were purchased from the Micron International Trading House PLC (Addis Ababa Ethiopia). Similarly, isoflurane, and formaldehyde were purchased from Neway Chemicals PLC (Addis Ababa, Ethiopia). Ascorbic acid was obtained from Food Sciences Department of AAU, which was purchased from Micron International Trading House PLC, Addis Ababa, Ethiopia. Ethylene glycol (EG) and ammonium chloride (NH_4Cl) were purchased from Pharma PLC (Addis Ababa, Ethiopia). EDTA tubes, serum separator tubes, capillary tubes and surgical blades were purchased from Micro Pharma PLC (Addis Ababa, Ethiopia). Furthermore, kits for liver and kidney function tests were purchased from a Roshi PLC (London, England). The oxalate (oxalic acid) colorimetric assay kits and **c**itrate colorimetric/fluorometric assay kits were purchased from BioVision Incorporated (Milpitas, USA).

2.7.1. Standard Drugs Used in the Study

Potassium citrate powder was obtained from the Black Lion Referral Hospital (Addis Ababa, Ethiopia). Cystone (polyherbal formulation) was purchased from Mumbi, India.

Ascorbic acid, which was purchased from Micron International Trading House PLC (Addis Ababa, Ethiopia).

2.7.2. Preparation of Plant Extracts and Standard Drugs

The test extracts/drugs of different concentrations were dissolved using appropriate vehicles. All plant extracts and potassium citrate were dissolved in distilled water at different concentrations. The procedure used to dissolve cystone was similar to the methods of Phatak and Hendre (2015) and Garimella *et al.* (2001) with some modifications. Cystone tablets were powdered using a Mortar and Pestle (size: 0.23 m), and suspended in distilled water containing 3% Tween 80 (i.e.,750 mg/ml). Then, they were kept for 3 hours to dissolve, centrifuged at 1000 rpm for 5 minutes, and filtered through 0.22 mm pore size filter paper. The clear supernatant was collected and used for calcium oxalate nucleation, and aggregation assays. Extracts were collected using Falcon tubes (45 ml), air tighten and stored in the refrigerator until experimental use. The test extracts and standard drugs were prepared daily, shortly prior to testing administrations at dose 200 mg/kg of extracts, 750 mg/kg of cystone, and 2.5 g/kg of potassium citrate. The test substance dosing volume was 2 ml/100 g of body weight. Deionized water was used to prepare all extracts/drugs and testing solutions of the *in vitro* CaOx crystallization assays.

2.8. *In vivo* Toxicity Study

2.8.1. Experimental Rats

Adult albino Wistar non-pregnant female rats weighing 200-220g, and 8 to 10 weeks of age were used. The previous reports suggested that female rats are more susceptible than male rats for toxicants (Ferreira *et al.,* 2014). As a result, female rats were purchased from the Ethiopian Public Health Institute (EPHI) animal breeding center for the experiment of this

study. The experiments were carried out in Biomedical laboratory, Addis Ababa University (AAU), and in Traditional Medicine and Modern Drug Research Laboratory, EPHI, Addis Ababa.

Additionally, the experimental animals were housed in groups under a controlled room temperature (22 ± 2°C) with a constant humidity (55 ± 5%), ventilation, no noise, and light (12 hours light/12 hours dark) cycles. Moreover, the rats were fed with pellets (standard diet) produced by Alema Farms PLC (Bishoftu, Ethiopia), and had access to tap water *ad libitum*. They were also acclimatized to standard laboratory conditions (in plastic cages with stainless steel top) for seven days prior to the experiment. The *in vivo* toxicity and the urolithiatic studies were conducted in accordance with the standard guidelines of the Organization for Economic Cooperation and Development for the use and care of laboratory animals in scientific research (OECD, 2001). It was also conducted in compliance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments.

2.8.2. Toxicity Tests

Prior to conducting extract administration, the rats did not eat any food for 12 hours, but they had access to water. The weight of each rat was recorded just before test extract administrations and the dose of extracts was calculated in relation to the body weight of each rat. The extracts were reconstituted as homogeneous suspensions in distilled water (vehicle) and were administered using an oral gavage feeding needle (PY252-4215, 16 gauge, Harvard). The dosing volume of test substances was 2 ml/100 g of body weight, and the time of dosing was at 6:30 am throughout the study. After the test substance was administered, food was withheld for 4 hours (OECD, 2001).

The starting dose of plant extracts could be selected from one of the four fixed levels (5, 50, 300 and 2000 mg/kg body weight) for an acute toxicity test. Ideally, it is most likely to produce mortality and some measurable toxic effects in the dosed animals (OECD, 2001). The previous studies administered extracts at 2000 and 5000 mg/kg dose levels for 28 days to determine sub-acute toxicity (Unuofin *et al.*, 2018). Similarly, we chose 2000 mg/kg dose to test acute and sub-acute toxicity in rats. When rats tolerate and no death occurs at the maximum dose of 2000 mg/kg per body weight, then, $1/10^{th}$ of the tolerated dose (that is, 200 mg/kg per body weight) was taken for pharmacological studies (Kishore *et al.*, 2013). Furthermore, in sub-chronic toxicity study (90 days), the body weight changes are indicators of adverse effects of drugs and chemicals if the weight loss is more than 10% from the initial weight (Teo *et al.*, 2002).

2.8.2.1. Acute Toxicity (14 days)

In acute toxicity tests, a total of 33 Wistar female rats were used. In the first experimental setup, 18 rats were randomly allocated into 6 groups consisting of 3 rats per group. That is, Group I (normal control), Group II (*Rumex abyssinicus*), Group III (*Satureja punctata*), Group IV (*Chenopodium murale*), and Group V (*Aloe pulcherrima*) and Group VI (*Achyranthes aspera*). In the second experimental setup, 15 rats were randomly categorized into 5 groups consisting 3 rats per group. That is, Group I (normal control); Group II (*Chenopodium ambrosioides*); Group III (*Inula confertiflora*), Group IV (*Commiphora myrrha*) and Group V (*Gomphocarpus fruticosus*). Each group of rats was administered with a single fixed dose of the respective plants extracts with a dosing volume of 2 ml/100 g body weight. The control groups received distilled water (vehicle), whereas the test groups received extracts dose 2000 mg/kg body weight.

Any indications of clinical toxicity were closely monitored within 4 hours of treatment periods up to 24 hours. Thereafter, observations for toxic manifestations (rising fur, draping, tremors, excitability, twitching, salivation and mortality) were made until the end of the 14 days period (OECD, 2001). After 14 days, they were all sacrificed.

2.8.2.2. Sub-acute Toxicity (28 days)

As recommended by OECD (2001), the selection of the starting dose of the sub-acute toxicity test was based on a dose, which will not cause mortality or severe toxic effects. The 2000 mg/kg body weight of the extracted material was the highest dose determined, which did not to induce acute toxicity in rats in the first phase of the study.

Parallel design (different groups received different extracts) was employed in the sub-acute toxicity study. A total of 30 female Wistar rats were randomly divided into 5 groups consisting of 6 rats each with matching body weights. That is, Group I (normal control), Group II (*Rumex abyssinicus*), Group III (*Satureja punctata*), Group IV (*Aloe pulcherrima*), and Group V (*Achyranthes aspera*). Group II to V were given a single dose of 2000 mg/kg body weight once daily for 28 days, consecutively. The dosing volume was 2 ml/100 g of body weight. The control group received distilled water once daily throughout the experiment.

2.8.2.3. Body Weight Measurements

The weights of the control and experimental rats were recorded using Mettler PE 1600 analytical balance with $\pm$0.01g (Switzerland) precision on the first day of the study (prior to the administration of test extracts), and at the end of the experiment on day 14th (acute), and on day 28th (sub-acute).

2.8.2.4. Hematological Studies

At the end of the experimental period, euthanasia was induced using isoflurane ($C_3H_2ClF_5O$, forane) inhalant followed by gentle cervical dislocation. Euthanized rats were placed in a sealed glass chamber where high levels of anesthetic gas (3%) were introduced, which depresses the central nervous system resulting in peaceful death. Blood samples were collected once unconsciousness was achieved. About 1 ml blood samples were collected from the rats in ethylenediamine tetraacetic acid (EDTA) anticoagulant tubes from the retro-orbital vein by a glass capillary tube puncture. Hematological examinations were carried out using Automated Hematology Analyzer (XT-1800i, Japan).

2.8.2.5. Estimation of Biochemical Markers

Liver enzymes such as Alkaline phosphatase (ALP), Aspartate aminotransferase (AST), and Alanine aminotransferase (ALT) were assayed as indicators of liver toxicity. This was done by enzyme marker kits using Clinical Chemistry Auto-analyzer (Cobas 6000 analyzer, Germany). The manufacturer's instruction was followed in the course of each biochemical analysis.

2.8. 2.6. Histopathological Examination

Histopathological examination was done for liver and kidney tissues of the experimental rats. All rats were sacrificed in a humane manner using isoflurane anesthesia at the end of the 14^{th} day for acute toxicity and at the end of the 28^{th} day for sub-acute toxicity studies. Liver and kidneys were examined macroscopically for their gross pathological changes (lesions developed) due to exposures of test extracts compared to control groups in toxicity studies. The tissues were microscopically examined for sub-acute toxicity test. In sub-acute toxicity, the tissues were fixed by 10% buffered neutral formalin solution, and subsequently

embedded in paraffin wax. The sections (5µm thick) were cut using Rotary Microtome 4060 E (Germany), and mounted on glass slides and stained with Hematoxylin and Eosin to study the histopathological changes (Doddola *et al*., 2008). All fields of the tissue morphology were examined under a light microscope (100x magnification) (Wagtech Thatcham, Berkshire, RG19 4QD, United Kingdom), and the photomicrographs were captured using a digital Camera manually mounted (Sony Cyber-shot DSC-W180 10.1MP with 3x optical Zoom, New Jersey, USA) for further reference.

2.9. DPPH Radical Scavenging Activity

The DPPH radical scavenging potentials of plant extracts were determined using the method described by Dinnimath and Jalalpure (2018) and Mittal *et al.* (2012) with minor modifications on the concentrations, in which 0.5 mM DPPH was used. In the DPPH colorimetric assay, the original deep violet colour of DPPH solution converts into a stable yellow-white color in the presence of potent antioxidants (Brand-Williams *et al.,* 1995). The basic chemical reaction of The DPPH in the presence of an electron donating antioxidant is represented as: DPPH + AH → DPPH-H + A* (Brand-Willians *et al.,* 1995; Huang *et al.*, 2005).

The antioxidant activities of the various concentrations (0.20, 0.39, 0.78, 1.56, 3.13, 6.25, 12.50, 25, 50, 100 mg/ml) of plant extracts vis-a-vis standard drugs were examined. Ascorbic acid was used as a reference drug. The working solution was prepared by diluting the stock solution to a series of concentrations. A solution of 0.5 mM DPPH ($C_{18}H_{12}N_5O_6$, molecular weight: 394.32) (0.19 mg/ml) in methanol was prepared. Then, 2 ml of DPPH solution was mixed with 1 ml of plant extract prepared in methanol. The mixture was vortexed thoroughly, was left in the dark chamber at room temperature for 30 minutes, and

was measured spectrophotometrically at 517 nm for light absorbance. These were analyzed in a quartz cuvette (optical path: 10 mm, and size: 4 ml) at room temperature. The ability of the plant extract to scavenge the DPPH radical was calculated as follows.

The DPPH radical scavenging activity (%) = [(Control - Sample)/Control] × 100, whereby control refers to the absorbance of the DPPH radical plus methanol; Sample = the absorbance of the DPPH radical plus the extract/ascorbic acid. Methanol was used as a blank reference, and each test was performed in triplicate.

The antioxidant activities of *Achyranthes aspera, Satureja punctata, Aloe pulcherrima, Gomphocarpus fruticosus* and *Commiphora myrrha* extracts were assessed at various concentrations ranging from 0.02 to 100 mg/ml. The reactions between the antioxidant molecules and the DPPH free radicals results in a noticeable colour change from purple to yellow. The DPPH radical scavenging potentials of these medicinal plants aqueous extracts were presented by plotting their inhibition percentages, respective to the various concentrations.

According to the United States Food and Drug Administration (FDA), the half maximal inhibitory concentration (IC_{50}) represents the concentration of a drug required for 50% inhibition under the *in vitro* test as reported by Hoetelmans (2017). The dose-response experiments were performed 3 times for each extract/standard control, and the mean IC_{50} ± SD was calculated. The percent of a control was calculated as absorbance units in the presence of ascorbic acid, and the dose response curves were drawn and IC50 values were obtained using the Graph Pad Prism software (San Diego, CA, USA) version 6.

2.10. *In vitro* Models of Urolithiasis

Although it is difficult to mimic the human urinary tract under the *in vitro* condition**,** the synthetic urine, which is a static environmental model, is used to study kidney stone nucleation and aggregation inhibitions of medicinal plant extracts **(**Saha and Verma, 2013). The synthetic urine was supersaturated with calcium chloride and sodium oxalate in a buffer solution to produce CaOx crystals**.**

The effect of extracts on the *in vitro* CaOx crystallization was determined by the light absorption measurements of turbidity for crystal nucleation and aggregation formation. The spectrophotometer UV-Vis-NIR was used to measure the turbidity of each mixture placed in quartz cuvette (4 ml). The light absorption measurements of CaOx nucleation and aggregation were determined at 620 nm as used by Sasikala *et al.* (2013). The absorbance mean values were calculated in percentages taking absorbance as a function of dose at a given time. The effects of plant extracts on CaOx crystallization compared with the standard drugs (potassium citrate and cystone) were plotted on graphs. The measurements of turbidity changes of the solution were examined with inhibitors (modifiers, example: extracts and potassium citrate) and without inhibitors. Deionized water was used to prepare all solutions and extracts/drugs for *in vitro* assay, and these were prepared daily before testing administrations.

The lower the IC_{50} is, the more potent the molecule is. Extract concentrations were tested in the range of 12.5 μg/ml to 3200 μg/ml so as to evaluate efficacy in a dose-dependent manner. The working solutions were prepared by diluting the stock solution into a series of concentrations. The CaOx crystal nucleation and aggregation inhibitory effects of the test

extracts/standard drugs would give a mechanistic insight for the mode of actions. Cystone and potassium citrate were used as a positive control (Sasidharan *et al.,* 2018).

2.10.1. Nucleation Inhibition Assay

The nucleation assay was similar to the *in vitro* experimental procedures described by Hennequin *et al.* (1993), and Atmani and Khan **(**2000) with minor modifications. That is, the selected stoichiometric concentration of 5 mmol/L was used due to their closure physiological concentration to the human urine. Calcium oxalate (CaOx) homogeneous precipitations were generated by mixing equimolar metastable solutions of calcium chloride monohydrate ($CaCl_2.H_2O$), and sodium oxalate ($Na_2C_2O_4$). Deionized water (17.5 micro-siemens, µS) was used to dissolve these solutions as used by Polat (2019).

Calcium chloride and sodium oxalate solutions were prepared at a final concentration of 5 mmol/L each in a buffer containing Tris-HCl (0.05 mol/L) and NaCl (0.15 mol/L) at pH 6.5. The pH (6.5) was adjusted to mimic to urine samples collected from people who formed calcium stones (Li *et al.,* 2017). Both solutions were filtered using vacuum filtering through 0.45 µm filter membrane, followed by filtering through 0.22 µm filter membrane pressing via syringe three times, and stored at 4 ºC until used for the experiment.

During the *in vitro* test extract experiment, 1.5 ml of calcium chloride monohydrate solution was transferred into a glass test tube (10 ml), and mixed with 500 µl of the plant extract/standard drugs at different concentrations (ranging from 12.5 µg/ml to 3200 µg/ml). The process of crystallization was initiated by adding 1.5 ml of sodium oxalate solution into the test tube to evaluate CaOx nucleation inhibitory efficacies. For the control, 500 µl of deionized water was added instead of the plant extract/standard drug and measured as blank.

These mixtures were capped with airtight stoppers after adding solutions into test tubes and stirred briefly at 800 rpm using a vortex. The crystallization reactions were allowed to proceed for 30 minutes incubation at temperature of 37°C as used by Sharma *et al.* (2017a). Then, 3.5 ml of the mixtures was transferred into 10 mm light path quartz cuvette and measured for absorbance of optical density at wavelength of 620 nm (OD_{60}) using Cary Series UV-Vis-NIR spectrophotometer (Agilent Technologies, Australia). Each test was performed in triplicates. An increase in turbidity of synthetic urine was suggestive of nuclei formation, whereas its reduction indicates the inhibitory effects of test extracts or standard drugs. The percentage inhibitions of CaOx nucleation were determined by comparing the presence of plant extracts with the controls. This was calculated using the following formula:

% Nucleation inhibition = [(SNc-SNi)/SNc] ×100, or Nucleation (N) inhibition percentage: 1 - [SNi/ SNc] × 100; Where; the slope of nucleation (SN); SNi - represents the slope of nucleation in the presence of the inhibitor (test extracts/drugs) of the sample set; SNc - represents a slope of nucleation for the control experiment (without inhibitor) of the sample set.

2.10.2. Aggregation Inhibition Assay

The method used for CaOx crystal aggregation assay was similar to that of Hess *et al.* (1989) and Atmani and Khan (1999). The CaOx monohydrate (COM) 'seed' crystals were prepared by mixing calcium chloride and sodium oxalate solutions at 50 mmol/L for each in a glass beaker (250 ml). This solution was equilibrated at 60^0C in a water bath for 1 hour, and get cooled to 37^0C overnight in an incubator. The CaOx crystals were harvested by centrifugation, and allowed to evaporate at 37^0C.

COM crystals were used at a final concentration of 0.8 mg/ml, buffered with 0.05 mol/L Tris-HCl, and 0.15 mol/L sodium chloride at pH 6.5. The plant extract/ standard drug (500 µl) was used at various concentrations ranging from 12.5 µg/ml to 3200 µg/ml. For the control experiment, 500 µl of deionized water was added and measured as blank. In the presence or absence of the plant extracts, the mixture was stirred in glass test tubes, and incubated for 30 minutes at 37^0C. The glass test tubes were capped with airtight stoppers after adding mixtures of solutions. The light absorbance of the mixtures were measured in the quartz cuvette at wavelength of 620 nm using Cary Series UV-Vis-NIR Spectrophotometer (Agilent Technologies, Australia). Each test was performed in triplicates. The percentage inhibition of aggregation was calculated by comparing turbidity due to CaOx formation in the presence of a plant extract with the control using the following formula:

% Aggregation inhibition = [(SAc-SAi)/SAc] ×100, or Aggregation (A) inhibition percentage: 1 - [SAi/ SAc] × 100; Where; SAi - represents a slope of aggregation in the presence of the inhibitor (herbal extracts/Standard drugs) of the sample set; SAc-represents a slope of aggregation for the control experiment (without inhibitor) of the sample set.

2.10.3. *In vitro* Decrystallization Assay of Kidney Stones Surgically Removed from Human Patients

Surgically removed stones were obtained from patients of St. Paul's Hospital. These were analyzed on the basis of color and appearance of stones to differentiate its type (Varsha *et al.,* 2012). The solubility of CaOx stones by the addition of *G. fruticosus* aqueous crude extracts and solvent-solvent fractionations were examined. In the present study, stones of a patient were measured for their initial weight and kept in a separate sample tube after proper

labeling. Then, each calculi was subjected to plant extracts and positive controls at different concentrations (in triplicates) to investigate decrystallization activities. After the 7^{th} days of soaking in testing agents at 37^0C in the dark, the stones were taken out and placed on the filter paper until it got dry. Then, measured for its final weight and compared with the initial weight changes.

2.11. *In vivo* Urolithiatic Pharmacological Investigations

2.11.1. Target Animals

Healthy adult male albino Wistar rats of the same age group between 8 to 10 weeks and weighing (220-280g) were used. There were purchased from the Ethiopian Public Health Institute (EPHI), and breed at the Biomedical Sciences animal laboratory, AAU. The experiment was conducted at the Biomedical Sciences Laboratory of AAU and EPHI, in accordance with internationally accepted standard guidelines for the use of animals in scientific research. Prior to starting the experiment, rats were acclimatized to standard laboratory conditions (6 rats per polypropylene cages) for 7 days. They were kept under a controlled environment of temperature (27 ± 2^oC), relative humidity (55 ± 5%), and light (12 h light/dark cycles). These rats were fed with regular pellets (standard diet) and were allowed for free drinking water (*ad libitum*) for 28 days.

2.11.2. Urolithiasis Induction

Kidney stones were induced using ethylene glycol (EG) along with ammonium chloride (NH_4Cl) administered to rats in drinking water. In this hyperoxaluria model, 1% (w/v) NH_4Cl was given with 0.75% (v/v) EG with water for the first 5 days to accelerate lithiasis, then switched by 0.75% EG alone for the next 25 days (Bashir *et al.*, 2010; Khan *et al.*, 2012; Khan, 2018). The rats will die if they are given NH_4Cl for long time. Exposure of

these dose levels were sufficiently tolerable in animal studies (Hess *et al*., 2004). EG administration results in hyperoxaluria, which in turn leads to CaOx deposition in the kidneys (Tzou *et al.,* 2016). The experimental rats assigned as kidney stone preventive and curative groups received stone inducing agents for 14 days and 28 days, respectively.

2.12. Preventive and Curative Effects Against Urolithiasis

2.12.1. Preventive effects

For the evaluation of preventive effect, a total of 42 albino Wistar male rats were divided randomly into 7 groups 6 each with matching body weights. Each extract was administered orally from day 1 to 14 along with induction of urolithiasis to determine preventive effect (Baheti and Kadam, 2013a; Vijaya *et al.,* 2013). Rats were assigned as Group I (Normal control/vehicle), Group II (Lithiatic control), Group III (K-Cit), Group IV (Cystone), Group V (*Satureja punctata*), Group VI (*Aloe pulcherrima*) and Group VII (*Gomphocarpus fruticosus*), which showed the highest *in vitro* activities among all plants tested. Group I and Group II did not received extracts/drugs. Previous investigators also used potassium citrate (2.5 g/kg) for positive controls (Renata *et al.*, 2003; Chow *et al.*, 2004). The dosing volume was 2 ml/100 g of body weight. The vehicle control group received distilled water once daily throughout the experiment. At the end of 14^{th} days, rats were sacrificed for biochemical and histopathological studies.

2.12. 2. Curative effects

For the evaluation of curative effect, a total of 78 albino r male Wista rats were divided randomly into 13 groups 6 each with matching body weights. Urolithiasis was induced priory from day 1 to 14. Then, each extract/drug was administered orally from day 15 to 28 along with induction (EG) of urolithiasis to determine curative effects (Baheti and Kadam,

2013a; Vijaya *et al.*, 2013). At the end of 28th day, rats were sacrificed for biochemical and histopathological studies. Rats were assigned as Group I (Normal control), Group II (lithiatic control), Group III (K-Cit), Group IV (Cystone), Group V(*Satureja punctata*), Group VI (*Aloe pulcherrima*), Group VII (*Gomphocarpus fruticosus* aq. crude extract), Group VIII (Mixed extracts), Group IX (*G. fruticosus* PET fraction), Group X (*G. fruticosus* Chl. fraction), Group XI (*G. fruticosus* EtOAc fraction), Group XII (*G. fruticosus* BuOH fraction), and Group XIII (*G. fruticosus* aq. fraction). The dosing volume was 2 ml/100 g of body weight. Group I and Group II did not received extracts/drugs. The control group received distilled water once daily throughout the experiment. At the end of the 28th day, the rats were sacrificed for biochemical and histopathological studies.

2.12.3. Urine Collection and Analysis

2.12.3.1. Urine Microscopic Examinations

Urine, serum and histological profiles are indicators of stone formation as well as recurrences. The abundance and morphology of calcium oxalate crystals formed under the *in vitro* models and the *in vivo* stone inductions were examined using a light microscope (Wagtech Thatcham Berkshire RG194QD, United Kingdom, 40× magnification).

Rats were placed in separate metabolic cages and were subjected to 24 hour urine collection on day 14th for preventive and on day 28th for curative test (Baheti and Kadam, 2013a). In crystalluria analysis, about 3 ml of the fresh urine samples collected were put in a glass tube and centrifuged at 3000 rpm for 10 minutes to remove debris, and supernatants were discarded. These urine sediments were used to determine CaOx crystal formation. About 10 µl of the vortexed sediments were placed onto a microscope glass slide (covered with a cover slip), and examined for CaOx crystals considering its number and size under a light

microscope (40×). The crystals formed from chemicals in the urine were carefully examined from other urine artifacts. The photographs of microscopic observations were taken using a digital camera (Sony Cyber-shot DSC-W180 10.1MP with 3× optical zoom, New Jersey, USA) manually mounted on the top of it.

2.12.3.2. Urine Biochemical Analysis

At the end of the respective treatment periods, the animals were individually housed in metabolic cages, and 24 hour urine (acidified and non-acidified) samples were collected. Urine was acidified with 1 ml of 6N HCL (hydrochloric acid) to dissociate oxalate ions and stored at 4^0C for 5 days. Then, these were centrifuged at 3000 rpm for 10 minutes (REMI, R24), and the supernatants of acidified urine were used to estimate excretions such as oxalate, calcium, magnesium and phosphate contents by clinical chemistry automated analyzer. In non-acidified urine sample contents such as citrate, creatinine and uric acid, and total protein concentrations were analyzed using commercially available diagnostic kits by the automated clinical chemistry analyzer. Both oxalate and citrate concentrations were analyzed using multi-well spectrophotometer (ELISA reader) as per the manual provided with kits. For the purpose of quantifying, a calibration curve was prepared using oxalate and citrate kits as standard.

2.12.4. Serum Collection and Analysis

At the end of the experimental period (day 14^{th} and 28^{th}), the rats were anesthetized using isoflurane and blood samples were collected from the retro-orbital vein by capillary puncturing. Serum was separated after centrifuged at 3000 rpm, 20°C for 15 minutes. The collected serum was investigated for biochemical parameters like creatinine, blood urea nitrogen, uric acid, urea, calcium, magnesium, and phosphate by Clinical Chemistry Auto-

analyzer (Cobas 6000 analyzer, Germany) with the respective diagnostic kits. The oxalate and citrate concentrations were determined using multi-well spectrophotometer (ELISA reader) as per the manual provided with kits. For the purpose of quantifying, a calibration curve was prepared using oxalate and citrate kits as standard.

2.12.5. Histopathological Study

2.12.5.1. Kidney Homogenate Analysis

At the end of the experimental period (day 14^{th} and 28^{th}), rats were sacrificed under isoflurane anesthesia, followed by cervical dislocation. Then, the abdomen was opened and both kidneys were collected from each rat. The isolated kidneys were carefully removed, and cleaned (washed/rinsed) from extraneous tissues with an ice cold physiological saline solution (0.15 M NaCl). The left kidneys from each animal was preserved in 10% buffered neutral formalin and used for histological studies. The right kidneys were sliced into two equal halves using a blade, and one-half dried at 80°C in a hot air oven. The method used was similar to Ashok *et al.* (2010) with some modifications, in which a fixed weight of 200 mg (29%) of the total kidney weight (0.67g) was further heated separately in 10 ml of 1N hydrochloric acid (1% HCl), which was placed in a boiled water bath of 100°C for 30 minutes. Then, it was finely chopped into pieces using a blade, crashed by pestle and mortar, and further homogenized for 10 minutes using Ultra-Sonicator. The homogenate was centrifuged at 3000 rpm for 15 minutes, and the supernatant was collected using labeled cryotubes. Finally, the supernatants were used for estimations of calcium, oxalate, and phosphate contents with commercially available biochemical kits (BioVision PLC,USA), according to the manufacturer's protocol (Ghelani *et al.*, 2016; Sharma *et al.*, 2017b).

2.12.5.2. Histopathological Examination

Histopathological examinations were done for kidney tissues of the experimental rats. All the rats were sacrificed in a humane manner using Isoflurane anesthesia at the end of the 14^{th} day (urolithiasis prevention studies) and at the end of 28^{th} day (urolithiasis curative studies). The tissue pieces were taken from kidneys and analyzed for urolithiatic prevention and curative potentials of plant extracts. The detailed technical approaches were used as described in section 2.8.2.6**.**

2.12.5.3. Calcium Oxalate Crystal Deposition

In the kidneys, crystal depositions were determined using semi-quantitative assays, which is a microscopic scoring method (Vanachayangkul *et al.,* 2011; Khan *et al.,* 2012). The microscope filar micrometer **(**0.1 mm) eyepiece (10×; Wide field of 23.3 mm**,** Olympus Optics OSM 212422, made in Japan) was used. That is, crystal deposits in the kidney tissues were counted using a light microscope. The severity grades of crystal deposits were assigned as 0 = <1 crystal (no crystal deposition), 1=1-10, 2= 11-30, 3=31-50, 4=51-75 and 5= >75 crystal counts, taking the mean values (Khan *et al.,* 2012).

For counting CaOx crystal deposits, a sagittal section of each renal specimen was divided into four equal sized regions by two virtual lines, and readings of an average of 4 microscopic fields were reported (Tsai *et al*., 2008; Bashir and Gilani, 2011). A field of 100× was then randomly selected from each region and the CaOx deposits were counted. The image of one of the four regions under a light microscope was randomly captured (Tsai *et al.,* 2008) using a digital camera manually mounted on top of the microscope.

2.13. Qualitative and Quantitative Determination of Active Compounds from Extracts

2.13.1. Column Chromatography Fractionation

A glass gravity column was clamped upright and packed with silica gel (internal diameter: 20 mm, reservoir volume: 500 ml, and length: 350 cm, Sigma-Aldrich) mixed with the mobile phase of chloroform to methanol solvents in 4.5:0.5 ratio, and poured into the column. The extract was mixed with a small amount of the mobile phase, and introduced as a thin band on the top of the silica gel. Once the extract was loaded onto the silica gel, the mobile phase was added at a constant flow rate. The different types of compounds to be separated was initiated by elution of chloroform: methanol (4.5 to 0.5), and maintained to the end, which passed through the column by gravity.

2.13.2. Thin Layer Chromatography (TLC) Analysis

The fractioned solutions were transferred into TLC plates using capillary tubes so as to screen the organic components of the extract. The TLC aluminium plate (20×20 cm) was marked up at 1.5 cm line at the bottom, and up to 10 cm was marked in pencil. The solvent system used was Chloroform : Methanol, in the ratio of 4.5:0.5. To this plate 20 µl of the fractionated sample extract was loaded, and placed in the mobile phase solutions until it reached up the marked line. Then, it was allowed to dry and get exposed to UV light (UVP MultiDoc-It, cabinet with an ENF-28^{0}C lamp, Germany) at 254 nm (SW) and 365 nm (LW). If the spots alignment on TLC were found to be the same, it was combined (collected) altogether in one test tube, and was allowed to concentrate on a water bath at 37°C. Subsequently, the *in vitro* anti-urolithiatic activities of the fractions were evaluated**.**

2.13.3. Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

The bioactive volatile components (metabolites) of the extract in a mixture were identified and quantified using the methods of Gavamukulya *et al*. (2015), Kalaisezhiyen and Sasikumar (2012), and Sivakumar and Dhivya (2015). GC-MS analysis of bioactive compounds from the extract was carried out using GC systems (Agilent Technologies, Santa Clara, CA, USA) equipped with HP-5MS column (30 m in length×250 µm in diameter ×0.25 µm in thickness of film). GC system comprising Gas Chromatograph interfaced to Mass Spectrometer (GC-MS) instrument employing the following conditions: Column Elite-1 fused silica capillary column (30×0.25 mm ID×1EM df, composed of 100% Dimethyl poly siloxane), an electron ionization system, which utilized high energy electrons (70eV), and pure helium (99.99%) was used as carrier gas that passed through at a flow rate of 1 ml/minute.

The initial oven temperature was set at 50^0C for 2 minutes, and the temperature gradually increased up to 280°C at holding time of 30 minutes with increasing rate of 10°C/min. Sample (1 µl) was dissolved in chloroform (1% of the extracts diluted) was injected into the gas chromatogram. The sample injector ion source and mass transfer line temperature were maintained at 250°C and 280°C, and at the split ratio of 10:1 throughout the experimental period of 30 minutes of total run time. Mass spectra were taken at a scan interval of 0.5 second and fragments from 40 to 550 dalton (Da), unified atomic mass unit.

The volatile components of the extract were identified from GC-MS spectrum peaks, using data of the corresponding components. That is, the mass spectra of unknown components were compared or matched with a database of known spectral components stored in the GC-MS library using National Institute of Standard and Technology (NIST, 2014) library based

on their molecular mass. The chemical compounds present in the extract were expressed as a percentage (%) based on the peak area observed in the chromatogram. The biochemical constituents were also identified based on retention time on the GC column as described by Casuga *et al.* (2016). Mass-Hunter software was used to integrate the area of each compound in the chromatogram of GC-MS data analysis.

2.14. Statistical Analysis

The data were analyzed using Graph Pad Prism version 6 Software (Graph Pad Software, San Diego, CA, USA). One-way analysis of variance (ANOVA) followed by post-hoc Dunnett's test comparisons were performed to compare between treated and untreated groups. Comparisons were performed between the positive control (ascorbic acid) and plant extract groups in antioxidant evaluations. Nonlinear regression analysis was used to calculate the IC_{50} values for all dose-responses. The data values were expressed as mean ± standard deviation (SD). The retrospective data were analyzed using descriptive statistics. Values of $p<0.05$ was considered statistically significant.

2.15. Ethical Considerations

Animal experiments were conducted in compliance with internationally accepted standard guidelines for scientific research (OECD, 2001). The research protocol was approved by the College of Natural Sciences Institutional Review Board (CNS-IRB) (Approval Minute number: IRB/020/2016), Addis Ababa University. Furthermore, ethical approval was obtained from the St. Paul's Hospital, Millennium Medical College (SPHMC) Institutional Review Board (Ref. Number: PM23/285/2016) to conduct retrospective study. To ensure confidentiality, the patients' name was assigned to code identifiers, and data were used only for the intended study.

3. Results

3.1. Trend in the Prevalence of Urolithiasis in Ethiopia

3.1.1. Stone Disease Prevalence

Among 32,370 patients who underwent open surgical treatment, 757(2.3%) of patients had stone diseases. In terms of gender, urolithiasis was more prevalent among males accounting for 68.2% (516) as compared to 31.8% (241) in females.

3.1.2. Trend in the Prevalence of Urolithiasis

During the thirteen year retrospective study period, the prevalence of urolithiasis showed an increasing trend from 2012 to 2017 (Figure 7; Figure 8).

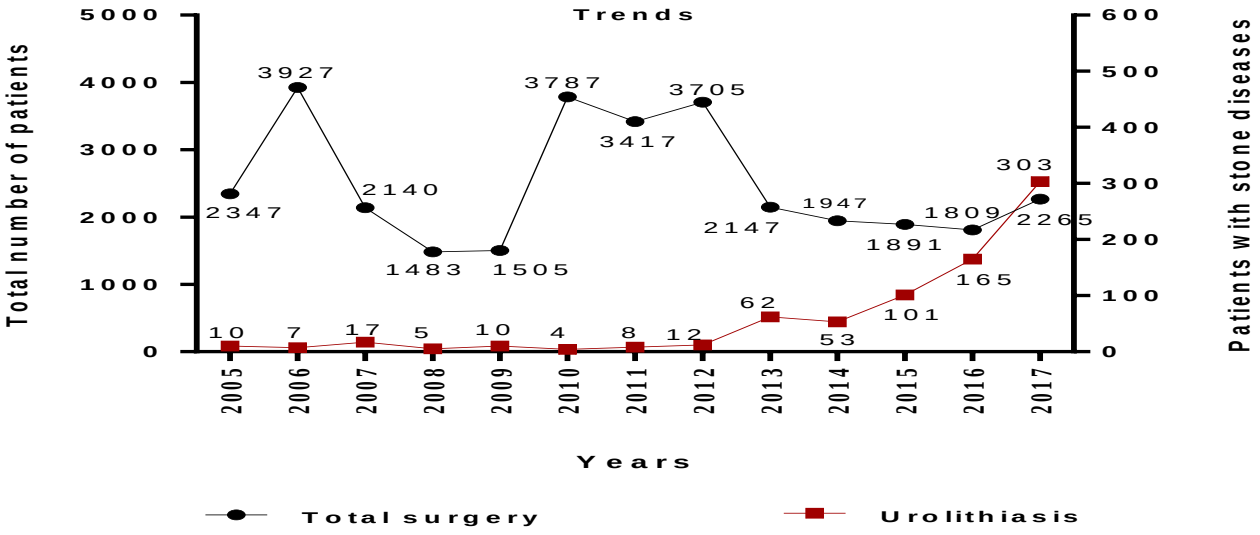


Figure 7. Trend in urolithiasis prevalence from September 2005 to 2017 at St. Paul's Hospital, Millennium Medical College.

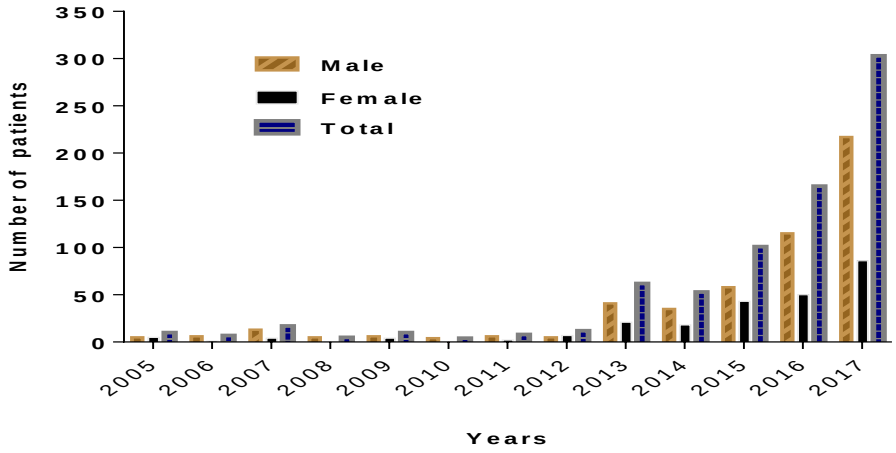


Figure 8. Proportions in stone surgery as it relates to gender at St. Paul's Hospital, Millennium Medical College, September 2005 - 2017.

3.1.3. Prevalence of Urolithiasis in Relation to Age Group

The age of the study population ranged from three to 84 years and the mean age (SD) was 42.5± 23.7 years. The majority of stone patients (22.2%) were in the age group of 30 to 39 years. In this group, the hospital prevalence of urolithiasis was 20.5% among males and 25.7% among females. In general, most cases with urolithiasis were between 20 and 49 years of age (Figure 9).

Figure 9. Age related frequency of urolithiasis among 757 patients at St. Paul's Hospital, Millennium Medical College, September 2005 - 2017.

3.1.4. Anatomical Locations of Urolithiasis

In 46.5% of the cases, the stones were located in the kidneys. The location of stones was more on the left kidney (47.7%) than the right kidney (37.5%). There were some patients whose stone locations not defined or recorded in the registry books (Table 4).

Table 4. The frequency of urolithiatic anatomical locations among 757 cases at St. Paul's Hospital, Millennium Medical College, September 2005 - 2017.

Urolithiatic locations	Number of cases (%)
Urolithiasis (n=757)	
Kidneys	352 (46.5)
Ureter	314 (41.5)
Bladder	89 (11.7)
Urethra	2(0.3)
Kidney stones (n=352)	
Right kidney	132(37.5)
Left kidney	168(47.7)
Not defined	52(14.8)
Ureteric stones (n=314)	
Right ureter	153(48.7)
Left ureter	126(40.1)
Not defined	35(11.2)

3.1.5. The Major Comorbidities Associated with Stone Diseases

Among 757 stone patients, 13.61% (103) of the urinary stones were associated with comorbidities or complications. The major comorbidity was benign prostatic hyperplasia (BPH), and major complications include decreased urinary output (UOP) and hydronephrosis. Stone surgeries due to recurrent stone formation constituted 1.32% (Table 5).

Table 5. Common co-morbidities and complications associated with urolithiasis among 757 cases at St. Paul's Hospital, Millennium Medical College, September 2005-2017.

No	Co-morbidities/Complications	Urinary stones
		Number of Patients(%)
1	Benign prostatic hypertrophy	24(3.17)
2	Reduced UOP	58(7.66)
3	Hydronephrosis	6(0.79)
4	Kidney failure	4(0.53)
5	*Diabetes mellitus* (Type 2)	1(0.13)
6	Recurrent	10(1.32)
7	Null	654 (86.39)

3.2. Results of Phytochemical Screening

Preliminary phytochemical analysis of the selected medicinal plant aqueous extracts is shown in Table 6. The percent yield results were presented in Table 7.

Table 6. Phytochemical screening of the crude aqueous extracts of medicinal plants.

	Chemical Constituents								
Extracts	**phenols**	**Flavonoids**	**Tannins**	**Steroids**	**Terpenoids**	**Alkaloides**	**Saponins**	**Resins**	**Glycosides**
A. aspera	+	+	+	+	+	+	+	_	_
R. abyssinicus	+	_	+	+	+	_	_	+	_
S. punctata	+	+	+	+	+	_	+	+	_
C. murale	+	_	+	+	+	_	+	+	+
A. pulcherrima	+	+	+	_	+	_	_	_	+
C. ambrosioides	+	+	+	+	+	+	_	+	+
I. confertiflora	+	_	+	+	_	+	+	_	+
G. fruticosus	+	+	+	+	_	+	+	_	+
C. myrrha	_	_	_	+	+	_	_	_	+

Note: "+" indicates presence of phytoconstituents; "-" indicates absence of phytoconstituents.

Table 7. The percentage yield of plants aqueous crude extracts.

No.	Plant Extracts	Yield of Extracts	
		Weight (g)	Percent(%)
1	*Achyranthes aspera* L. -Leaves	38	13.01
3	*Rumex abyssinicus* Jacq. -Rhizome	24	7.25
4	*Satureja punctata* (Benth.) Briq. -Aerial parts	21	6.71
2	*Chenopodium murale* L. -Leaves	32	11.87
5	*Aloe pulcherrima* Gilbert & Sebsebe - gel	12	5.47
6	*Chenopodium ambrosioides* L.- Leaves	28	13.5
7	*Inula confertiflora* A.Rich.-Leaves	42	18.2
8	*Gomphocarpus fruticosus* (L.) Ait.f.- Leaves	43	22.0
9	*Commiphora myrrha* (Nees) Engl.- resins	17	9.50

3.3. Acute and Sub-acute Toxicity Profiles of Antiurolithiatic Medicinal Plant Extracts in Albino Wistar female Rats

3.3.1. Acute Toxicity Profile

Administrations of 70% ethanol extracts of the leaves of *A. aspera* (Aa), *G. fruticosus* (Gf), *C. murale* (Cm), *C. ambrosioides* (Ca), and *I. confertiflora* (Ic), the rhizomes of *R. abyssinicus* (Ra), the aerial parts of *S. punctata* (Sp), the resins of *C. myrrha* (Cm) and the gel of *A. pulcherrima* (Ap) to rats did not show mortality at a single dose of 2000 mg/kg body weight. Moreover, there were no visible signs of acute toxicity, i.e., food and water consumptions were unaffected; and salivation, aggression, rising furs, and writhing were not observed for 14 days.

3.3.1.1. Acute Effect on Body weight

The extracts of *S. punctata*, *A. pulcherrima*, and *A. aspera* did not acutely affect body weight at dose of 2000 mg/kg body weight in rats (Figure 10A). Similarly, the leaves extracts of *G. fruticosus* and *C. myrrha* resins were associated with increased body weight

(Figure 10B). However, administrations of *C. murale* and *R. abyssinicus* extracts (Figure 10A), and *C. ambrosioides* and *I. confertiflora* extracts (Figure 10B) at a dose of 2000 mg/kg were associated with weight loss compared to their initial weight, although it was not significant.

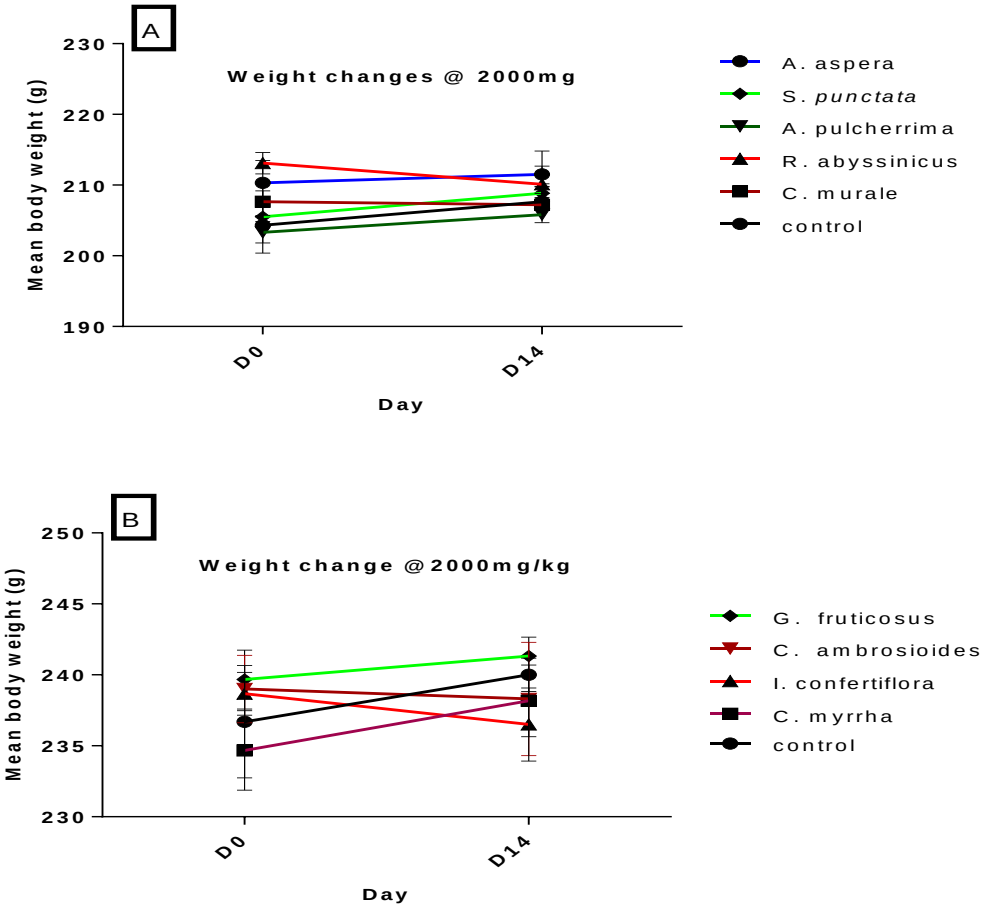


Figure 10. Acute toxicity effects of extracts (2000 mg/kg) on body weight changes in non-pregnant female Wistar albino rats. (A) *A. aspera*, *R. abyssinicus*, *S. punctata*, *C. murale*, *A. pulcherrima*, and the control; (**B**) *C. myrrha*, *G. fruticosus*, *C. ambrosioides*, *I. confertiflora* and the control. D0 = day zero, and D14 = day 14. Comparisons were made with a control group before and after the experiment. The data illustrated the mean ± SD of 3 rats per treatment groups (n = 3).

3.3.1.2. Hematological Parameters of Acute Toxicity

The administrations of *C. murale* and *R. abyssinicus* extracts significantly ($p<0.05$) reduced HGB concentrations(13.11±1.31 g/dl) and 14.10±2.52 g/dl, respectively, compared with the normal control rats (18.23±1.00 g/dl). Clinically meaningful increment of RBC concentration was observed, although it did not reach statistical significance compared with the controls (4.75 $\times 10^6$/mm3 Cells). However, most of the hematological biomarkers remain within the normal limit in comparison with the control group ($p>0.05$), although the extracts of *C. murale*, *R. abyssinicus* and *S. punctata* revealed a significant reduction in platelet count compared with the control ($p< 0.05$) (Table 8**)**.

Table 8. Hematological parameters of non-pregnant female Wistar rats exposed to extracts (dose 2000 mg/kg) on14th day follow up.

Hematological Parameters	Normal control	Cm	Ra	Ap	Sp	Aa
WBC (Cell x 10^3/mm3)	6.69±1.21	5.44±1.23	6.10±2.67	5.99±0.31	6.12+1.47	6.67±0.91
RBC (Cell x 10^6/mm3)	4.75±0.40	4.46±0.67	4.52±1.39	4.55±0.28	5.00±0.16	4.94±0.53
HGB (g/dl)	18.23±1.00	13.11±1.31**	14.10±2.52*	16.25±1.20	17.36±0.20	17.36±1.06
HCT (%)	48.23±3.37	44.80±4.03	43.46±7.33	46.65±2.75	50.50±0.43	50.46±2.68
MCV (fl/cell)	56.36±1.25	57.33±1.07	56.50±1.21	54.50±1.41	56.13±1.43	56.46±0.61
MCH (pg/cell)	19.43±0.25	19.20±0.34	19.03±0.20	18.95±0.77	19.26±0.49	19.43±0.40
MCHC (g/dl)	34.50±0.30	33.40±0.10	33.63±0.32	34.85±0.49	34.36±0.25	34.40±0.43
RDW-SD (fl)	28.96±2.80	27.76±0.60	27.60±1.67	26.95±0.63	27.93±1.25	28.20±0.85
RDW-CV(%)	17.53±1.49	15.06±1.19	15.76±2.56	17.20±1.13	18.43±0.20	17.33±1.58
PDW (fl)	8.70±0.20	8.53±0.45	8.73±0.25	8.30±0.28	9.06±0.32	8.80±0.30
MPV (fl)	8.00±0.20	7.96±0.35	7.90±0.34	7.55±21.21	8.06±0.23	7.90±0.30
Platelet (10^3/m l)	846.66 ±17.22	586.33±28.00***	653.00±37.6**	842.50±36.42	787.33±17.32*	809.66±29.44
P-LCR (%)	10.83±1.20	10.13±2.41	10.06±2.40	8.40±1.41	11.14±1.70	10.33±1.70
PCT (ng/ml)	0.67±0.13	0.35±0.14	0.43±0.31	0.75±0.03	0.35±0.11	0.63±0.17

NEUT (%)	0.83±0.08	0.73±0.02	0.70±0.27	0.78±0.26	0.84±0.18	1.06±0.26
LYM (%)	5.39±0.98	4.09±1.00	5.08±2.29	5.01±0.69	4.83±1.05	5.15±0.84
MONO (10^3/ml)	0.17±0.17	0.12±0.05	0.20±0.16	0.14±0.14	0.24±0.13	0.22±0.05
EO(x10^3/ml)	0.08±0.00	0.06±0.00	0.06±0.01	0.05±0.02	0.19±0.05	0.08±0.02

Note: WBC = White Blood Cells; RBC = Red Blood Cells; HGB =Hemoglobin; HCT = Hematocrit (called Packed Cell Volume, PCV); MCV = Mean Corpuscular Volume; MCH = Mean Corpuscular Hemoglobin; MCHC= Mean Corpuscular Hemoglobin Concentration; RDW-SD = Standard Deviation in Red Cell Distribution Width; RDW-CV= Coefficient of Variation in Red Cell Distribution Width; PDW= Platelet Distribution Width; MPV= Mean Platelet Volume; PLT = Platelet; P-LCR= Platelet Larger Cell Ratio; PCT= Procalcitonin; NEUT=Neutrophils; LYM=Lymphocyte Count; MONO= Monocytes; EO= Eosinophils; pg (pictograms); Cm=*C. murale*- leaves; Ra=*R. abyssinicus*-rhizome; Ap=*A. pulcherrima*- gel; Sp= *S. punctata*- aerial parts and Aa=*A. aspera*-leaves. Values are represented as mean ±SD of triplicates (n = 3). *p < 0.05, **p < 0.01,***p < 0.001 indicate significant changes in comparison with the normal control.

3.3.2. Sub-acute Toxicity Study

No mortality was observed for extracts of *A. aspera* leaves, *R. abyssinicus* rhizome, *S. punctata* aerial parts, and *A. pulcherrima* gel at a dose of 2000 mg/kg body weight. The sub-acute toxicity study was not conducted for other extracts.

3.3.2.1. Sub-acute Effect on Body Weight

Results revealed no significant weight changes for all extracts at a dose of 2000 mg/kg (Figure 11).

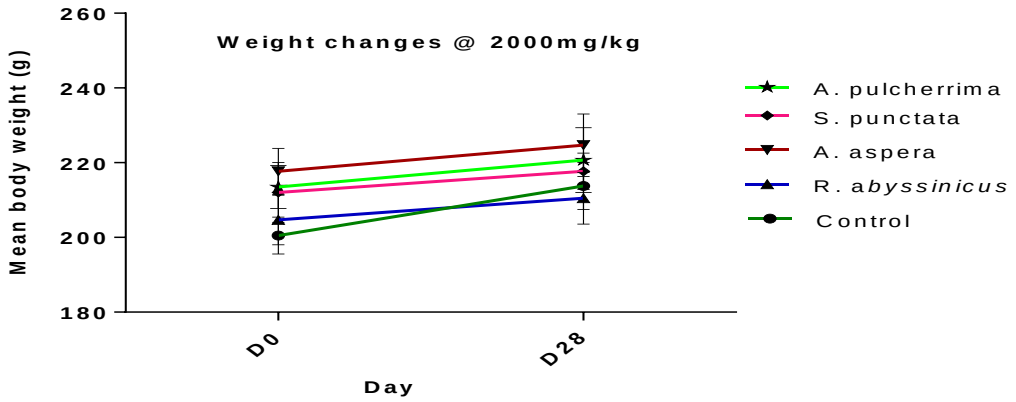


Figure 11. Effects of extracts on body weight changes in non-pregnant female Wistar rats during sub-acute toxicity study. Note: Aa=*A. aspera*-leaves, Sp=*S. punctata*-aerial parts, Ra=*R. abyssinicus*-rhizomes, Cm=*C. murale*-leaves and Ap=*A. pulcherrima*- gel, D0= day zero, and D28 = day 28**.** Comparisons were made with a control group before and after the experiment. The data illustrated the mean ± SD of 6 rats per treatment groups (n = 6).

3.3.2.2 Hematological Markers of Sub-acute Toxicity

Comparisons made between the control and the treated groups showed no significant difference for the Ra, Sp, Aa and Ap extracts (Table 9).

Table 9. Hematological parameters of sub-acute toxicity in non-pregnant female Wistar albino rats exposed to plant extracts at a single dose of 2000 mg/kg given once daily for 28 days.

Hematological parameters	Normal control	Ra	Sp	Aa	Ap
WBC(cell × 10^3/ml)	7.46±0.96	6.99±1.04	6.57±1.13	7.28±0.54	6.92±1.38
RBC (cell× 10^6/ml)	5.68±0.77	4.37±0.23	4.49±0.44	4.63±0.05	4.50±1.12
MCV (fl/cell)	60.50±2.40	61.63±1.23	61.23±1.19	60.56±0.80	60.53±2.99
MCH (pg/dl)	18.00±0.56	18.10±0.17	17.76±0.15	17.60±0.10	17.93±0.70
MCHC (g/dl)	29.80±0.28	29.36±0.40	29.00±0.36	29.10±0.43	29.60±0.65
PLT (10^3/µl)	762.00±94.75	688.33±7.09	642.33±11.80	818.66±81.05	580.00±278.26
RDW-SD (fl)	38.90±0.84	39.86±1.80	41.13±0.90	39.83±1.40	39.00±1.92
RDW-CV (%)	22.35±1.34	21.33±0.60	22.06 ±0.50	21.83±0.20	21.16±0.87
PCT (ng/ml)	0.86±0.01	0.61±0.02	0.58±0.11	0.75±0.06	0.53±0.24
MONO (x10^3/ml)	0.37±0.31	0.19±0.05	0.30±0.06	0.49±0.22	0.45±0.10
EO x (10^3/ml)	0.05±0.01	0.34±0.02	0.05±0.01	0.04±0.01	0.07±0.04

Note: WBC = White Blood Cells; RBC = Red Blood Cells; MCV = Mean Corpuscular Volume; MCH = Mean Corpuscular Hemoglobin; MCHC= Mean Corpuscular Hemoglobin Concentration; PLT = Platelet; RDW-SD = Standard Deviation in Red Cell Distribution Width; RDW-CV= Coefficient of Variation in Red Cell Distribution Width; PCT= Procalcitonin; MONO= Monocytes; EO= Eosinophils; pg = pictograms; Ra=*R. abyssinicus*-rhizome, Sp= *S. punctata*-aerial parts, Aa=*A. aspera*-leaves and Ap=*A. pulcherrima*-gel. All extracts were with EtOH (70%). Values are represented as mean ±SD (n=6).

The PDW (9.98 fl/cell) and a platelet larger cell ratio (P-LCR) concentrations reduced significantly (18.33%) following exposures to *R. abyssinicus* extracts ($p<0.05$). Although it was not statistically significant, there was a decrease in the mean platelet volume (MPV) for all EtOH (70%) extracts (Ra, Sp, Aa and AP) administered compared with the control (9.86 fl/cell) (Figure 12A-E).

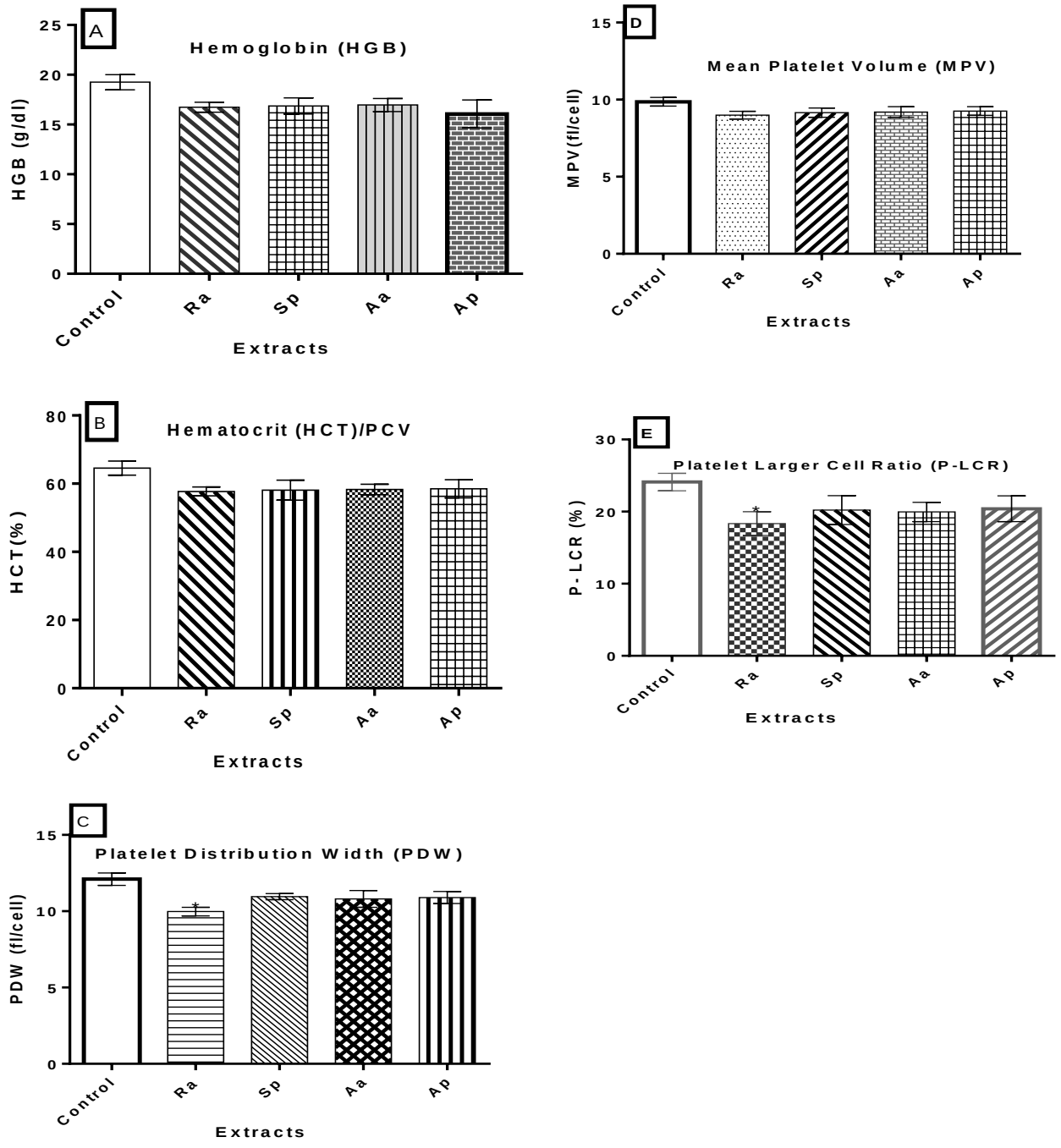


Figure 12. Hematological markers of toxicity in non-pregnant female Wistar albino rats exposed for 28 days. Values are expressed as mean ± SD (n=6). (A) HGB, (B) PCV, (C) PDW, (D) MPV, and (E) P-LCR. Note: Aa=*A. aspera*-leaves, Sp=*S. punctata*-aerial parts, Ra=*R. abyssinicus*-rhizome, and Ap=*A. pulcherrima*-gel. *p<0.05 statistically significant compared to the control group.

3.3.2.3 Effects of plant Extracts on Biochemical Markers of Liver Injury

Following an exposure to *A. aspera* extract, the level of ALP decreased (126U/L) compared to the controls (148U/L). Similarly, ALT levels increased significantly ($p<0.05$) in the administration of *R. abyssinicus* extracts (71U/L) compared to the controls (63.65U/L). However, all tested extracts did not alter AST levels (Figure 13A-C). Renal toxicity was reduced significantly ($p<0.05$) the levels of creatinine due to exposures for *A. aspera* extract compared to the normal control (Figure 14A-C**).**

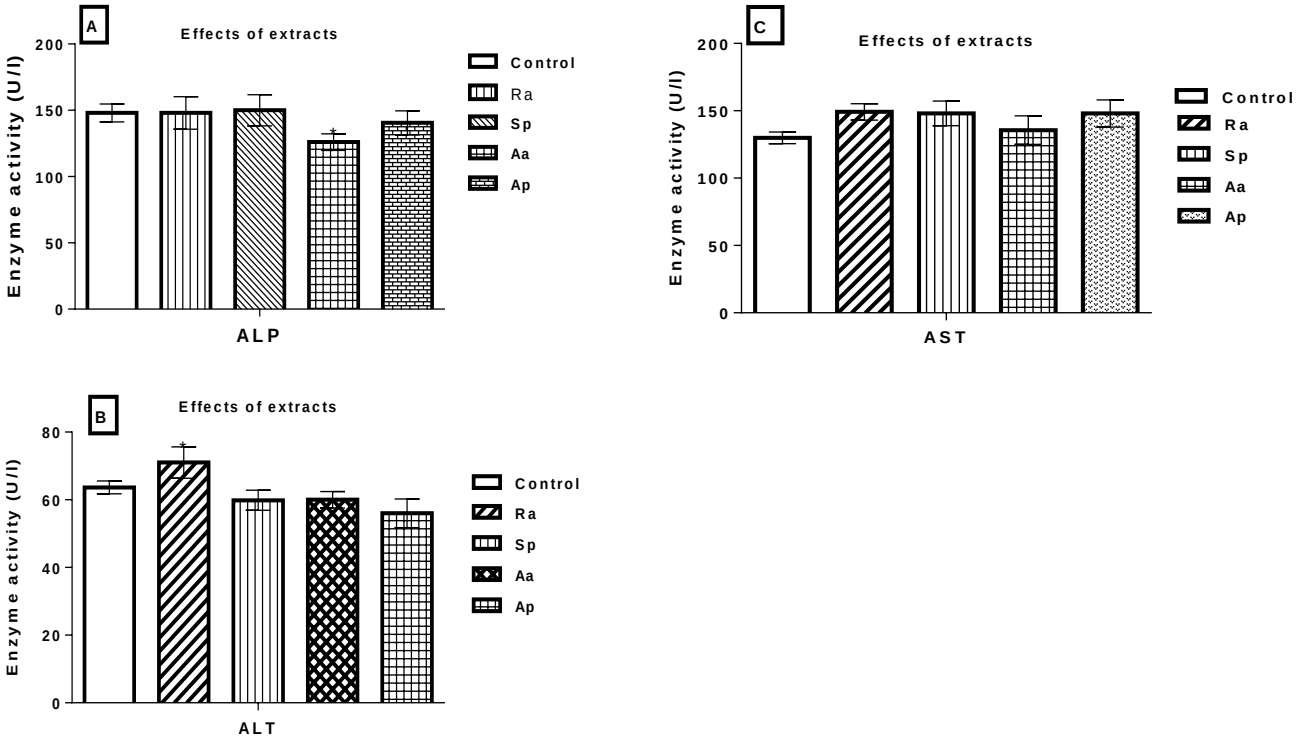


Figure 13. Estimation of ALP (A), ALT (B), and AST (C) in serum indicating the effects of plant extracts on liver functional indices of non-pregnant female Wistar rats exposed for 28 days. Note: ALP= Alkaline phosphatase, ALT=alanine aminotransferase, AST=aspartate aminotransferase, Aa=*A. aspera*-leaves, Sp=*S. punctata*- aerial parts, Ra=*R. abyssinicus*-rhizome, and Ap=*A. pulcherrima*- gel. The data illustrated the mean $\pm$ SD of six rats per treatment groups (n = 6). *$p<0.05$ statistically significant compared to the control group.

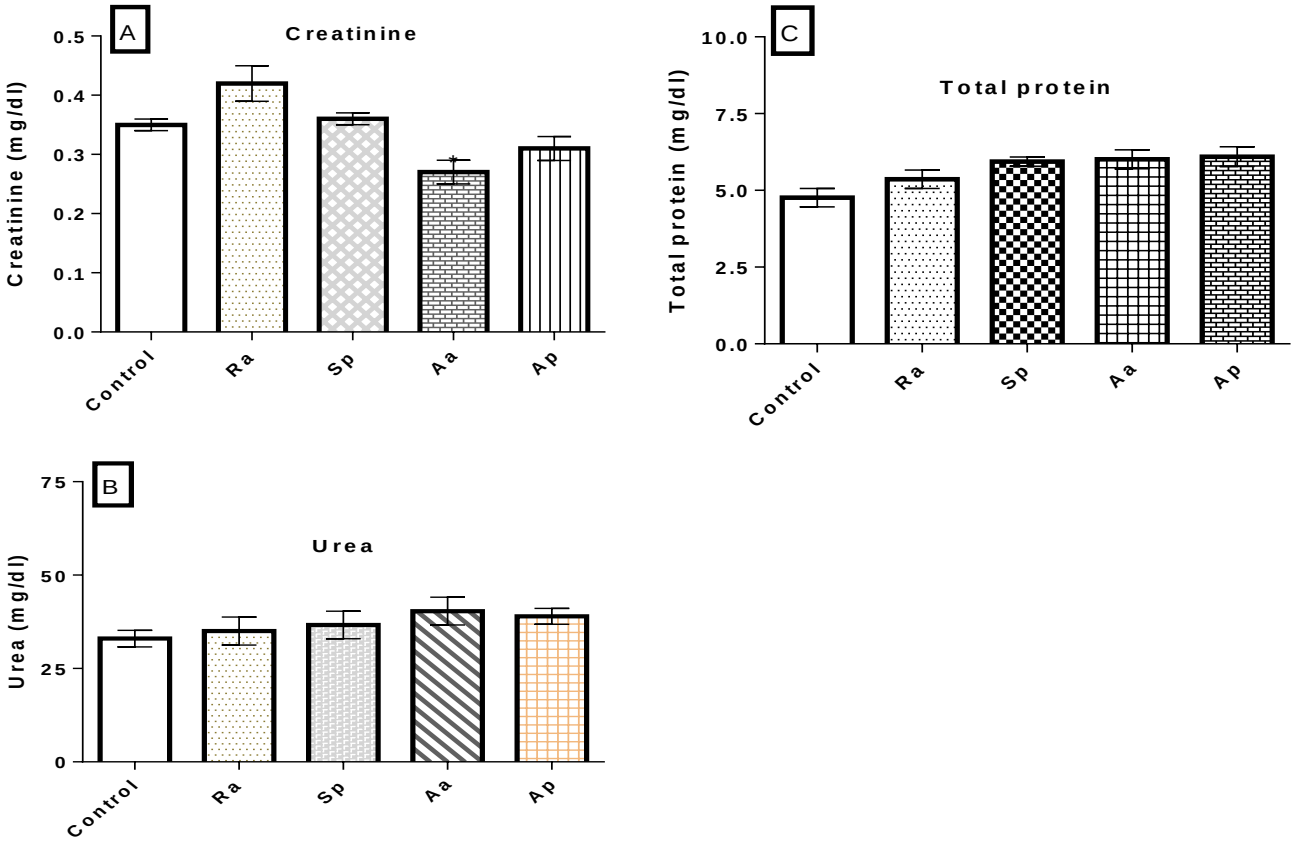


Figure 14. Renal toxicity following administrations of *R. abyssinicus* (Ra), *S. punctata* (Sp), *A. aspera* (Aa) and *A. pulcherrima* (Ap) crude extracts at a dose of 2000 mg/kg exposed for 28 days. (A) Serum creatinine, (B) Serum urea, and (C) Serum total protein concentrations. The data illustrated the mean ± SD of six rats per treatment groups (n = 6). *p<0.05 statistically significant compared to the control group.

3.3.2.4. Effect of the Extracts on Histopathology of the Liver and Kidneys

3.3.2.4.1. Liver Histopathology

Histopathological studies of liver demonstrated that the extracts of *R. abyssinicus*, *A. aspera* and *S. punctata* showed mild acute injury on liver tissues at a dose of 2000 mg/kg body weight in comparison with the control group. Indications of sub-acute toxicity in the liver, following exposure of rats to extracts, were characterized microscopically by fine septa of extracellular matrix (chiefly collagen) that divided hepatocyte plates into small

clusters of individual hepatocytes. An exposure to *A. pulcherrima* extract was not associated with any toxicity (Figure 15B).

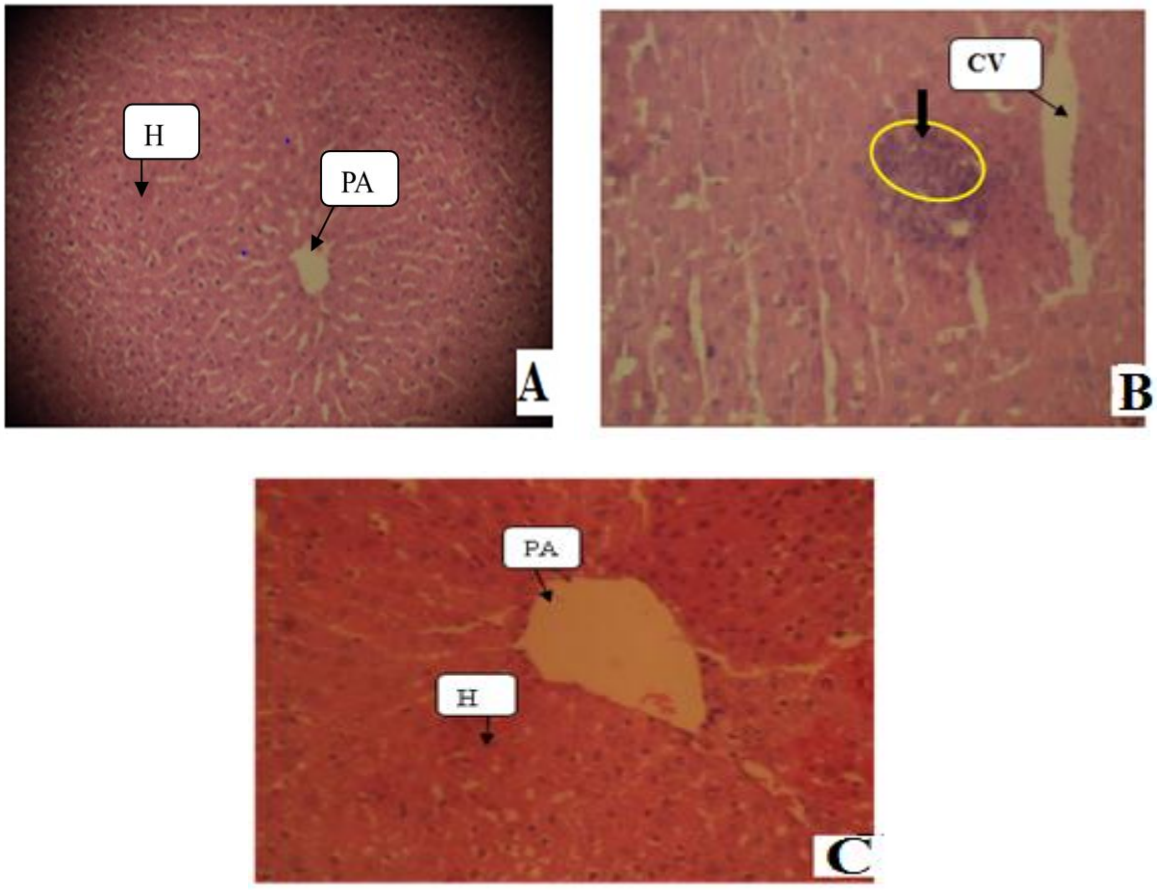


Figure 15. Effects of extracts on liver tissues of non-pregnant female rats exposed for 28 days. **(A)** Liver tissues from control rats had normal architecture with hepatocytes arranged around the central vein; (**B**) Liver tissue revealed local inflammatory infiltrations (*R. abyssinicus*, *A. aspera* and *S. punctata* extracts), and (**C**) *A. pulcherrima* extracts. Photomicrographs were at 100x magnification using light microscope, and 5µm thick paraffin sections, Hematoxylene and Eosin stain. Arrows indicate tissues affected as a result of herbal extracts; the yellow circle shows lobular hepatitis**.** Note: PA=Portal area; H= Hepatocytes; and CV=Central vein.

3.3.2.4.2. Kidney Histopathology

Some minor tubular inflammations were observed following exposure to *R. abyssinicus*, *A. aspera* and *S. punctata* extracts (Figure 16).

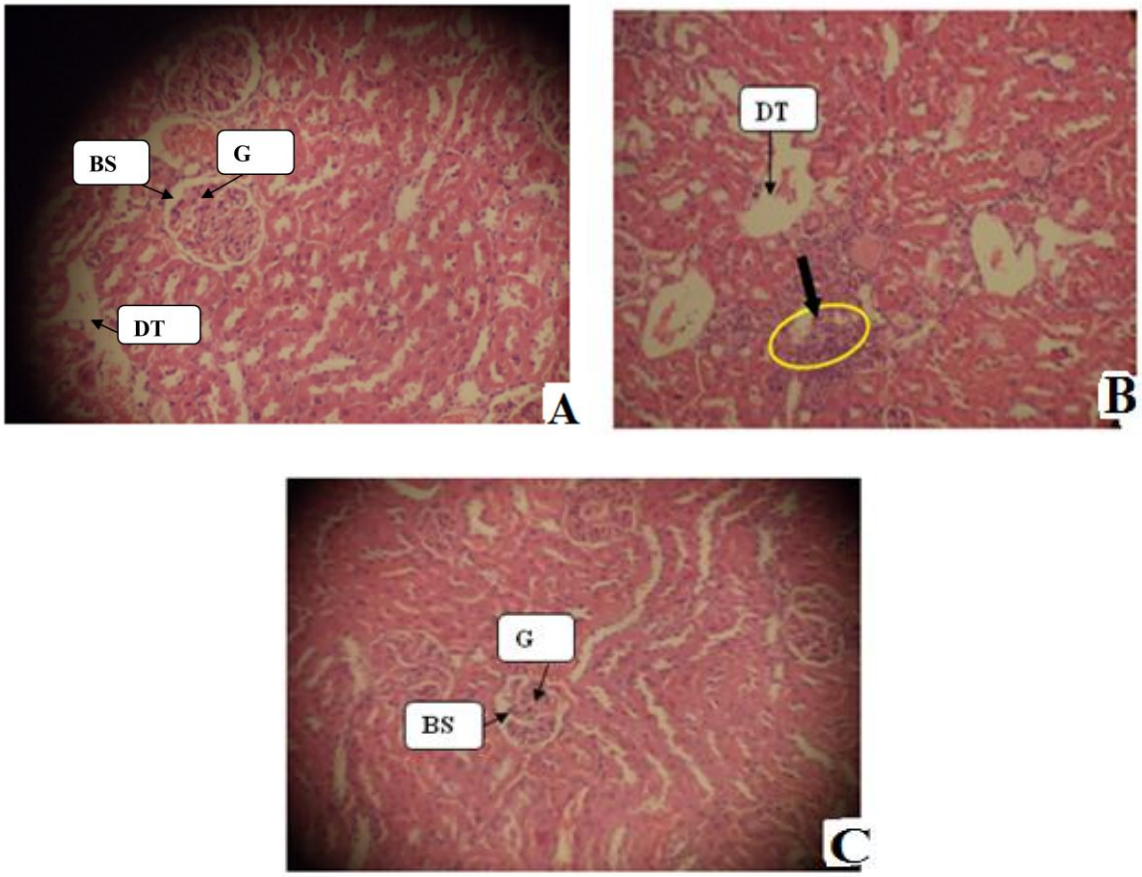


Figure 16. Sub-acute toxic effects of extracts on kidney tissues of non-pregnant female rats after exposure for 28 days. (A) Kidney tissues of control group showing normal morphology of the glomeruli and tubules; (**B**) Mild tubular inflammations (*R. abyssinicus*, *A. aspera* and *S. punctata* extracts), (**C**) *A. pulcherrima* extract. Photomicrographs were at 100x magnification using a light microscope, and 5µm thick paraffin sections, Hematoxylene and Eosin stain. Arrows indicate tissues affected as a result of herbal extracts. Note: G=Glomerulus, BS=Bowman's space, DT= Distal convoluted tube.

3.4. Evaluations of *In vitro* Antioxidant Activities of the Extracts

3.4.1. DPPH radical scavenging assay

The antioxidant activity of *S. punctata* extract was 92.3% inhibition at 0.20 mg/ml, which was higher than the effect of ascorbic acid (vitamin C) (87.6%) at 0.20 mg/ml. The antioxidant activity of *S. punctata* at dose 100 mg/ml was 88.5%, which was lower than ascorbic acid (98.4%), although not statistically significant. In general, *S. punctata* extract was a strong antioxidant equivalent to ascorbic acid. The antioxidant activity of *A. pulcherrima* extract was 72.3% at 0.78 mg/ml, which was significantly different from that of ascorbic acid (94.5%) ($p<0.01$). However, as the concentrations of *A. pulcherrima* extract increased beyond 6.25 mg/ml (88.4%), its antioxidant activity declined to 69.7% at 100 mg/ml significantly lower than ascorbic acid ($p<0.001$). *G. fruticosus* showed a scavenging potential of 81.6% at 3.13 mg/ml, which was significantly different from ascorbic acid (92.3%) ($p<0.05$) at the same concentration. In addition, *G. fruticosus* scavenged free radicals by 93% at 12.50 mg/ml, which was not significantly different from the positive control, ascorbic acid (92.8%) at the same concentration.

The antioxidant activity of *A. aspera* leaves extract (48.9%) at 12.50 mg/ml was significantly lower than ascorbic acid (95.6%) at the same concentration. Similarly, the antioxidant activity of *A. aspera* inflorescences was 54.9% at 6.25 mg/ml compared to ascorbic acid (94.1%) ($p<0.001$). The DPPH scavenging potentials of *A. aspera* inflorescences was 71.6% compared to ascorbic acid (95.5%) at 25 mg/ml ($p<0.001$). The antioxidant activity of *A. aspera* leaves increased in a concentration dependent manner and it was 94.6% at 100 mg/ml equivalent to ascorbic acid (98.4%). However, *A. aspera* roots extract did not possess antioxidant activity. The DPPH radical scavenging activity of *C.*

myrrha extract was 15.9% at 100 mg/ml, which was significantly lower than that of ascorbic acid ($p<0.001$). The IC_{50} antioxidant values of *S. punctata*, *A. pulcherrima*, *G. fruticosus*, *A. aspera* and *C. myrrha* extracts were 0.01±0.003 mg/ml, 0.42±0.047 mg/ml, 1.64±0.147 mg/ml, 13.51±1.08 mg/ml and nill, respectively, compared to ascorbic acid (0.03 ±0.007 mg/ml) (Figure 17).

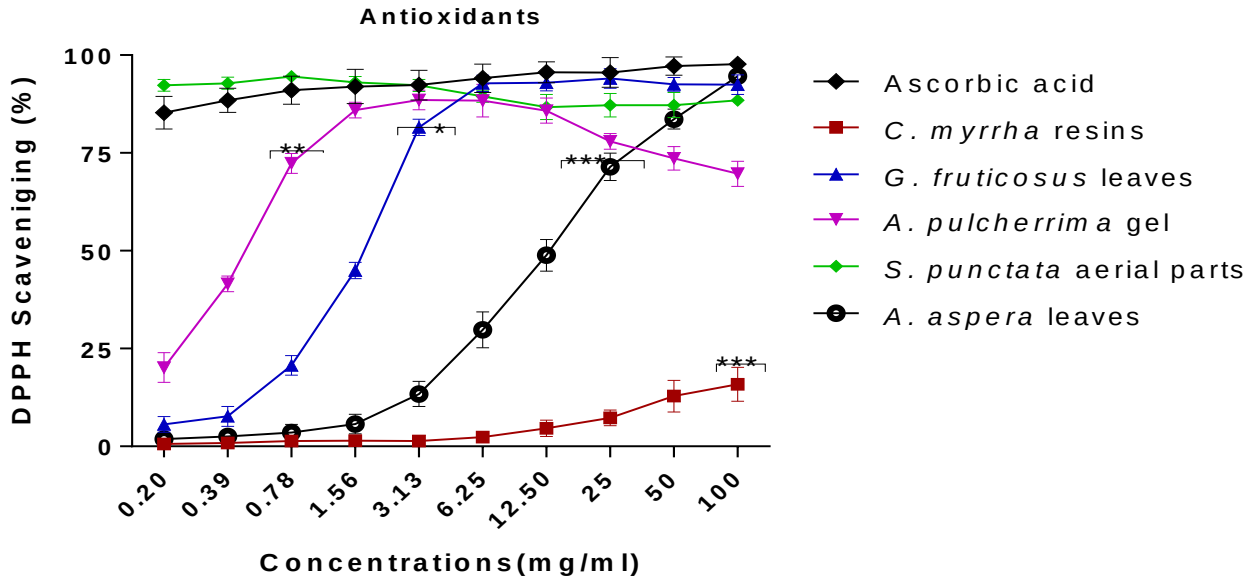


Figure 17. DPPH radical scavenging activities (%) of aqueous extracts of test plants at various concentrations in comparison with that of the standard. *C. myrrha*, *G. fruticosus*, *S. punctata*, *A. pulcherrima* and *A. Aspera* extract as compared to the positive controls-ascorbic acid. Data presented were based on mean± SD of three independent observations. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ statistically significant compared to the control group.

3.5. *In vitro* Evaluations of the Antiurolithiatic Effect of the Extracts

3.5.1. Effects of Extracts on Calcium Oxalate Crystallization

The findings for the *in vitro* effects of *S. punctata*, *A. pulcherrima*, *G. fruticosus*, *C. myrrha* and *A. aspera* extracts on calcium oxalate crystallization was recorded after 30 minutes incubation.

3.5.1. 1. Nucleation Inhibition

The percentage nucleation inhibitions of *S. punctata* extract (26.0%) was higher than cystone (20.3%), but significantly lower than K-Cit (44.0%) ($p<0.001$) at 3200 μg/ml for 30 minutes incubation period. The present findings indicated that *A. pulcherrima* extract reduced calcium oxalate nucleation by 25.5%, which was closely similar to K-Cit (21.9%), but significantly higher than that of cystone (14.1%) ($p<0.01$) at a dose of 200 μg/ml. As indicated in Table 10, *A. pulcherrima* extract inhibited crystal nucleation at lower concentrations than the standard drugs. The extract of *G. fruticosus* revealed a higher nucleation inhibition (12.5 μg/ml to 50 μg/ml) than the standard drugs (K-Cit and cystone). The inhibitory effects of *G. fruticosus* extract (37.6±0.44%) was significantly higher than cystone (20.3±2.03%), but lower than K-Cit (44.2±0.95%) at 3200 μg/ml ($p<0.05$). *G. fruticosus* extract inhibited CaOx nucleations by 14.9%, which was similar to K-Cit (11.3%), but significantly higher than cystone (7.9%) at 50 μg/ml ($p<0.01$) (Table 10).

The aqueous extract of *C. myrrha* had a significant crystal nucleation inhibition (19.9%) than its ethanol extracts (5.9%) at a dose 800 μg/ml ($P<0.001$). Effect of turbidity reduction by aqueous extracts of *C. myrrha* (19.9%) was almost the same as that of cystone (19.0%), but significantly lower than K-Cit (32.9%) ($P<0.001$) at a dose of 800 μg/ml. The CaOx nucleation inhibition activities of aqueous extract of *C. myrrha* (12.8%) was higher than the ethanol (EtOH) extract (8.4%) at 400 μg/ml. The latter was significantly lower than the effects of cystone (15.9%) ($p<0.05$) and K-Cit (26.9%) ($p<0.001$) at 400 μg/ml. An increase in the concentration of *C. myrrha* aqueous extract showed a decrease in the inhibition of CaOx nucleation. The extract of *A. aspera* leaves prevented nucleation by 17.2%, which

was closely similar to cystone (20.3%), but significantly lower than K-Cit (44.0%) ($p<0.01$) at a dose of 3200 μg/ml. The nucleation inhibition capacity of *A. aspera* roots (13.2%), and inflorescences (8.5%) were significantly lower than K-Cit at a concentration of 3200 μg/ml (Table 10).

Table 10. Effect of *S. punctata* aerial parts, *A. pulcherrima* gel, *G. fruticosus* leaves, *C. myrrha* resins and *A. aspera* leaves aqueous extracts on CaOx nucleation inhibition at various concentrations in synthetic urine incubated for 30 minutes.

Conc. (μg/ml)	S_N % inhibition						
	Cystone	**K-Cit**	***S. punctata***	***A. pulcherrima***	***G. fruticosus***	***C. myrrha***	***A. aspera***
12.5	3.6±0.65	6.6±1.86	0.8 ±0.25##	8.1±1.71**	10.4±0.56***#	3.0±1.00#	4.2±0.79
25	4.5±1.06	7.7 ±1.57	0.7±0.16**###	9.9±1.59***	14.0±0.45***#	3.8±1.53#	5.7 ±0.80
50	8.0±1.61	11.3±1.52	4.7±2.28#	16.2±0.26***##	14.9±0.59**#	6.1±1.53#	6.8 ±0.81#
100	10.7±1.60	17.0 ±1.00	6.7±2.51##	22.2±2.98***#	17.2±0.64**	6.7±2.52##	7.6 ±1.28##
200	14.1±2.19	21.9±2.11	13.4±2.91#	25.5±3.01***	17.7±0.45*#	10.6±2.00##	8.6 ±1.25*###
400	15.9±2.79	26.9±2.24	14.4±2.29##	24.8±2.31***	19.8±0.55*##	12.8±1.00###	11.5 ±2.11###
800	19.0±2.02	32.9±2.11	17.3±2.91##	22.7±2.75##	27.1±1.60***#	19.9±1.73##	14.3 ±2.79###
1600	20.0±2.28	39.4±1.41	22.9±2.80###	23.2±2.63###	32.2±0.65***##	17.0±1.00###	16.6 ±2.95###
3200	20.3±2.03	44.0±0.95	26.0±3.43###	22.5±2.29###	37.6±0.44***#	13.0±1.73***###	17.2 ±2.25###

Note: S_N represents the slope of nucleation. Values were expressed as mean ±SD (n = 3). *p < 0.05, **p < 0.01,***p < 0.001 indicate significant changes when compared with cystone, #p < 0.05, ##p < 0.01, ###p < 0.001 indicate significant changes when compared with potassium citrate (K-Cit).

3.5.1. 2. Aggregation Inhibition

Effects of extract of *S. punctata* incubated for 30 minutes on crystal aggregation inhibition decreases as concentrations increased. The crystal aggregation inhibitory effects of *S. punctata* extract (13.9%) was significantly higher than that of cystone (8.1%) ($p<0.01$), but lower than that of K-Cit (18.6%) at a dose of 12.5 μg/ml. Similarly, the extract of *S.*

punctata promoted crystal aggregations as concentrations increased to 3200 μg/ml in a linear manner, whereas cystone and K-Cit prevented aggregations significantly by 33.7% and 43.2%, respectively ($p<0.001$). The findings indicated that *S. punctata* extracts promoted crystal aggregations at higher concentrations; whereas it inhibited at lower concentrations (Table 11).

The crystal aggregation inhibitions of *A. pulcherrima* extract (33.1±3.00%), were significantly higher than cystone (19.9%) ($p<0.01$), and K-Cit (38.7%) at dose 100 μg/ml. Moreover, *A. pulcherrima* inhibited crystal aggregation at lower concentrations compared to the standard controls. The number and size of crystals were reduced as crystal inhibitors of *G. fruticosus* extract added. The aqueous extract of *G. fruticosus* showed better aggregation inhibitory effects (33.3±2.31%) than cystone (22.3±1.80%) ($p<0.05$), but lower than K-Cit (41.2±1.44%) ($p<0.05$) at the concentration of 200 μg/ml. However, as the concentration of *G. fruticosus* extract increased, crystal aggregation inhibitions declined faster than at its lowest concentrations (Table 11).

The aqueous extracts of *C. myrrha* interfered crystal aggregations (18.6%) significantly higher than its ethanol extracts (10.6%) ($p<0.05$) at a dose of 100 μg/ml. The effect of the former extract was close to the effects of cystone (19.9%), but significantly lower compared to K-Cit (38.7%) at dose 100 μg/ml ($p<0.001$). The aqueous extract of *C. myrrha* decreased the slope of turbidity linearly up to 100 μg/ml. The crystal aggregations inhibited by *C. myrrha* EtOH extract (12.2%) was closely similar to its aqueous extract (16.2%) at 200 μg/ml. The effects of the aqueous extract of *C. myrrha* was lower than cystone (22.3%), and significantly less than K-Cit (41.3%) ($p<0.001$) at 200 μg/ml. *A. aspera* leaves extract that

demonstrated aggregation inhibitory effects of 27.4% at dose 800 μg/ml, which was almost the same to cystone (28.6%), and significantly lower than K-Cit (46.1%) ($p<0.001$). On the other hand, the aggregation inhibition effects of *A. aspera* roots and inflorescences extracts were significantly less than that of *A. aspera* leaves extract ($p<0.01$) (Table 11).

Table 11. Effect of *S. punctata* aerial parts, *A. pulcherrima* gel, *G. fruticosus* leaves, *C. myrrha* resins and *A. aspera* leaves aqueous extracts on CaOx crystal aggregation inhibition at various concentrations in synthetic urine incubated for 30 minutes.

Conc. (μg/ml)	S_A % inhibition						
	Cystone	**K-Cit**	***S. punctata***	***A. pulcherrima***	***G. fruticosus***	***C. myrrha***	***A. aspera***
12.5	8.1±2.06	18.6±2.67	13.9 ±0.79*#	19.9 ±1.38***	18.2 ±2.31***	5.9±1.15##	13.9±2.20*#
25	12.2±2.26	26.3 ±2.21	10.3 ±0.79###	27.5 ±2.88***	27.5 ±2.08***	9.0±1.73###	15.3 ±2.45##
50	15.7 ±3.00	32.1 ±2.87	7.3 ±0.98**###	31.0 ±1.37***	29.8 ±2.31***	13.0±2.31###	16.6 ±1.81##
100	19.9 ±2.64	38.7 ±1.53	5.8 ±0.28***###	33.1 ±3.00***#	32.2 ±2.08***#	18.6±2.31###	21.0 ±2.31##
200	22.3±1.80	41.2 ±1.44	3.8 ±0.24***###	32.9 ±2.93***#	33.3 ±2.31***#	16.2±2.30*###	27.2±2.85##
400	25.7±2.70	45.0 ±1.94	2.9 ±0.47***###	30.8 ±1.69##	32.0 ±3.46##	13.7±2.89***###	27.3 ±3.61##
800	28.6 ±2.66	46.1 ±2.01	2.3 ±0.38***###	27.3 ±2.12###	29.4 ±2.89##	9.8±4.04***###	27.4 ±2.29###
1600	33.1±2.03	44.3 ±3.28	1.3 ±0.29***###	25.2 ±2.03*##	27.6 ±1.73##	5.5±2.31***###	25.6 ±2.87*##
3200	33.7 ±2.68	43.2 ±2.15	0.5 ±0.31***###	23.2 ±1.93**###	24.0 ±2.89###	2.8±1.73***###	23.8 ±1.95**###

Note: S_A represents the slope of aggregation. Values were expressed as mean ± SD (n = 3). *p < 0.05, **p < 0.01,***p < 0.001 indicate significant changes when compared with cystone, #p < 0.05, ##p < 0.01, ###p < 0.001 indicate significant changes when compared with potassium citrate (K-Cit).

3.6. *In vitro* Effects of *Gomphocarpus fruticosus* Successive Solvent Extracts on Calcium Oxalate Crystallization

3.6.1. Nucleation Inhibition

The aqueous (aq.) extract of *G. fruticosus* interfered with nucleation processes by 10.9%, which was significantly less active compared to Cystone (20.3%) ($p<0.01$) and K-Cit

(44.0%) ($p<0.001$) at 3200 µg/ml for 30 minutes incubation. *G. fruticosus* EtOAc fraction inhibited CaOx nucleation by 56.9% at 3200 µg/ml, which was significantly higher than cystone (20.3%) ($p<0.001$) and K-Cit (44.0%) ($p<0.05$). The *G. fruticosus* BuOH fraction inhibited nucleation by 26.9%, which was significantly higher than cystone (10.7%) ($p<0.001$) and K-Cit (17.0%) at 100 µg/ml. The nucleation inhibition of *G. fruticosus* chloroform fraction was 19.4%, which was not different from that of cystone (20.3%), but significantly lower than that of K-Cit (44.0%) ($p<0.001$) at 3200 µg/ml. There was no nucleation inhibitory activity for *G. fruticosus* petroleum ether (PET) fraction. It has been noted that a promising nucleation inhibition activity was found for *G. fruticosus* EtOAc fraction followed by its BuOH fraction for 30 minutes incubation (Figure 18).

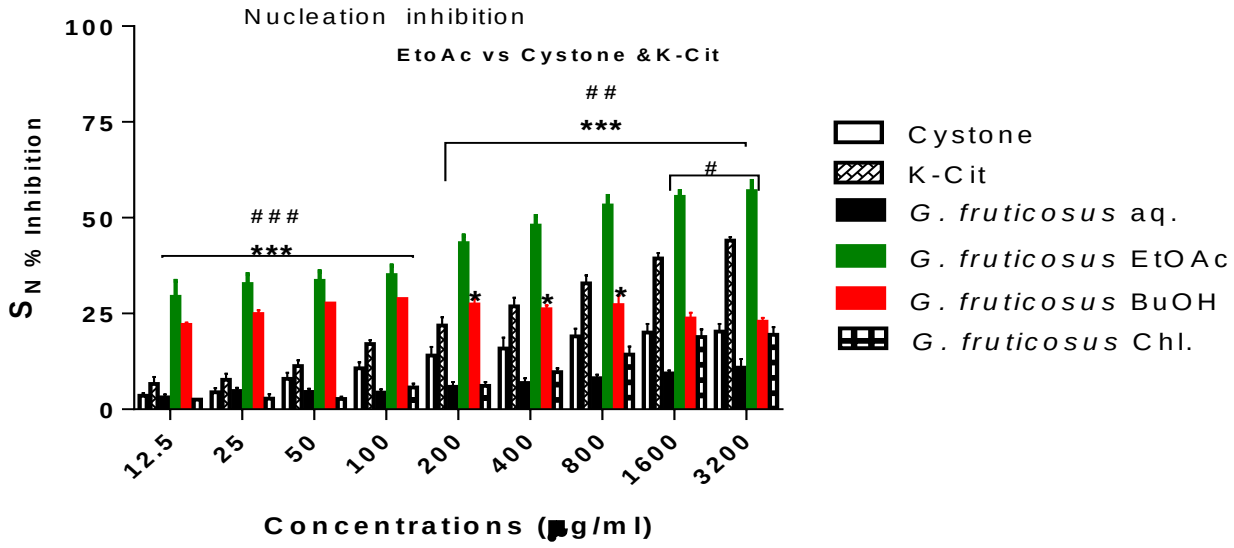


Figure 18. Effects of various concentrations of Ethyl acetate (EtOAc), Butanol (BuOH), Chloroform (Chl) and *G. fruticosus* aqueous (aq.) solvent-solvent fractions on CaOx crystal nucleation inhibitions in synthetic urine. S_N represents the slope of nucleation. Values were expressed as mean ±SD (n=3). *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared with cystone. # $p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ when compared to K-Cit.

3.6.2. Aggregation Inhibition

The aggregation inhibitions of *G. fruticosus* EtOAc fraction (16.0%) was close to that of cystone (19.9%), but significantly lower than that of K-Cit (38.7%) ($p<0.001$) at 100 µg/ml in 30 minutes incubation. As the concentration of the fraction increased, its aggregation inhibition effect decreased in comparison with the control. The aggregation inhibition effects of *G. fruticosus* BuOH fraction (8.1%) was similar to that of cystone (9.5%), but significantly lower than that of K-Cit (18.6%) ($p<0.01$) at 12.5 µg/ml. There was no aggregation inhibition for *G. fruticosus* Chl. extract, *G. fruticosus* PET fraction, and its aqueous fraction. In the present study, a promising aggregation inhibition activity was found for *G. fruticosus* EtOAc fraction followed by its BuOH fraction (Figure 19).

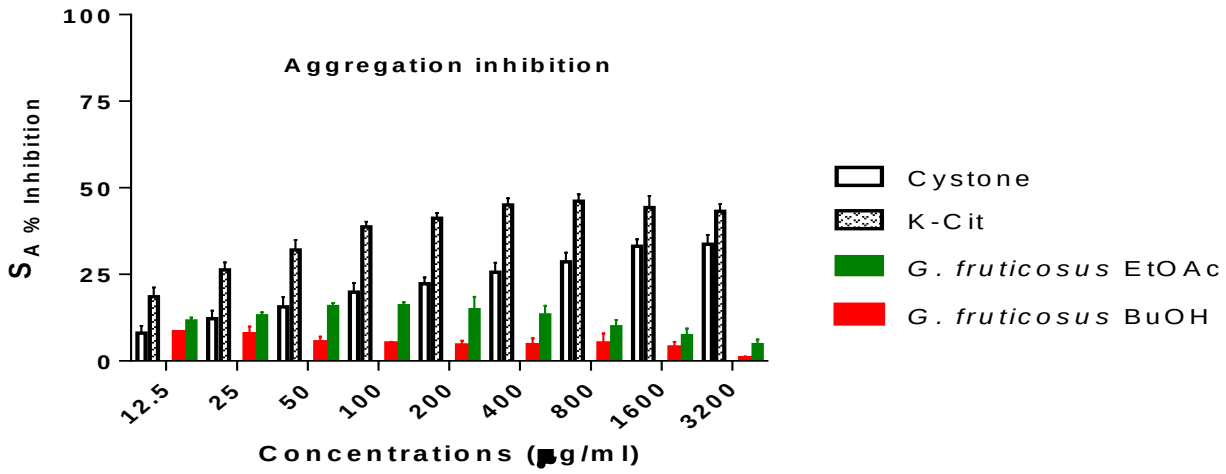


Figure 19. Effects of various concentrations of *Gomphocarpus fruticosus* ethyl acetate (EtOAc), and Butanol (BuOH) fractions on CaOx aggregation inhibitions in synthetic urine. S_A represents the slope of aggregation. Values were expressed as mean ±SD (n = 3).

Evaluation of *in vitro* dissolution of surgically removed calcium oxalate stones, following exposure to *G. fruticosus* successive solvent fractions showed no effect.

3.7. Antiurolithiatic Activities of *Gomphocarpus fruticosus* Fractions

3.7.1. Nucleation Inhibition

Among the eight (I-VIII) column chromatographic fractions isolated from EtOAc fractions of *G. fruticosus*, fractions II and III showed CaOx nucleation and aggregation inhibitions in a dose dependent manner (Appendix 3.4). The effect of fraction II (37%) was significantly higher than that of cystone (20.3%) ($p<0.001$) at 3200 μg/ml in 30 minutes incubation. However, the effect of fraction II was significantly lower than that of K-Cit (44.0%) ($p<0.05$). There were no nucleation inhibitory effects for the rest of *G. fruticosus* fractions (I, IV-VIII). A promising effect of the *in vitro* CaOx nucleation inhibition was shown by *G. fruticosus* fraction II followed by fraction III (Figure 20A).

3.7.2. Aggregation Inhibition

The crystal aggregation inhibitions of *G. fruticosus* fraction II (58.9%) was significantly higher than that of cystone (33.7%) ($P<0.01$), and K-Cit (43.2%) ($p<0.05$) at 3200 μg/ml for 30 minutes incubation. Similarly, fraction III reduced crystal aggregations by 18.1%, which was lower than that of cystone (25.7%), and K-Cit (45.0%) ($p<0.001$) at 400 μg/ml. There were no aggregation inhibition effects for the rest of *G. fruticosus* fractions (I, IV-VIII). Thus, promising effects of the *in vitro* CaOx aggregation inhibition showed by *G. fruticosus* fraction II followed by fraction III (Figure 20B).

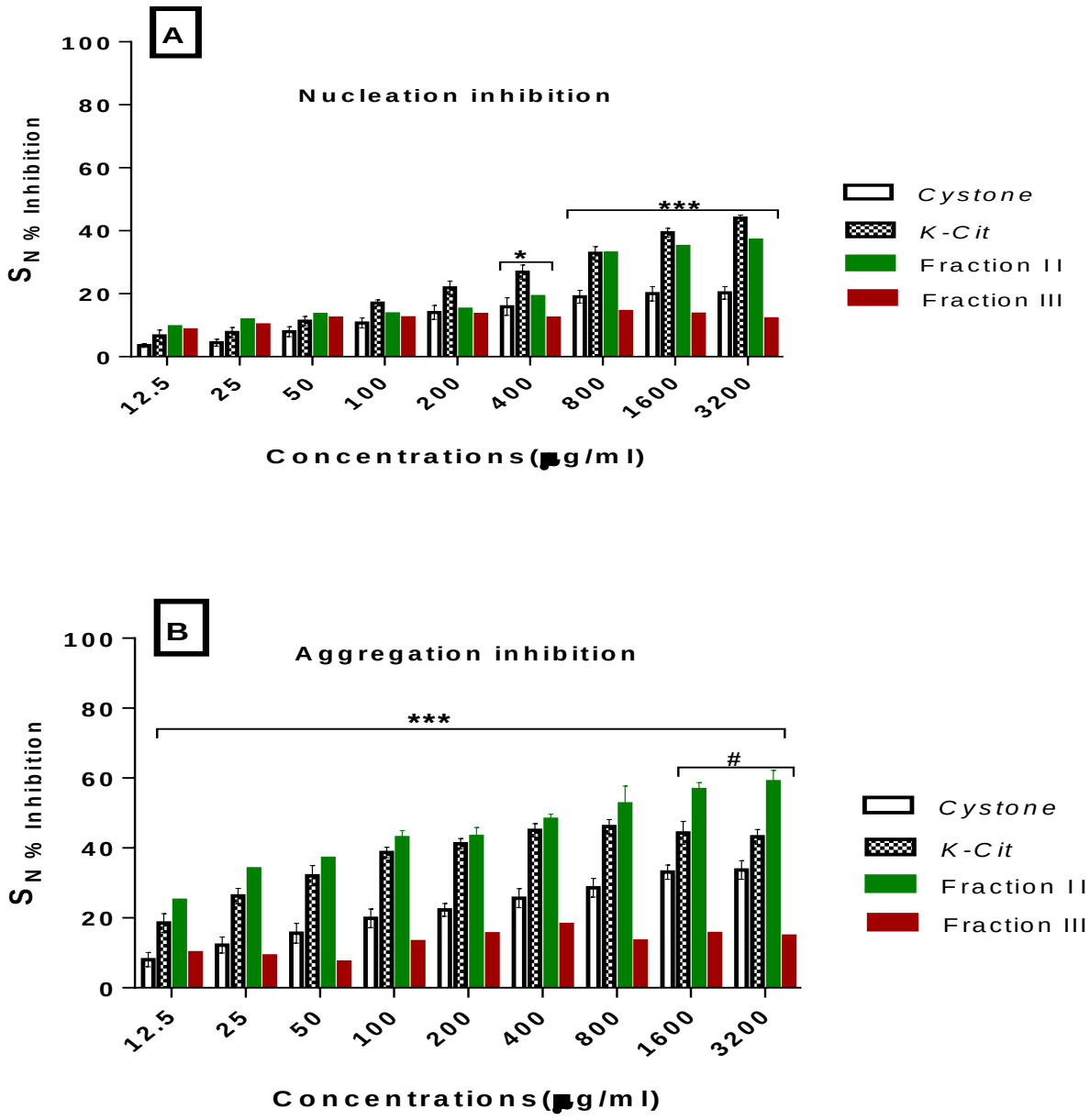


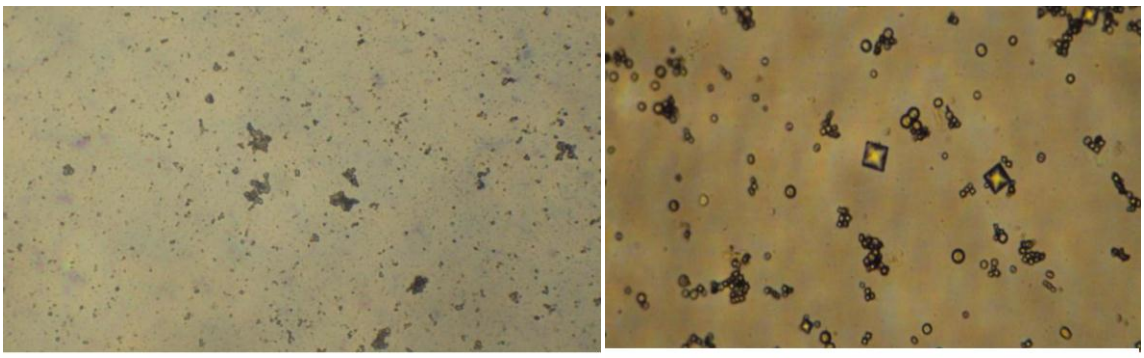
Figure 20. Effects of various concentrations of EtOAc fractions of *G. fruticosus* on CaOx nucleation (A), and aggregation (B) inhibitions for 30 minutes incubation. S_N represents the slope of nucleation. S_A represents the slope of aggregation. Values were expressed as mean ± SD (n = 3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared with cystone. # $p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ when compared to K-Cit.

3.8. Preventive Effects of Extracts in Experimentally Induced Nephrolithiatic Male Wistar Rats

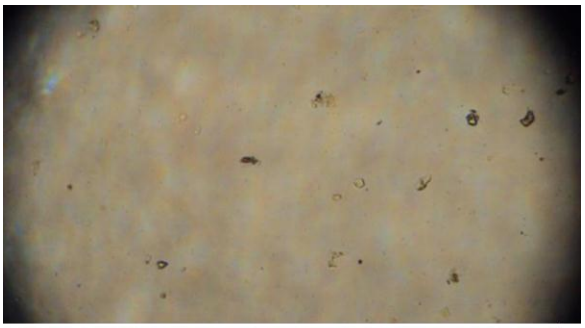
In the preventive and the curative (therapeutic) studies, the selected extracts dose was 200 mg/kg body weight of rats, which was one-tenth of the maximum tolerated dose 2000 mg/kg b.w. (Bahuguna *et al*., 2009b). This was chosen based on the prior acute and/or sub-acute toxicity studies revealing its safety up to a dose of 2000 mg/kg. The induction of kidney stones by administration of 0.75% EG combined with ammonium chloride (1%) in drinking water was confirmed in male Wistar rats.

3.8.1. Preventive Effects of the Extracts

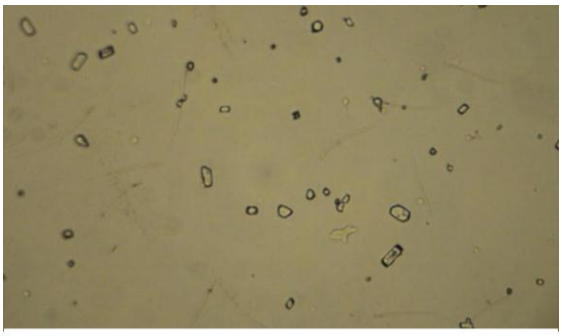
Each rat was kept under individual metabolic cage and urine samples were collected in the following morning without any preservative a day before sacrifice. The 24 hour urine samples, were analyzed for its chemistry, and the representative photomicrographs were taken in the presence and absence of extracts. The treatment with K-Cit (C), *S. punctata* (E), *A. pulcherrima* (F) and *G. fruticosus* (G) were associated with reductions in frequency and size of CaOx crystals in the urine compared to lithiatic control (Figure 21).



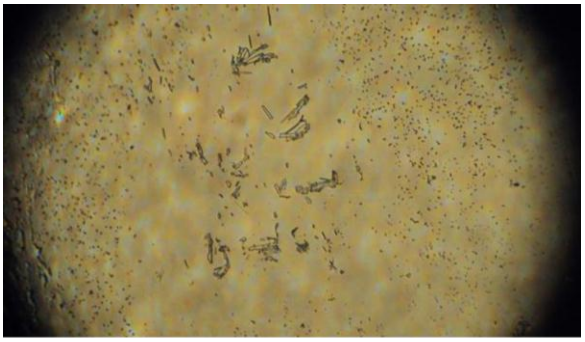
(**A**) Normal control (**B**) Lithiatic control



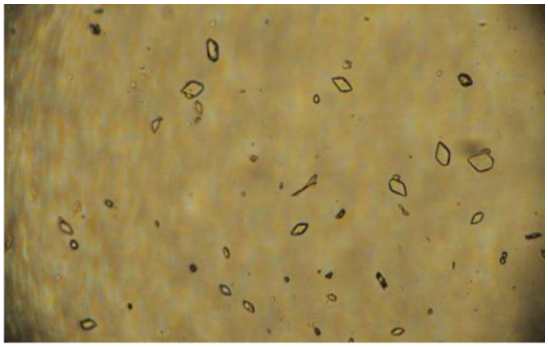
(**C**) Potassium citrate (K-Cit)



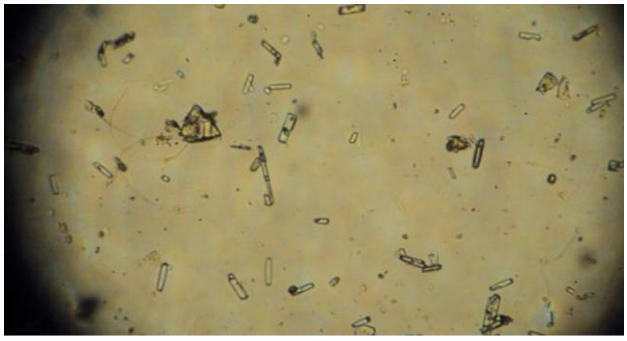
(**D**) Cystone



(**E**) *S. punctata*



(**F**) *A. pulcherrima*



(**G**) *G. fruticosus*

Figure 21. Urine microscopy (40×) of CaOx crystals in the prevention study. Calcium oxalate crystal morphology and number viewed under the light microscope of male Wistar rats' urine (**A**) Normal control/vehicle, (**B**) Lithiatic control, treatment with (**C**) Potassium citrate (K-Cit), (**D**) Cystone, (**E**) *S. punctata*, (**F**) *A. pulcherrima* and (**G**) *G. fruticosus* crude extracts (dose of extracts 200 mg/kg**).**

3.8. 2. Effect on Urine and Serum electrolytes and kidney function markers

In the preventive study, there were elevations of urinary electrolytes (solutes) among EG induced rats than the normal control and treated groups. The levels of sodium and uric acid were reduced by *G. fruticosus* extract than lithogenic group ($p<0.01$). The concentration of potassium decreased with treatment of *S. punctata* and *A. pulcherrima* extracts compared to the untreated lithiatic group ($p<0.01$) (Table 12).

In serum analysis, *G. fruticosus* extract reduced the level of sodium in comparison to lithiatic control ($p<0.05$). The level of potassium was reduced by *S. punctata* and *A. pulcherrima* extracts compared to lithiatic control ($p<0.01$). Similarly, the level of uric acid was reduced by K-Cit treatment compared to lithiatic control. The serum creatinine and blood urea nitrogen (BUN) levels markedly increased in disease control (Group II). Treatment with *A. pulcherrima* showed significant reduction in creatinine level compared to the untreated lithiatic group II (negative control) ($p<0.001$), but similar to K-Cit (Table 12).

Table 12. Effects of extracts on urine (24 hour) chemistry and serum levels of electrolytes and kidney function markers on the 15th day of the experiment in the preventive study.

Groups	Treatments	Electrolytes (mg/dl)			Kidney Function Test (mg/dl)			
		Sodium	Potassium	Chloride	Creatinine	Total proteins	Uric Acid	BUN
	Urine							
I.	Normal control (DH_2O)	103±10.34	53.9±0.44	113±9.13	0.77 ±0.05	2.15 ± 0.20	1.23±0.14	-
II.	Lithiatic control	184±21.05	89.3±0.13	147±13.17	3.54±0.09	8.30 ± 2.14	3.41±0.86	-
III.	K-Cit (2.5 g/kg)	120±11.11##	67.4±0.33#	116±14.11#	1.09±0.04#	5.07 ± 1.01*#	1.98±0.25	-
IV.	Cystone(750 mg/kg)	128±10.07**##	74.6±0.92	140±17.05*	1.86±0.07#	6.11 ± 1.26**	1.45±0.61#	-
V.	*S. punctata* (200 mg/kg)	156 ±4.96***#	55.70±0.85##	130±13.11	2.17±0.11*	3.43±0.47##	1.74±0.33	-
VI.	*A. pulcherrima*(200 mg/kg)	171±7.50***	58.73±0.36##	153±12.10*#	2.35±0.93*	3.98 ±0.34##	1.88 ±0.36	-
VII.	*G. fruticosus*(200 mg/kg)	119±6.03##	69.3±1.03#	117±12.06#	1.70±0.19#	7.12± 2.11**	0.96±0.05#	-
	Serum							
I.	Normal control (DH_2O)	142± 7.89	2.61±0.72	67± 3.65	0.69±0.05	2.24±0.13	0.85±0.12	132± 14.81
II.	Lithiatic control	163 ± 9.63	7.37±2.44	98±6.55	3.73±0.27	7.78±2.50	4.30±2.40	213± 41.20
III.	K-Cit (2.5 g/kg)	124±4.67	4.02±1.13**	60± 3.43	0.78 ±0.11###	4.68±1.25**#	1.73±0.15###	146± 15.82##
1V.	Cystone (750 mg/kg)	130±7.45	3.30±0.67	84± 4.03	1.14±0.31	4.06±1.07**#	2.69±0.07	174 ± 18.90
V.	*S. punctata* (200 mg/kg)	147±7.11	2.36±0.38##	95±8.22	1.18±0.22	3.89±1.07#	3.62±0.30**	178± 16.23
VI.	*A. pulcherrima*(200 mg/kg)	153±7.63	2.22±0.77##	87±9.97	0.98±0.15###	3.75±0.84#	2.15±0.40	192±26.04*
VII.	*G. fruticosus*(200 mg/kg)	136±6.54#	3.67±0.65	73±8.03	2.74 ±0.55*	5.51±1.20***	2.01±0.76	198±11.60*

Note: The data were presented as mean ± SD for 6 rats in each group (n=6). Comparisons between means were made against Group I (vehicle control) and Group II (lithiatic control). *$p < 0.05$, **$p < 0.01$,***$p < 0.001$ indicate significant changes in comparison with the group I (vehicle control); #$p < 0.05$, ##$p< 0.01$, ###$p<0.001$ indicate significant changes in comparison with group II (lithiatic control).

3.8. 3. Effect on Urine and Serum Crystal Formation

In lithiatic induced rats, the urinary concentrations of oxalate, calcium and phosphorus significantly increased ($p<0.05$), whereas magnesium and citrate decreased compared to the normal group. *S. punctata, A. pulcherrima* and *G. fruticosus* extracts increased urinary magnesium levels significantly ($p<0.05$), whereas *G. fruticosus* and K-Cit increased citrate

levels significantly compared to lithiatic control ($p<0.01$). *S. punctata* and K-Cit reduced oxalate level significantly than the lithiatic control ($p<0.05$). It was also observed that *G. fruticosus* extract reduced the level of calcium significantly in the urine ($p<0.01$) and reduced phosphate excretions, although statistically not significant (Table 13).

In the serum analysis of the preventive study, the concentrations of crystal promoters in Group II were about 2 to 3 times higher than the normal control (Group I). The *G. fruticosus* extract elevated the level of magnesium, which was close to the effects of the normal control, but different from the disease control (Group II) ($p<0.05$). The extracts of *G. fruticosus* reduced the levels of calcium compared to the lithiatic control in the urine analysis. Moreover, the concentration of phosphate was reduced by *A. pulcherrima* and *G. fruticosus* extract treatments ($p<0.01$) (Table 13).

Table 13. Effect on Urinary excretion (24 hour) and Serum biochemical markers of kidney stone crystal formation in hyperoxaluric rats on 15th day post-prevention compared to the control.

Groups	Treatments	Crystal formation inhibitors (mg/dl)		Crystal formation promoters (mg/dl)		
		Magnesium	Citrate	Calcium	Phosphate	Oxalate
	Urine					
I.	Normal control (DH_2O)	3.67±0.42	5.83 ± 1.24	3.90±0.26	4.10±0.86	3.22 ± 0.23
II.	Lithiatic control	1.24±0.08	2.41 ± 0.03	9.56±2.31	7.20±2.17	9.13 ± 2.17
III.	K-Cit (2.5 g/kg)	2.18±0.13	7.34 ± 1.32##	5.75±1.60#	5.52±0.92	5.56 ± 1.31#
IV.	Cystone (750 mg/kg)	2.67±0.35	3.72 ± 0.25*	6.13±1.02**	6.70±2.84*	6.24 ± 2.33*
V.	*S. punctata* (200 mg/kg)	3.66±0.93#	4.98±0.65#	5.48±1.93*#	5.21±4.49	5.34±0.84#
VI.	*A. pulcherrima* (200 mg/kg)	3.21±0.61#	4.65±1. 67#	5.37±1.91*#	5.18±2.67	6.14 ±2.34*
VII.	*G. fruticosus* (200 mg/kg)	3.51.±0.77#	4.61±0.72##	4.10±0.83##	5.41±0.84	6.01 ± 1..91*
	Serum					
I.	Normal control (DH_2O)	2.15 ±0.37	3.02±0.75	2.64±0.40	1.39±0.31	0..98±0.05
II.	Lithiatic control	1.35±0.04	1.13±0.82	7.09±1.59	3.57±0.72	9.11 ±2.18
III.	K-Cit (2.5 g/kg)	2.05±0.20	2.41±0.63	3.73±0.92#	2.63±0.13	5.13±1.50*#
IV.	Cystone (750 mg/kg)	1.98±0.19	2.19±0.32	3.21±1.43#	2.11±0.50	4.25±0.71**##
V.	*S. punctata* (200 mg/kg)	2.21±0.33	2.11±0.51	4.21±1.25*	2.33±0.30	6.55±3.73**
VI.	*A. pulcherrima* (200 mg/kg)	1.83±0.06	1.58±0.43*	3.24±0.31#	1.50±0.12#	5.86±0.55*#
VII.	*G. fruticosus* (200 mg/kg)	2.24±0.25#	2.30±0.85	4.5 ±1.50*	1.38±0.27#	6.39±0.97**

Note: The data were presented as mean ±SD for 6 rats in each group (n=6). Comparisons between means were made against Group I (vehicle control) and Group II (lithiatic control). *p < 0.05, **p < 0.01,***p < 0.001 indicate significant changes in comparison with the group I (vehicle control), #p < 0.05, ##p < 0.01, ###p < 0.001 indicate significant changes in comparison with group II (lithiatic control).

In the lithiatic induction, serum excretions of ALT, AST and ALP significantly increased compared to the healthy control (p<0.05). ALT level decreased by *G. fruticosus*, and *A. pulcherrima* treatments similar to K-Cit compared to lithiatic control (p<0.01). Similarly, *S. punctata* extract significantly increased ALT concentrations compared to the normal control (p<0.05). The level of AST also reduced when treated with *A. pulcherrima* extract, which

was close to the effects of cystone. However, *G. fruticosus* extract elevated the level of AST ($p<0.01$) compared to the vehicle group, and lowered the elevated levels of ALP significantly ($p<0.01$) compared to the untreated lithiatic group (Table 14).

Table 14. Effect of extracts on serum enzymes in nephrolithic rats on 15th day post-experimental treatment in preventive study.

Groups	Treatments	Enzyme Activity (IU/L)		
		ALT	**AST**	**ALP**
I.	Normal control (DH_2O)	55.8±5.12	96.7 ±9.13	162± 13.06
II.	Lithiatic control	87.2±7.33	137.3±18.62	217± 29.51
III.	K-Cit (2.5 g/kg)	51.4±6.41##	112.7±9.78	190± 11.43*
IV.	Cystone (750 mg/kg)	56.5±5.44#	93.9±7.45##	178±11.08#
V.	*S. punctata* (200 mg/Kg)	85.4±5.00*	101.2±7.20#	144±5.17#
VI.	*A. pulcherrima* (200 mg/kg)	47.3±6.23##	88.6±5.11###	162±7.31#
VII.	*G. fruticosus* (200 mg/kg)	51.9±5.76##	152.3±11.52**	118± 6.03**##

Note: The data presented as mean ± SD for 6 rats in each group (n=6). Comparisons between means were made against Group I (vehicle control) and Group II (lithiatic control). *$p < 0.05$, **$p < 0.01$,***$p < 0.001$ indicate significant changes in comparison with group I (vehicle control), #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ indicate significant changes in comparison with group II (lithiatic control).

3.8. 4. Effect on Kidney Homogenate

The level of calcium and phosphorus decreased significantly following administrations of *S. punctata* compared to lithiatic control ($p<0.01$). Similarly, the oxalate level showed a significant reduction following administrations of *S. punctata* extract compared to disease control (Group II) ($p< 0.05$), which was similar to the effects of K-Cit and cystone (Table 15).

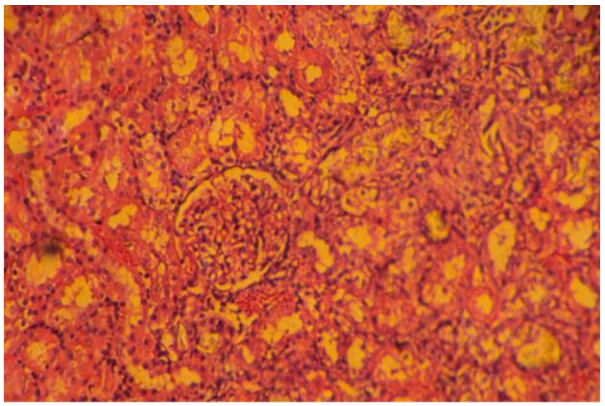
Table 15. Effects of extracts on kidney stone promoting chemical agents in hyperoxaluric rats in the preventive study.

Groups	Treatments	Kidney homogenate (mg/dl)		
		Calcium	**Phosphate**	**Oxalate**
I.	Normal control (DH_2O)	1.47±0.43	2.57±0.024	1.33±0.08
II.	Lithiatic control	5.98±1.54	6.12±1.73	5.43±1.15
III.	K-Cit (2.5 g/kg)	3.01±0.17*	4.38±1.07*	2.48±0.39##
IV.	Cystone(750 mg/kg)	2.65±0.39#	3.88±0.11#	3.29±0.76#
V.	*S. punctata* (200 mg/kg)	1.04±0.16##	2.43±0.06##	3.35±1.04#
VI.	*A. pulcherrima* (200 mg/kg)	3.76±0.22*	3.62±0.12#	4.01±0.05*
VII.	*G. fruticosus* (200 mg/kg)	2.50±0.72#	4.45±1.29*	3.80 ±0.14

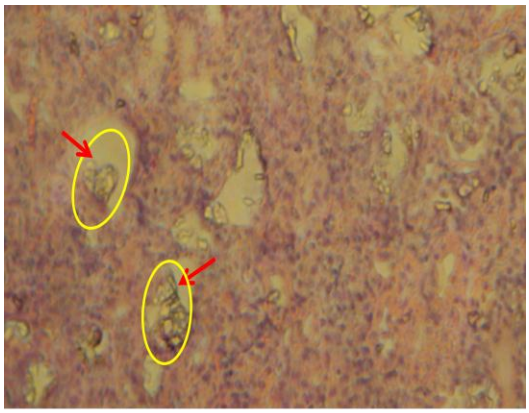
Note: The data were presented as mean ±SD for 6 rats in each group (n=6). Comparisons between means were made against Group I (vehicle control) and Group II (lithiatic control). *$p < 0.05$, **$p < 0.01$,***$p < 0.001$ indicate significant changes in comparison with the group I (vehicle control). #$p < 0.05$, ##$p < 0.01$, ###$p< 0.001$ indicate significant changes in comparison with group II (lithiatic control).

3.8. 5. Effect of Extracts on Kidney Histopathology in the Prevention of Urolithiasis

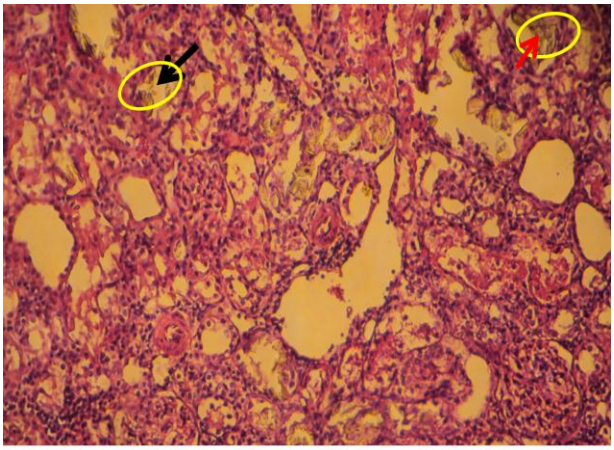
The antiurolithiatic effect of extracts was further confirmed by kidney histopathological studies. The histologic sections from the kidney of untreated rats showed abundant CaOx crystal depositions mainly in the lumen of the proximal convoluted renal tubules of rats. The proximal tubules showed tubular injury characterized by tubular dilations and flattening of epithelium (Fig. 22B). In the rats treated with extracts, minimal crystal depositions with limited tubular injury were noted compared to the untreated group (Fig. 22C-G). The normal control groups retained normal morphological views of nephrons, and there were no intra-tubular crystal depositions **(**Fig. 22A**).** The effects of *S. punctata* (E) and *G. fruticosus* (G) were comparable to K-Cit, suggesting their preventive effect on urolithiasis. Figure 22 shows photomicrographs on representative histological images of kidney tissues from experimental rats.



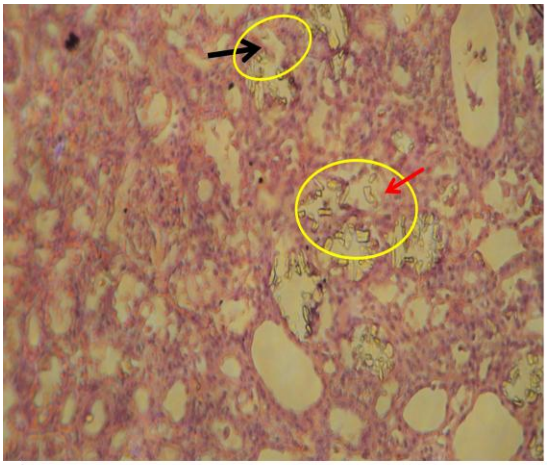
(A) Normal control



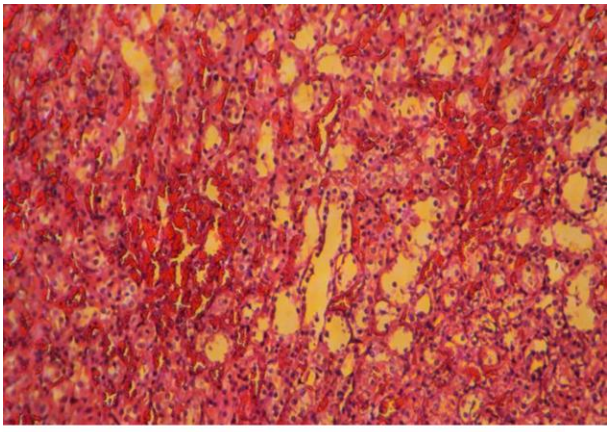
(B) Lithiatic control



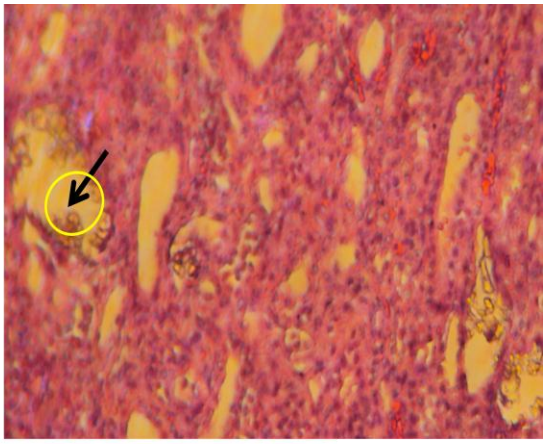
(C) Potassium citrate(K-Cit)



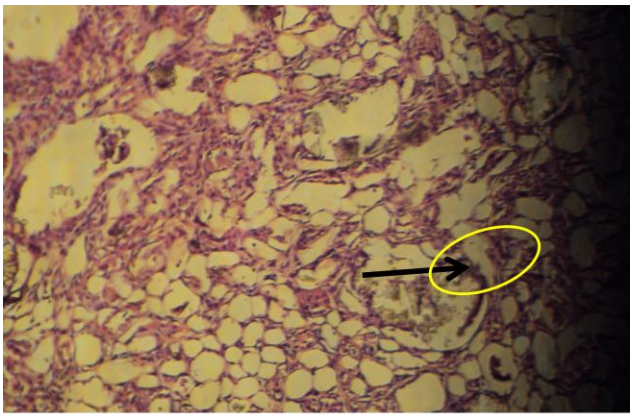
(D) Cystone



(E) *S. punctata*



(F) ***A. pulcherrima***



(G) *G. fruticosus*

Figure 22. Histopathology of male Wistar rats kidney tissue microscopic appearance (100× magnification) in the prevention of CaOx crystals (dose of extracts 200 mg/kg). Images were 5 µm thick paraffin sections. Microscopic images of kidney sections viewed under light microscope after Hematoxylin-Eosin staining from male Wistar rats. Arrows (circles) indicate the presence of Calcium oxalate crystals deposited in the renal tubules.

3.8.6. Preventive Effects of Extracts on CaOx Crystals Deposition on Rats Kidney Tissues

The number of crystal deposits was counted via a sagittal section or longitudinal plane of each renal tissue specimen divided into 4 equal sized regions (two virtual lines) and taking the mean of all fields (Figure 23).

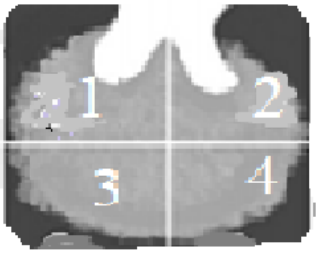


Figure 23. Sagittal section of the kidney tissue slide divided into four equal parts for crystal deposition count.

Kidney tissue histopathological examination showed a uniform distribution of CaOx crystals throughout the tissues. Treatment with extracts of *S. punctata* ($p<0.001$) and *G. fruticosus* ($p< 0.01$) showed a significant reduction in CaOx depositions in renal tissues. Their effect was similar to that of K-Cit ($p<0.01$), which was significantly different ($p<0.01$) from the lithiatic control. It has been noted that *S. punctata* extract showed a promising activity in preventing kidney stones followed by *G. fruticosus* and *A. pulcherrima* extracts (Figure 24).

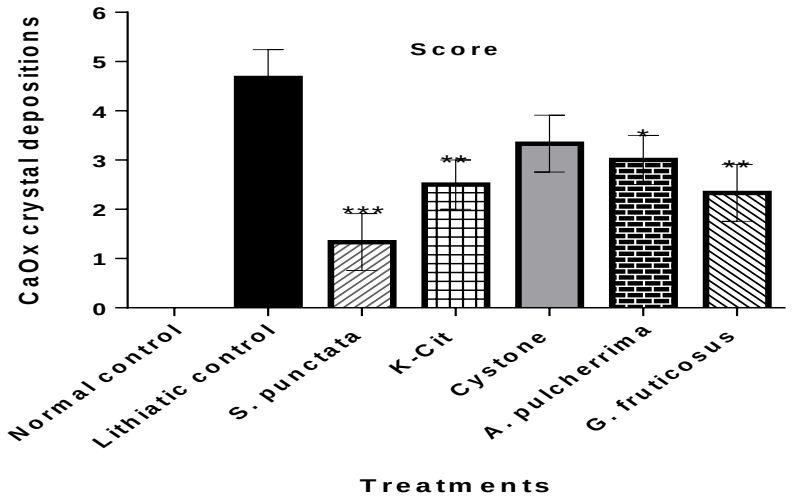


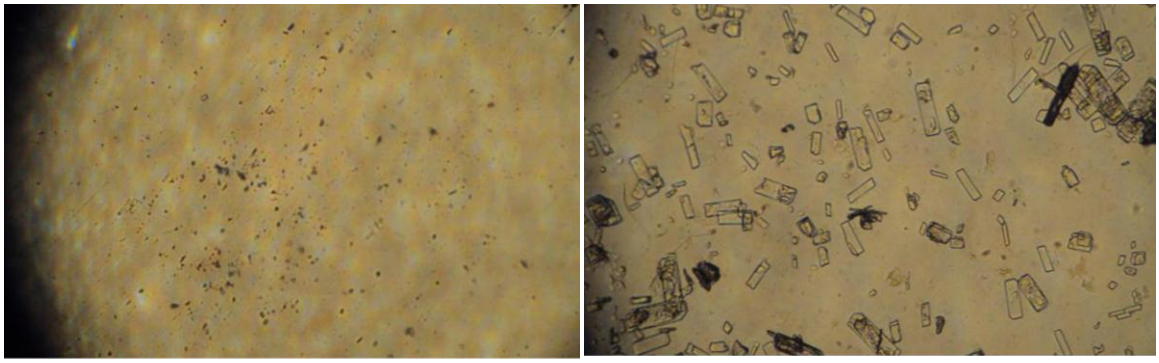
Figure 24. Effect of various 200 mg/kg extracts on CaOx crystals deposition in the prevention study. Data were expressed as mean ±SD of n=6 rats per group. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ indicate significant changes in comparison with lithiatic control (hyperoxaluric group).

3.9. Curative Effect of Extracts on Experimentally Induced Nephrolithiatic Male Wistar Rats

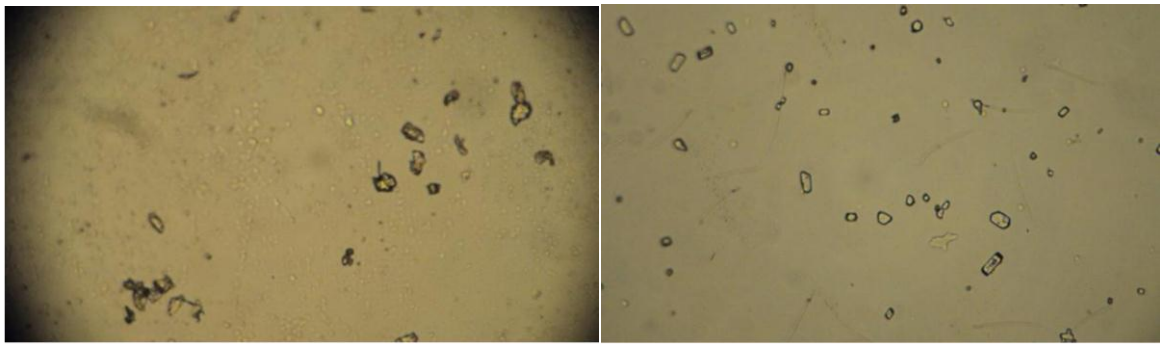
3.9.1. Urine (24hr) Photomicroscopy of Curative Effects of Extracts

The analysis of urine samples showed variations in crystal density and size. Treatment with K-Cit (Fig. 25C), *A. pulcherrima* (Fig. 25F), and *G. fruticosus* crude extracts (Fig. 25G) minimized the number of crystals and sizes compared to lithiatic control (Fig. 25B). Among

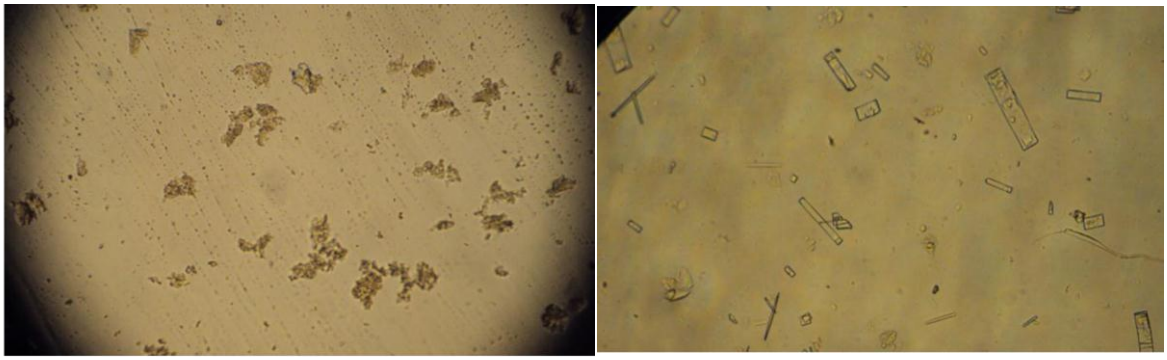
the *G. fruticosus* successive solvent fractions, *G. fruticosus* EtOAc fraction (Fig. 25K), and *G. fruticosus* BuOH fraction (Fig. 25L) also reduced crystal numbers and sizes (**Figure 25**).



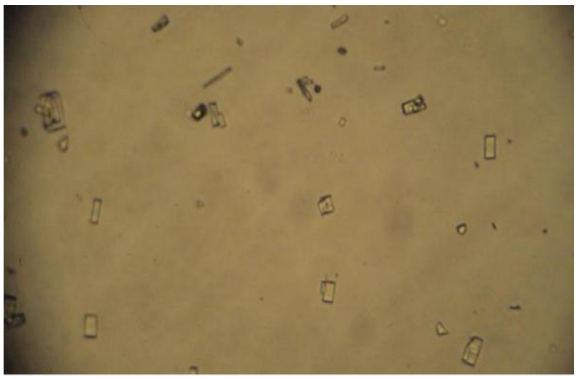
(A) Normal control **(B) Lithiatic control**



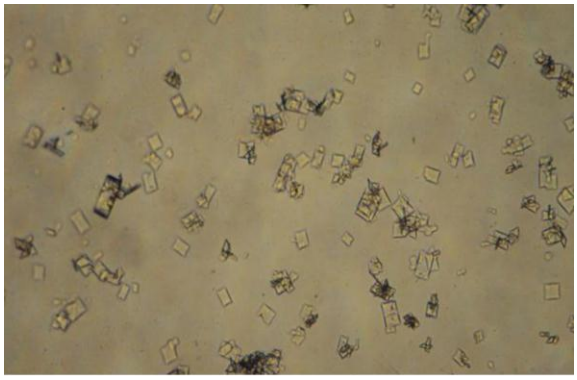
(C) Potasssium citrate (K-Cit) **(D) Cystone**



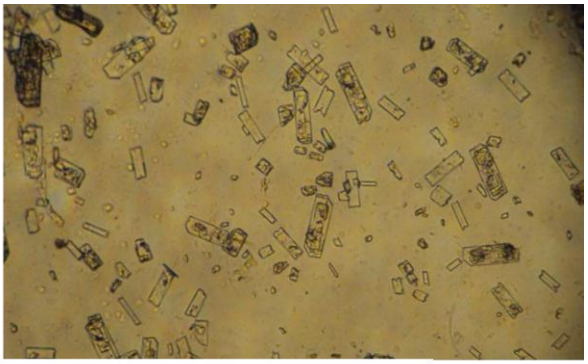
(E) *S punctata* **(F) *A. pulcherrima***



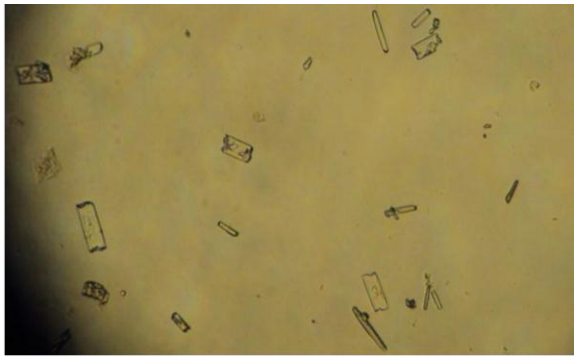
(G) *G. fruticosus* crude extract



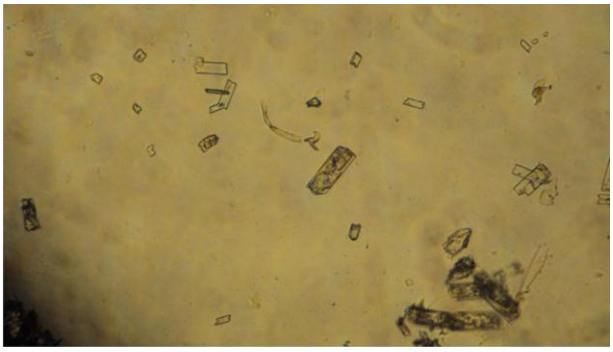
(H) Mixed extracts



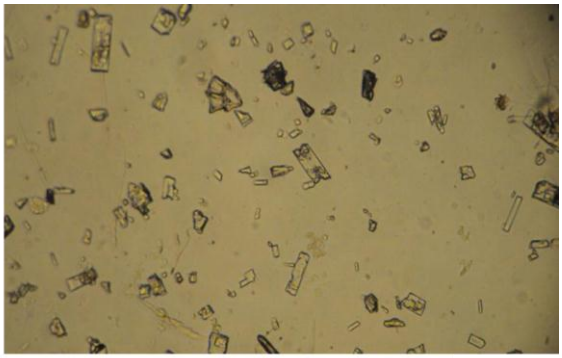
(I) *G. fruticosus* PET extract



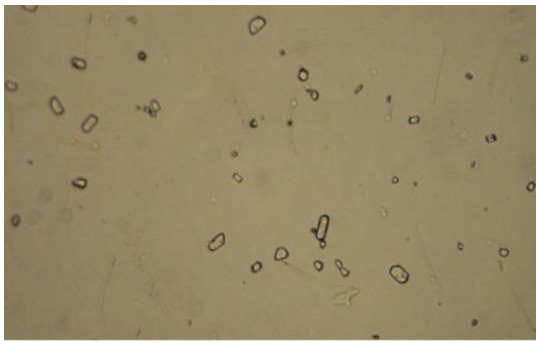
(J) *G. fruticosus* Chl. extract



(K) *G. fruticosus* EtOAc extract



(L) *G. fruticosus* BuOH extract



(M) *G. fruticosus* aqueous fraction

Figure 25. Urine photomicrographs (40×) of CaOx crystals following Curative treatment at 200 mg/kg extracts dose. The calcium oxalate crystal morphology and number viewed under a light microscope (40×), in morning urine from male Wistar rats (**A**) Normal control/vehicle; (**B**) Lithiatic control; treatment with (**C**) Potassium citrate (K-Cit); (**D**) Cystone; (**E**) *S. punctata;* (**F**) *A. pulcherrima;* (**G**) *G. fruticosus* crude extract; (**H)** Mixed extract (combination of *A. aspera*, *S. punctata* and *R. abyssinicus* extracts in 1:1:1 ratio); (**I**) *G. fruticosus* PET extract; (**J)** *G. fruticosus* Chl. extract; (**K**) *G. fruticosus* EtOAc extract; (**L**) *G. fruticosus* BuOH extract; and **(M)** *G. fruticosus* aqueous fraction.

3.9.2. Effect on Urine and Serum Electrolytes and Renal Function Markers in Curative Study

In the urinary analysis, aqueous extracts of *A. pulcherrima*, the mixed aqueous extracts (*A. aspera* leaves, *S. punctata* aerial parts and *R. abyssinicus* rhizomes), and *G. fruticosus* EtOAc fraction reduced the level of sodium significantly compared to lithiatic control ($p<0.001$). The level of potassium reduced by *S. punctata* aqueous extract and *G. fruticosus* BuOH fraction ($p<0.01$) in the urine. *G. fruticosus* aqueous extract lowered the level of potassium by half compared to EG induced control (Group II) and it was closely similar to the normal control ($p<0.05$). Similarly, *G. fruticosus* aqueous extract lowered the level of chloride compared to the untreated lithiatic group ($p<0.01$). Treatment with the aqueous

extract of *S. punctata*, EtOAc and BuOH fractions of *G. fruticosus* showed significant reductions in urinary protein excretions ($p<0.01$) in relation to lithiatic control. Sodium concentration was also reduced very significantly with the treatment of EtOAc extract of *G. fruticosus*, the aqueous extract of *A. pulcherrima* and the mixed extracts ($p<0.001$) in the urine. The treatment undertaken by mixing extracts increased uric acid level compared to the normal control ($p<0.01$), and the fraction of *G. fruticosus* BuOH lowered the level of uric acid compared to lithiatic group ($p<0.05$) in the urine. However, lithogenic induction may cause impairments of renal function as evidenced by raising the levels of creatinine, proteins and uric acids (Table 16)**.**

In the serum analysis, sodium level significantly increased by *A. pulcherrima* extract administrations in relation to the normal control ($p<0.01$). The level of potassium reduced by *G. fruticosus* EtOAc fraction in comparison to lithiatic control ($p<0.01$). It was also found that the effects of *G. fruticosus* BuOH fraction on the level of chloride was close to the normal control. Treatment with *S. punctata* extract similar to K-Cit reduced the level of creatinine compared to lithiatic control ($p<0.05$); however, it raised by *G. fruticosus* crude extract and PET extract ($p<0.01$) compared to the normal control. *S. punctata* extract significantly lowered the level of uric acid compared to lithiatic control ($p<0.01$) (Table 16).

Table 16. Effects of 200 mg/kg extracts and their fractions on urinary (24 hr) excretions and serum kidney stone-forming electrolytes and kidney function markers in treated on 28th day post-treatment.

Groups	Treatments	Electrolytes (mg/dl)			Kidney Function Test (mg/dl)		
		Sodium	Potassium	Chloride	Creatinine	Total protein	Uric acid
	Urine						
I.	Normal control (DH_2O)	135±33.85	25.33±6.44	142.56±17.71	1.11±0.09	3.50±0.74	2.14±0.33
II.	Lithiatic control	213±48.14	43.00±11.91	189.30±21.65	5.11±2.53	12.13±5.35	5.13±1.71
III.	K-Cit (2.5 g/kg)	154±32.32#	35.75±7.03	145.20±15.25#	2.51±0.76#	7.66±2.90*#	3.05±0.98
IV.	Cystone (750 mg/kg)	163± 18.14#	38.03±5.19	157.58±16.51	3.13±1.03*	8.50±1.57**	3.12±1.07
V.	*S. punctata*	168±46.25*#	19.66±5.50##	143.30±13.90#	2.17±1.06#	4.44±2.26##	4.11±1.91*
VI.	*A. pulcherrima*	118±22.14###	23.71±11.30#	146.48±15.40#	2.19±0.95#	6.42±1.54*#	3.53±1.50
VII.	*G. fruticosus* crude extract	131±10.08##	23.34±12.18#	136.19±17.11##	3.01±0.05*	7.10±1.56*#	4.23±0.64
VIII.	Mixed extract	127 ±19.13###	34.2±4.12	157.30±20.02	3.53±1.14*	6.16±0.87*	6.11±1.42**
IX.	*G. fruticosus* PET fraction	204±34.05**	43.98±7.11*	166.03±15.12*	4.15±0.33**	9.15±1.87**	5.11±0.33*
X.	*G. fruticosus* Chl. fraction	213±33.71**	39.83± 6.45	164.50±15.73*	3.68±0.66*	8.98± 3.32**	5.30±1.20*
XI.	*G. fruticosus* EtOAc fract	128.5±10.19##	31.08±4.50	144.21±20.06#	3.41±0.47*	4.66±1.09##	4.83±0.71
XII.	*G. fruticosus* BuOH fract	133±15.04##	21.41±3.82##	143.78±14.37#	3.36±0.87*	4.97±0.75##	3.91±0. 67#
XIII.	*G. fruticosus* aq. fraction	148 ±17.03#	27.50±6.73#	148.00±18.00#	3.35±0.12*	8.90±2.15*#	3.79±2.11
	Serum						
I.	Normal control (DH_2O)	157±6.25	5.17±1.25	82±4.33	1.03±0.05	3.50±0.74	0.97±0.08
II.	Lithiatic control	219±13.24	9. 14 ±2.31	113.±7.25	5.29±0.89	13.19±3.02	6.74±2.34
III.	K-Cit (2.5 g/kg)	16*.5±6.19#	7.3 ±1.77	93±6.19	2.68±0.50#	5.63±1.34##	2.50±1.05##
IV.	Cystone (750 mg/kg)	175±10.32#	6.1±2.03	88±6.22	3.19±0.39	7.85±2.55*#	4.21±0.89#
V.	*S. punctata*	174±6.34#	5.12±6.32#	97±5.55	2.94±0.72#	8.33±2.50**	3.56±0.54##
VI.	*A. pulcherrima*	240± 13.76**	5.98 ± 1.37#	115±10.12*	3.26±0.86	5.74±2.88##	3.98±0.15*#
VII.	*G. fruticosus* crude extract	173±8.65#	5.56±0.84#	89±7.56#	6.10±1.13**	8.15±1.07*	5.55±0.52
VIII.	Mixed extract	198±18.50*	5.61±1.25#	93±6.11	3.85±0.44	7.61±2.61*#	5.30±0.56*
IX.	*G. fruticosus* PET extract	203±17.16**	8.20±2.13*	129±6.51*	5.93±0.16**	7.82±1.20*#	4.18±0.78*
X.	*G. fruticosus* Chl. extract	193±12.50*	9.50±2.17*	122±9.54*	4.03±0.39*	6.93±1.54*#	5.40±1.33**
XI.	*G. fruticosus* EtOAc	169±8.34#	4.85±0.93##	94±5.78	3.67±0.04	5.04±1.43##	2.76±0.61##
XII.	*G. fruticosus* BuOH	174± 11.23#	8.22±2.07*	79±4.13#	3.19±0.07	7.98±2.17*#	5.22±1.27*
XIII.	*G. fruticosus* aq. fraction	185±8.37*	5.16±1.64#	87±4.84#	3.18±0.60	6.23±2.17*#	4.93±0.44*

Note: The mixed extract = the combination of *A. aspera*, *S. punctata* and *R. abyssinicus* extracts (1:1:1 ratio), Aqueous (aq.), Ethyl acetate (EtOAc), Butanol (BuOH), Chloroform (Chl), and

Petroleum ether (PET). The data were presented the mean ± SD for 6 rats in each group (n=6). Comparisons between means were made against Group I (vehicle control) and Group II (lithiatic control). *p< 0.05, **p < 0.01,***p < 0.001 indicate significant changes in comparison with Group I (vehicle control), #p < 0.05, ##p < 0.01, ###p< 0.001 indicate a significant change in comparison with Group II (lithiatic control).

3.9.3. Effect on Crystal Formation in Curative Study

As indicated in Table 17, the level of magnesium was significantly elevated by supplementation of the mixed extracts and *G. fruticosus* EtOAc fraction compared to lithiatic control ($p<0.01$) in the urine. Similar to K-Cit, the effects of *G. fruticosus* EtOAc extract increased the level of citrate significantly ($p<0.01$). *G. fruticosus* BuOH fraction, and the mixed extracts lowered the elevated levels of calcium and phosphate significantly in the urine compared to the diseased (lithiatic) control ($p<0.01$). It was also observed that *S. punctata* extract and *G. fruticosus* EtOAc fraction decreased the level of oxalate in the urine compared to lithiatic control ($p<0.001$) (Table 17).

In the serum analysis, the excretions of calcium, oxalate and phosphate grossly increased in lithiatic induced male Wistar rats. The aqueous extracts of *S. punctata, A. pulcherrima, G. fruticosus* extract, and EtOAc fraction of *G. fruticosus* increased the concentration of magnesium ($p<0.05$). The serum level of citrate increased after exposure to the aqueous extract of *S. punctata*, the mixed extracts, and BuOH fraction of *G. fruticosus* ($p<0.01$), suggesting similar effects to potassium citrate ($p<0.001$). *G. fruticosus* Chl. extract significantly reduced the citrate level compared to the normal control ($P<0.001$). The mixed extract ($p<0.01$), *A. pulcherrima* ($p<0.01$) and *G. fruticosus* aq. fraction ($p<0.001$) reduced the level of calcium in comparison to lithiatic group (Table 17).

Table 17. Effects of 200 mg/kg extracts and their fractions on changes in urinary excretion (24 hr) and serum crystal formation in male Wistar rats on the 28th day post-treatment.

Groups	Treatments	Crystal inhibitors		Crystal promoters (mg/dl)		
		Magnesium	Citrate	Calcium	Phosphate	Oxalate
	Urine					
I.	Normal control(DH_2O)	2.88±0.63	3.46±0.78	2.60±0.48	5.35±1.12	3.81±0.43
II.	Lithiatic control	0.96±0.14	1.83±0.51	11.40±3.14	9.71±2.89	12.76±3.62
II.	K-Cit (2.5 g/kg)	1.52±0.42	4.32±1.43##	6.28±1.15*#	6.73±1.34	7.53±2.02*#
IV.	Cystone (750 mg/kg)	1.70±0.34	2.21±0.46	5.84±1.23*#	5.31± 1.29#	8.29±2.11**
V.	*S. punctata*	2.02.±1.16#	2.15±0.75	5.32±0.65*#	6.68±2.52	4.55±0.95###
VI.	*A. pulcherrima*	2.43± 0.92#	2.04±0.19	6.21±0.11*#	6.18± 1.31	7.34±1.06*#
VII.	*G. fruticosus* crude extract	2.13± 0.62#	2.33±0.89	7.81±0.44	5.18±2.33#	4.86±0.74##
VIII.	Mixed extract	3.20±1.05##	2.33±0.97	4.20±0.92##	4.50±0.76##	5.74±0.63##
IX.	*G. fruticosus* PET fraction	1.87±0.65	1.98±0.23*	8.11±1.29*	7.04±2.17	7.32±0.91*#
X.	*G. fruticosus* Chl. fraction	1.57±0.28	2.37±0.04	7.07±1.16*	8.3±2.62*	7.35±1.40*#
XI.	*G. fruticosus* EtOAc fraction	3.30±1.21##	3.02±1.12##	6.24±1.35#	5.12±0.98#	4.25±0.53###
XII.	*G. fruticosus* BuOH fraction	2.61±0.74#	2.43±0.91	4.17±0.85##	4.10±0.65##	4.39±0.33##
XIII.	*G. fruticosus* aq. fraction	2.50±0.88#	2.95±0.66#	5.16±0.71#	5.41±1.02#	6.51±1.03*##
	Serum					
I.	Normal control (DH_2O)	3.07±0.51	5.11±1.33	3.17±0.97	1.78±0.64	1.24±0.21
II.	Lithiatic control	0.98±0.07	1.20±0.07	11.6±2.13	5.14±1.45	11.32±2.27
III.	K-Cit (2.5 g/kg)	1.72±0.12	5.41±1.13###	6.11±1.75*#	3.61±0.61*	4.06±0.83*##
IV.	Cystone (750 mg/kg)	1.50±0.30*	3.00±0.32#	8.24±2.88**	3.33±1.14*	3.57±0.67*###
V.	*S. punctata*	2.44±0.45#	4.00±0.53##	6.30±1.77*#	4.11±1.16*	5.11±1.22**##
VI.	*A. pulcherrima*	2.41±0.34#	3.75±1.23#	4.30±0.61**##	3.64±0.70*	7.10± 2.13**
VII.	*G. fruticosus* crude extract	2.14±0.52#	3.01±0.76#	7.60±2.05**	3.30±0.51*	4.88±1.13*##
VIII.	Mixed extract	1.77±0.34	4.11± 1.33##	4.28±0.55##	5.11 ±1.19*	5.50±1.95**##
IX.	*G. fruticosus* PET fraction	0.79±0.08**	2.43±0.79*#	7.44±2.50**	6.78±1.14*	6.75±1.29**#
X.	*G. fruticosus* Chl. fraction	1.44±0.19*	0.93±1.19***	8.30±2.11**	4.39±0.87*	7.92±2.31**
XI.	*G. fruticosus* EtOAc fraction	2.47±0.11#	1.68±1.27**	5.25±1.62#	3.06± 0.32*	4.72±0.68**##
XII.	*G. fruticosus* BuOH fraction	1.04±0.31**	4.58±1.54##	5.14±0.82#	4.48±1.89*	5.86±2.14**##
XIII.	*G. fruticosus* aq. fraction	1.87±0.15	2.50±0.92*#	3.42±0.84###	6.03±1.57*	7.10±2.05**

Note: The mixed extract = the combination of *A. aspera*, *S. punctata* and *R. abyssinicus* extracts (1: 1:1 ratio). The data were presented as mean ± SD for 6 rats in each group (n=6). Comparisons between means were made against Group I (vehicle control) and Group II (lithiatic control). *p<0.05, **p< 0.01,***p < 0.001 indicate significant changes in comparison with Group I (vehicle control), #p<0.05, ##p<0.01, ###p<0.001 indicate significant changes in comparison with Group II (lithiatic control).

The serum level of alanine aminotransferase (ALT), and aspartate aminotransferase (AST) increased in the lithiatic control compared to the normal (healthy) control ($p<0.05$). Although the concentrations of ALT grossly decreased for all treatments, it was significantly reduced by the aqueous extracts of *A. pulcherrima*, and *G. fruticosus*, and *G. fruticosus* EtOAc and BuOH fractions compared to the lithogenic group ($p<0.001$). This effect was similar to the normal control values. Similarly, the mixed extracts and *G. fruticosus* PET fraction increased serum AST level significantly ($p<0.001$) compared to the healthy control. The aqueous extract of *S. punctata*, and *G. fruticosus*, and the BuOH and EtOAc fractions of *G. fruticosus* significantly lowered the level of AST similar to K-Cit ($p<0.001$) as it was compared to lithiatic control (Table 18).

Table 18. Effects of 200 mg/kg extracts and their fractions on the serum enzyme activity on the 28th day post-treatment.

Groups	Treatments	Enzyme Activity	
		ALT (IU/L)	AST (IU/L)
I.	Normal control (DH_2O)	68.4±5.04	113.1±6.53
II.	Lithiatic control	135.3±14.13	211.0 ±23.10
III.	K-Cit (2.5 g/kg)	78.7±6.10##	126.7±8.11###
IV.	Cystone (750 mg/kg)	67.8±5.89##	161.3±10.32*##
V.	*S. punctata*	77.3±4.51##	133.1±9.39*###
VI.	*A. pulcherrima*	56.9±6.11###	176.7±14.25**##
VII.	*G. fruticosus* crude extract	87.2±6.35**###	154.4±19.35*##
VIII.	Mixed extract	121.2±8.37***	160.1±12.51*##
IX.	*G. fruticosus* PET fraction	123.1±11.05***	225.1±23.12***
X.	*G. fruticosus* Ch fraction	115.8±7.01***	208.7±23.13***
XI.	*G. fruticosus* EtOAc fraction	78.7±3.85*###	219.4±27.60***
XII.	*G. fruticosus* BuOH fraction	71.4±5.16###	139.3±9.54*###
XIII.	*G. fruticosus* aq. fraction	69.2±4.11###	120.6.±11.32**###

Note: The mixed extract = the combination of *A. aspera*, *S. punctata* and *R. abyssinicus* extracts (1:1:1 ratio). Data were presented as mean ± SD for 6 rats in each group (n=6). Comparisons between means were made against Group I (vehicle control) and Group II (lithiatic control). *p< 0.05, **p < 0.01,***p < 0.001 indicate significant changes in comparison with group I (vehicle control), #p < 0.05, ##p < 0.01, ###p < 0.001 indicate significant changes in comparison with group II (lithiatic control).

3.9.4. Curative Effect on Kidney Homogenate

The concentrations of calcium, phosphorus and oxalate increased in the lithiatic group (Group II) compared to the normal control (Group I). All treatments showed significant reductions in the level of phosphate ($p<0.05$), except treatment with the aqueous mixed extracts, BuOH, Chl. and PET fractions of *G. fruticosus*. The administration of the aqueous mixed extract and *G. fruticosus* EtOAc fraction reduced the level of oxalate significantly compared to the untreated lithiatic groups ($p<0.01$), whereas the oxalate level increased with

G. fruticosus aq. fraction similar to cystone significantly compared to the normal control ($p<0.01$). The kidney homogenate analysis showed that calcium level was significantly lower, following the administrations of *A. pulcherrima* compared to lithiatic control ($p<0.05$) (Table 19).

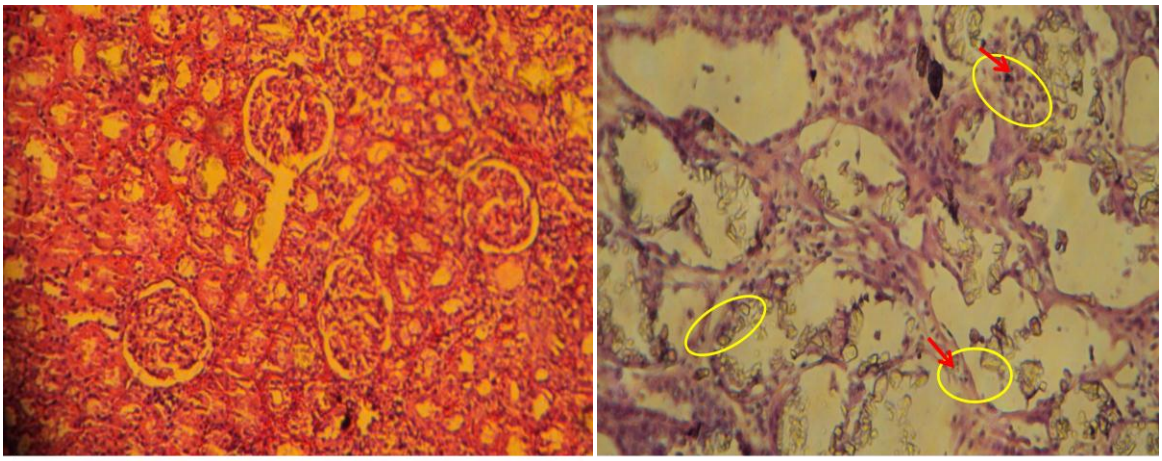
Table 19. Effect of 200 mg/kg extracts and the fractions on kidney homogenate constituents of phosphate, oxalate and calcium following administrations of curative test on the 28th day at dose.

Groups	Treatments	Kidney homogenates (mg/dl)		
		Phosphate	**Oxalate**	**Calcium**
I.	Normal control (DH_2O)	3.84±1.02	1.45±0.30	2.12±1.30
II.	Lithiatic control	9.44±2.31	7.67±2.25	9.50±2.47
III.	K-Cit (2.5 g/kg)	5.13±1.33#	3.33±0.87*#	3.33±2.24#
IV.	Cystone (750 mg/kg)	7.05±2.00	5.27±1.42**	4.22±2.10#
V.	*S. punctata*	5.74±1.19#	3.79±0.83*#	5.15±2.33
VI.	*A. pulcherrima*	4.31±0.58#	4.60±0.738*#	3.10±0.89#
VII.	*G. fruticosus* crude extract	3.74±1.13#	4.91±0.61*#	6.98±2.03*
VIII.	Mixed extract	7.03±2.71	2.39±0.18##	5.58±1.55*
IX.	*G. fruticosus* PET fraction	8.23±2.70*	4.52±0.56*#	5.03±1.98
X.	*G. fruticosus* Chl. fraction	7.50±2.08	4.09±0.78*#	5.11±1.69
XI.	*G. fruticosus* EtOAc fraction	3.89±0.42#	2.10±0.87##	7.05±2.56*
XII.	*G. fruticosus* BuOH fraction	6.11±1.55	3.31±0.32*#	7.21±2.37*
XIII.	*G. fruticosus* aq. fraction	4.52±0.63#	5.43±1.05**	5.53±1.88*

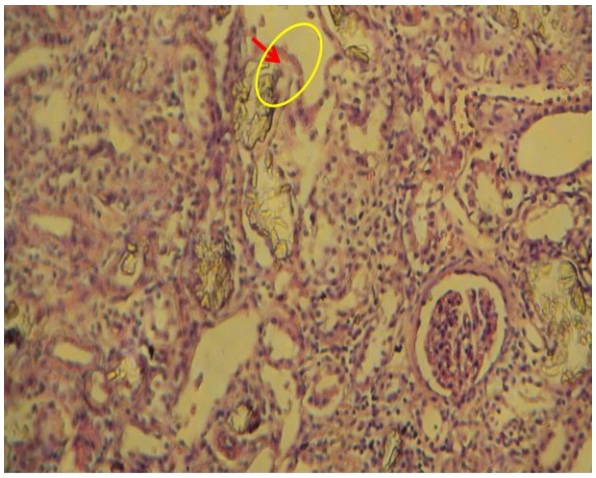
Note: The mixed extract = the combination of *A. aspera*, *S. punctata* and *R. abyssinicus* extracts (1: 1:1 ratio). Data were presented as mean ± SD for 6 rats in each group (n=6). Comparisons between means were made against Group I (vehicle control) and Group II (lithiatic control). *$p < 0.05$, **$p < 0.01$,***$p < 0.001$ indicate significant changes in comparison with the group I (vehicle control), #$p< 0.05$, ##$p< 0.01$, ###$p< 0.001$ indicate significant changes in comparison with group II (lithiatic control).

3.9.5. Effect of Extracts on Histopathology of Kidneys Against CaOx Cystal Deposition

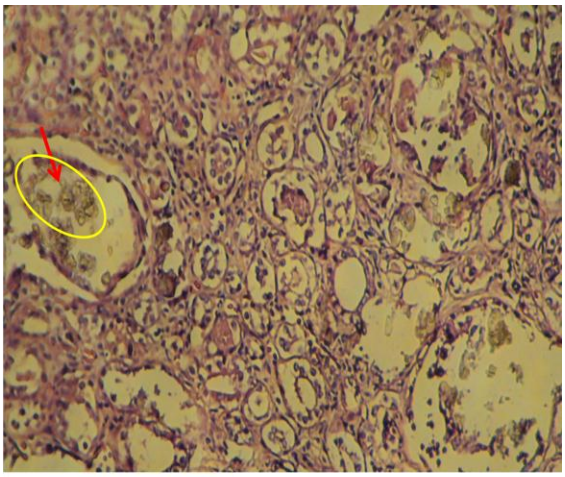
Light microscopic examination for CaOx crystals in kidney histologic sections revealed formation of CaOx crystals in the tubular lumen. The size and amount of CaOx deposits decreased among treated groups in comparison with lithiatic controls. The effects of *G. fruticosus* aqueous fraction (Fig. 26M) was higher than that of the standard control drugs (K-Cit and cystone). However, the effects of aqueous extract of *S. punctata* (Fig. 26E), *A. pulcherrima* (Fig. 26F), and the mixed extracts (Fig. 26H) were similar to the effect of K-Cit (Fig. 26C) ($p<0.05$). The effect of *G. fruticosus* EtOAc fraction (Fig. 26K), and its BuOH fraction (Fig. 26L) reduced the number of crystals compared to lithiatic control, whereas *G. fruticosus* aqueous crude extract was not protective against kidney tubular damage (Fig. 26G).



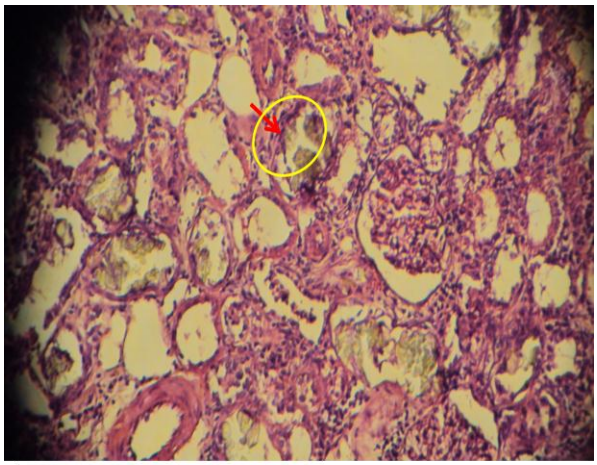
(A) Normal control **(B) Lithiatic control**



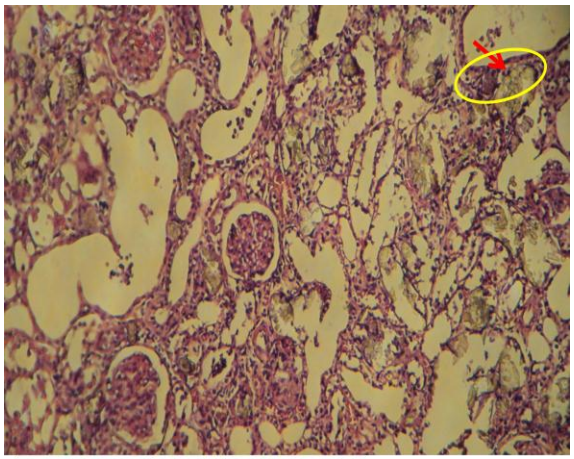
(C) Potassium citrate (K-Cit)



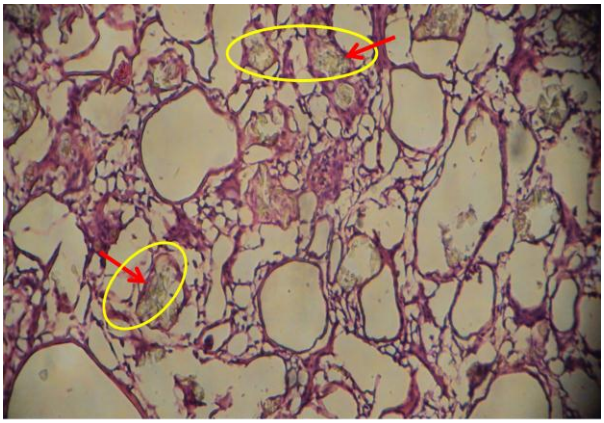
(D) Cystone



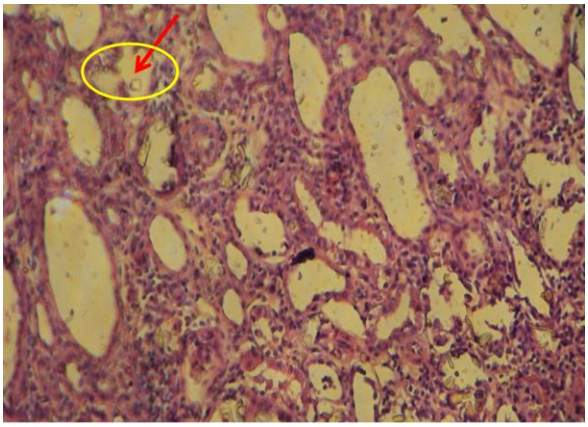
(E) *S. punctata*



(F) *A. pulcherrima*



(G) *G. fruticosus* crude extract



(H) Mixed extracts

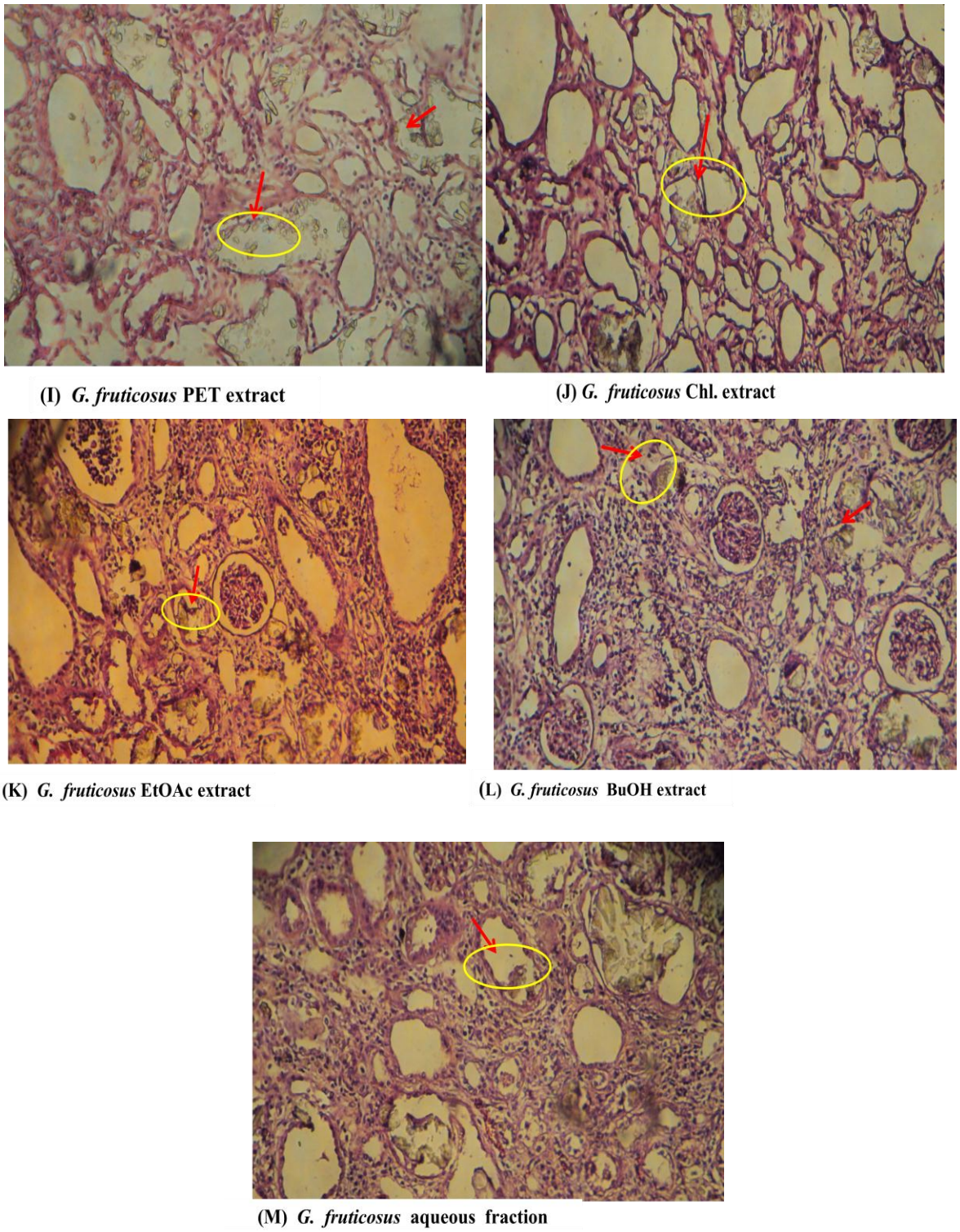
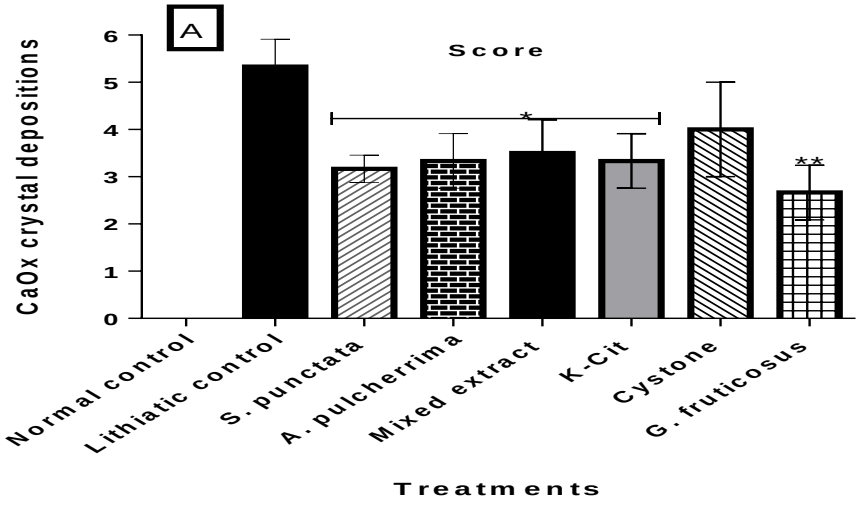


Figure 26. Representative photomicroscopic (100×) images of kidney sections of extracts treated male Wistar rats showing anti-CaOx crystal deposition effect (dose of extracts 200 mg/kg). Histopathology of kidney tissues (**A**) Normal control/vehicle, (**B**) Lithiatic control, treatment with (**C**) K-Cit, (**D**) Cystone, (**E**) *S. punctata*, (**F**) *A. pulcherrima*, (**G**) *G.*

fruticosus crude extract, (**H**) Mixed treatment, (**I**) *G. fruticosus* PET extract, (**J**) *G. fruticosus* Chl. extract, (**K**) *G. fruticosus* EtOAc extract, (**L**) *G. fruticosus* BuOH extract, and (**M**) *G. fruticosus* aq. fraction. The mixed extracts (the combination of *A. aspera*, *S. punctata* and *R. abyssinicus* extracts in 1:1:1 ratio). Polymorphic irregular CaOx crystals in the renal tubules (yellow circle/arrows). Images were 5µm thick paraffin sections with Hematoxylin-Eosin stain.

3.9.6. Curative Effects of Extracts on CaOx Deposition in the Kidneys

The number of crystal deposits was counted from the cortex, medulla and papilla by taking the mean of 4 microscopic fields. In comparison with lithogenic groups, CaOx crystal deposits in the kidneys reduced significantly by *G. fruticosus* crude extract ($p < 0.01$) (Figure 27A), and *G. fruticosus* EtOAc fraction ($p < 0.01$) (Figure 27B).



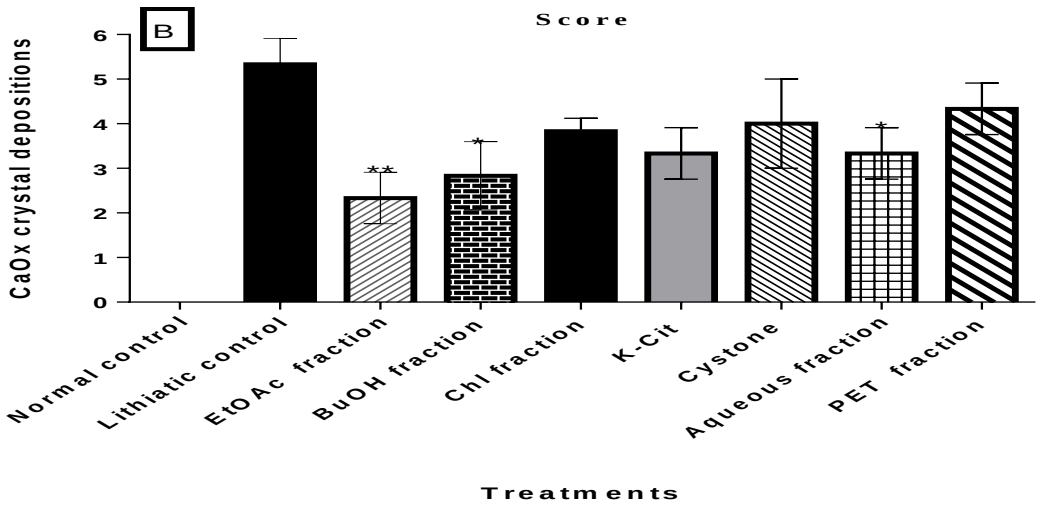


Figure 27. **CaOx crystal deposition scores in the curative study after treatment with various extracts (dose of extracts: 200 mg/kg).** (**A**) Plant crude extracts; (**B**) *G. fruticosus* successive solvent extracts. Data were expressed as mean ±SD of n=6 rats per group. *p < 0.05, **p < 0.01, and ***p < 0.001 indicates a significant change in comparison with lithiatic control (hyperoxaluric group).

It has been noted that *G. fruticosus* extract was a promising antiurolithiatic extract followed by *S. punctata* and *A. pulcherrima* extracts**.** Furthermore**,** *G. fruticosus* EtOAc fraction was the most potent agent in treating urolithiasis followed by BuOH fraction.

3.10. Characterizations of the Bioactive Compounds of *G. fruticosus* Extracts

3.10.1. Isolated Compounds Using Silica Gel Column Chromatography

Among the eight (I-VIII) fractions of *G. fruticosus* EtOAc fractions collected through silica gel column chromatography, the most active fractions (II, III) tested under the *in vitro* conditions were isolated (Appendix 3.4).

3.10.2. Bioactive Compounds Identified Using GC-MS Analysis

The bioactive compounds present in the ethyl acetate extract of *G. fruticosus* leaves was identified using Gas chromatography and Mass spectroscopy. The active principles with their retention time (RT), and concentration (peak area %) are presented in Table 20, which elucidated the presence of 29 bioactive phytochemical compounds (constituents), of these, 20 compounds possessed good abundance.

Among the phytocompounds identified, the major compounds with the highest composition were at peak 22 (di-isooctyl phthalate or 1,2-benzenedicarboxylic acid with peak area of 17.44%), peak 19 (n-hexadecanoic acid with peak area 12.12%); peak 26 (3-(1-Methylhept-1-enyl)-5-methyl-2,5-dihydrofuran-2-one with peak area 7.46%); peak 2 (benzoic acid with peak area 7.29%); peak 7(isolongifolene, 9-hydroxy-with peak area 6.17%); peak 20 (2-Buten-1-ol,2-ethyl-4-(2,2,3-trimethyl-3-cyclopenten-1-yl)-with peak area 5.23%); peak 14(4H-1,2,4-Triazol-3-amine,4-propyl-with peak area 4.66%); and peak 18 (2-Hydrazino-6-methyl-pyrimidin-4-ol with peak area 4.61%), while the rest compounds had less than 4% peak area compositions. GC-MS analyzed compounds in the extract were compared with the database of the spectrum of known components stored in the GC-MS library of the National Institute of Standards and Technology (NIST, 2014). In the present study, the relative % relatedness with the library was estimated qualitatively. Here, the major phytochemical was estimated to be oxygenated compound (Table 20).

The chromatogram of GC-MS spectral analysis showed peaks the number of compounds from the *G. fruticosus* fractions of ethyl acetate fraction. The X-axis represents the retention time of each compound identified in minutes, while the Y-axis represents the intensity/ the presence of various compounds with a corresponding peak area at different retention times.

GC-MS chromatogram analysis showed 10 major peaks, which indicated the presence of 10 major phytochemical constituents. The major bioactive compounds identified were 1-hexanol, 2-ethyl-, benzoic acid, benzeneacetic acid, phthalic anhydride, isolongifolene, 9-hydroxy-, 4H-1,2,4-Triazol-3-amine, 4-propyl-,2-Hydrazino-6-methyl-pyrimidin-4-ol, n-hexadecanoic acid, di-isooctyl phthalate, and 3-(1-Methylhept-1-enyl)-5-methyl-2,5-dihydrofuran-2- one were identified as major compounds from the sharp peak patterns of the head-space. Both n-hexadecanoic acid, and di-isooctyl phthalate were the most prevailing compounds with retention time (RT) of 21.14 with a peak area of 12.11, and RT of 22.49 with a peak area of 17.44, respectively, which could be an anti-oxidant (Figure 28).

Table 20. Compounds identified by GC-MS analysis from *G. fruticosus* EtOAc fractions.

Peak number(PK)	Name of Compounds (Chemical / Trivial Names)	Retention time (min)	% Area Pick Count	% Similarity (with library)
1	1-Hexanol, 2-ethyl-	7.8778	3.0196	83
2	Benzoic acid	11.6346	7.2864	97
3	Benzofuran, 2,3-dihydro-	12.7427	0.7233	74
4	Benzeneacetic acid	12.9203	2.366	93
5	Phthalic anhydride	13.9149	1.4095	94
6	4-Ethylbenzoic acid, 2-bromo-4-fluorophenyl ester	16.5702	0.5863	41
7	Isolongifolene, 9-hydroxy-	16.7866	6.1694	43
8	1-Methyl-5-nitro-1H-benzoimidazol-2-ol	16.8643	2.831	43
9	1,4-Butanediol, 3-methyl-1-phenyl-	16.9417	1.217	44
10	Drim-7-en-11-ol	17.1237	1.3971	52
11	Ibuprofen	17.2264	0.9764	25
12	Phenylacetic acid, 2-ethylhexyl ester	17.4088	1.4307	64
13	Bicyclo[5.2.0] nonane, 4,8,8-trimethyl-2-methylene-	18.022	1.7604	53
14	4H-1,2,4-Triazol-3-amine, 4-propyl-	18.6224	4.6649	46
15	2-Cyclohexen-1-ol, 2-methyl-5-(1-methylethenyl)-, cis-	18.9023	0.6094	38
16	Isolongifolol	20.2511	1.7613	43
17	2,3-Dimethyl-8-oxo-non-2-enal	20.4723	0.9385	46
18	2-Hydrazino-6-methyl-pyrimidin-4-ol	20.5756	4.6114	50
19	n-Hexadecanoic acid	21.144	12.1182	99
20	2-Buten-1-ol, 2-ethyl-4-(2,2,3-trimethyl-3-cyclopenten-1-yl)-	21.2416	5.2328	55
21	1-Formyl-2,2-dimethyl-3-trans-(3-methyl-but-2-enyl)-6-methylidene-cyclohexane	21.8738	2.3262	80
22	Di-isooctyl phthalate/ 1,2-Benzenedicarboxylic acid, diisooctyl ester/	22.4972	17.4404	91
23	3-Buten-2-one, 3-methyl-4-(1,3,3-trimethyl-7-oxabicyclo[4.1.0]heptan-1-yl)-	22.7567	2.0966	35
24	(-)-Globulol	22.999	1.4659	46
25	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(1-methylethenyl)-, [2R-	23.1265	2.938	74
26	3-(1-Methylhept-1-enyl)-5-methyl-2,5-dihydrofuran-2-one	24.6499	7.4623	38
27	Octadecanoic acid	24.8591	2.2458	97
28	1a,2,5,5-Tetramethyl-cis-1a,4a,5,6,7,8-hexahydro-gamma-chromene	25.2143	1.0397	70
29	7,8-Dimethoxy-1-methyl-3,5-dihydro-benzo[d] [1,2] diazepin-4-one	28.9268	1.8756	38

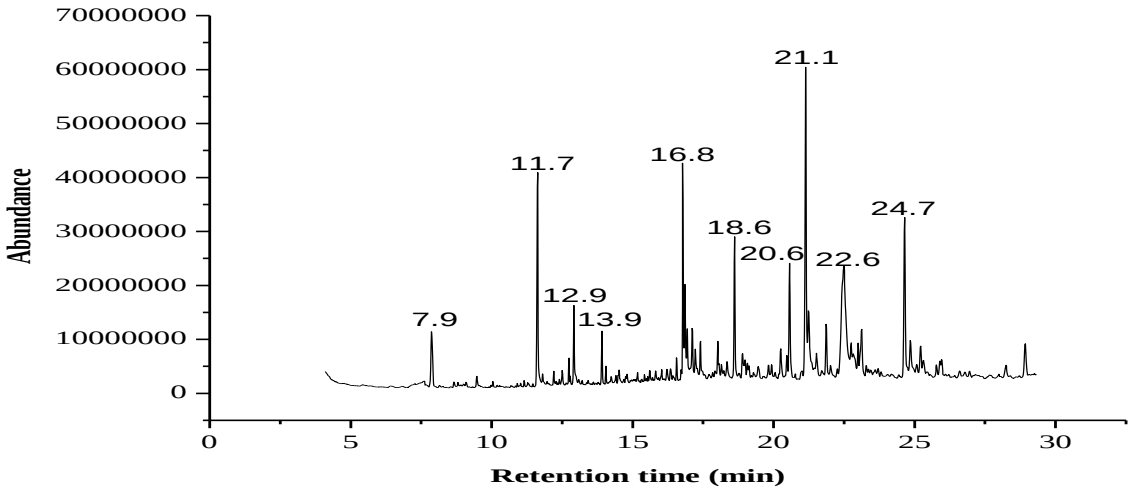


Figure 28. GC-MS chromatogram of *G. fruticosus* EtOAc leaves fraction showing abundance versus retention time (in minutes).

4. Discussion

The increasing trend in stone disease prevalence through years in Ethiopia may be a reflection of the changes in the lifestyle of the population such as diet and reduced daily activities. There are indications that the increase in prevalence of stone diseases may be associated with increasing consumption of animal proteins and salts, decrease in calcium-rich products, and climate change as reviewed by Alelign and Petros (2018). The analysis results of trends in urolithiasis prevalence were low and similar across the years 2005 to 2012, but started to rise thereafter until 2017. This might be due to improvements in diagnosis of the disease and availability of surgical services at the hospital.

Similarly, increasing trends in stone disease prevalence was reported from USA by Raheem *et al*. (2017). In Japan, the prevalence of renal stones rose from 4 to 5.4% within 10 years (1975-1985) (Yoshida and Okada, 1990). It was also shown that the recurrent urinary tract infections (urease-producing microorganisms) aggravated the prevalence of urolithiasis (Dursun *et al.,* 2008; Noshad *et al.,* 2014). The findings that most stones were located in the kidneys are similar to previous reports from Tunisia and Saudi Arabia (Alaya *et al.,* 2009; Ahmad *et al*., 2015). The retrospective prevalence of urolithiasis (2.3%) in Ethiopia was substantially higher than the prevalence (0.24%) from the USA (Masterson *et al.,* 2017), and in Iran (5.7%) (Safarinejad, 2007). The majority of stones were located in the left kidney, probably due to its larger size and lightly higher position close to the heart, which would increase the chance of crystal depositions. Patients with ureteric stones (41.5%) had surgical interventions, although the non-invasive treatment options such as ESWL were available, though there are problems with their functionality. Therefore, open surgery remains the mainstay of urinary stone treatment.

The finding that the prevalence of kidney stone in men was higher than women, may be explained by the fact that the female sex hormone (estrogen) inhibits calcium oxalate stone formation (Iguchi *et al.,* 1999), whereas male hormones, testosterone (Lee *et al.,* 1996) and androgen (Naghii *et al.,* 2014) promote stone formation. Similar studies from other countries indicated that urolithiasis was more frequent among men than women (Pak, 1998; Curhan, 2007; Scales *et al.,* 2012). Studies from the USA also showed that males have a three times higher urinary stone incidence compared to females and provided an explanation that urolithiasis mainly occurred in the third and fourth decades of life when the level of serum testosterone is the highest (Travison *et al.,* 2009). However, as Lieske *et al.* (2006) reported from Rochester, and Minnesota of the USA there is a decreasing trend during the past 30 years in the male-to-female ratio in kidney stone disease, in the developed countries. In the present study, the life expectancy in Ethiopia is in the 60's and a small number of patients in the 70's may come to the hospital for support. Moreover, one of the possible reasons for gender variation might be due to gender inequality, in which females may not have equal opportunity to go to health setup.

The mean age of 42.5 (±23.7) at the time of stone treatment was similar to that of the USA, which was 45 years in men and 41 years in women (Lieske *et al.,* 2006), but was different from what was reported from Iceland (30 to 79 years) (Indridason e*t al.,* 2006). However, there is also another study, which showed that younger age groups are not fully free from developing kidney stones (Alatab *et al.,* 2016). Thus, age does not appear to be a risk factor for stone disease formation in the kidneys. Although *Diabetes mellitus* and hypertension have been significantly associated with stone diseases, affecting children as

young as 5 years old (Schaeffer *et al.*, 2011), these were not associated with urolithiasis in the current study.

The body weight loss observed in the experimental rats, following *R. abyssinicus*, *C. murale, C. ambrosioides* and *I. confertiflora* extract administration during the acute toxicity study was similar to the reports on other plant extracts (Prasanth *et al.*, 2015; Bello *et al.*, 2016). The weight loss in the test groups may be due to disturbances in carbohydrate, protein or fat metabolism, which may have been affected by administering extracts (Ghelani *et al.*, 2016). In the present acute toxicity study, no toxic effects observed for both *C. myrrha* and *G. fruticosus* aqueous extracts at dose 2000 mg/kg body weight. These supported the safety of the extracts at a given dose in rats, which resulted in an increase in body weight over time similar to the controls. This is in agreement with what was reported for *C. myrrha* resin extract at dose 100 mg/kg b.w. given to the mice (Rao *et al*., 2001). On the other hand, although the dose was not specified, it was reported that consumption of significant quantities of *G. fruticosus* leaves extract was toxic to animals due to the presence of cardiac glycosides and pregnane glycosides (Wagstaff, 2008).

A significant reduction in platelets count subsequent to administration of *R. abyssinicus* and *S. punctata* extracts can be an indication of acute toxicity to the test rats. It has been reported that short-term exposure of erythrocytes to cytotoxic agents could result in hemoglobin reduction (da Silva *et al.*, 2014). Although earlier work on *S. punctata* essential oils revealed cytotoxicity and hemolytic properties of human monocytic leukemia cells (THP-1 cell lines) (Tarikua *et al.*, 2010) and erythrocytes (Tepe and Cilkiz, 2015), no toxic effects were detected in the present study, which used crude extracts of the plant's aerial parts.

Non-toxic compounds may be toxic on prolonged exposure due to their accumulation, effects on enzyme levels, and the disruption of physiological and biochemical homeostasis (Gandhare *et al.,* 2013). A significant reduction in the activities of alkaline phosphatase (ALP) following *A. aspera* administration is suggestive of anti-urolithic potential of the plant extract similar to a study on *Phyllanthus niruri* extract, which have demonstrated that the decrease in alkaline phosphatase level contributes to a reduction in the number of renal calculi (Pucci *et al.,* 2018). Furthermore, since it was reported that *A. aspera* extracts have hepatoprotective activity against paracetamol-induced toxicity (Kumar *et al.,* 2012b) and it is non-toxic when administered intraperitoneally in rats (Reddy and Kamble, 2014) are good indications that would justify further investigation on its use as an antiurolithic treatment. The previous study demonstrated the nephroprotective effects of *R. abyssinicus* extract (Jaganathan *et al.,* 2012) indicating the need for further evaluation of the effects of this plant extract.

The antioxidant constituents of plant extracts might restore the antioxidant enzymes of kidneys, which help prevent renal cell injury (Dinnimath and Jalalpure, 2018). Antioxidants have been reported to reconcile free radicals by directly reacting and quenching their catalytic metal ions (Robak and Marcinkiewicz, 1995). In other words, antioxidants neutralize the damaging effects of free radicals on tissues by donating electrons at the site of injury (Zhao *et al.,* 2019). The previous studies reported that DPPH scavenging potentials of whole leaves extract of *Aloe ferox* was concentration dependent in which hydrogen donating compounds in the extract might increase (Wintola and Afolayan, 2011). In the present study, *S. punctata* extract has scavenged DPPH free radicals as postulated that it may react with hydrogen donors (Sanchez-Moreno, 2002).

Previous phytochemical studies on other plants had reported that flavonoids (Sikarwar *et al.*, 2017), saponins and terpenoids (Rajesh *et al.*, 2011; Dinnimath and Jalalpure, 2018), and phenols (Bawari *et al.*, 2018) exhibited antioxidant activities. In line with these studies, it would be expected that *S. punctata* aqueous extract possesses phenols, flavonoids, saponins and terpenoids; whereas *A. pulcherrima* extract has phenols, flavonoids and terpenoids, while *G. fruticosus* extract possesses phenols and flavonoids to show antioxidant effects and DPPH radical scavenging activities. Therefore, targeting reductions in oxidative stress would be a therapeutic option for urolithiasis.

The antioxidant properties of test extracts may be potential contributors for lithotriptic effects (Pareta *et al*., 2011b), and *S. punctata* aqueous extract was found to be the most potent antioxidant comparable to ascorbic acid, the drug in use for treatment of urolithiasis. As reported earlier, the presence of antioxidant metabolites like flavonoids and phenols might be responsible for antiurolithiatic effects (Kifayatullah *et al.*, 2015). These were present in *S. punctata*, *A. pulcherrima* and *G. fruticosus* aqueous extracts phytochemical analysis that would further strengthen the traditional claim for their use to treat urolithiasis-induced oxidative stress. Furthermore, the antiurolithiatic activities of *S. punctata*,, *A. pulcherrima* and *G. fruticosus* aqueous extracts might be due to the disintegrations of mucoproteins by saponins and tannins found in the present study. In addition, the presence of hydroxyl groups may be responsible for the antioxidant properties by quenching free radicals as supported by earlier studies (Vinson *et al.*, 1998; Panigrahy *et al.*, 2017).

The kidneys filter waste products from the blood and void them into the urine. This is not possible if waste materials do not dissolve completely in the urine, leading to kidney stone formation. Kidney stone formation is not only a crystallization phenomenon, but it is also a

cellular process in the renal tissues (Bawari *et al.,* 2018). Therefore, the biomineralization processes involve successive physicochemical changes such as super-saturation, nucleation, growth, aggregation and retention within renal tubules (Akanae *et al.,* 2010; Alelign and Petros, 2018).

The significantly ($p<0.05$) high *in vitro* nucleation inhibition (37.6%) of *G. fruticosus* extract compared to that of cystone (20.3%) could be due to its ability to competitively block the formation of CaOx complexes with free calcium or oxalate ions as suggested by Bawari *et al.* (2018). Similarly, previous *in vitro* studies showed that potassium citrate prevents CaOx nucleation by reducing super-saturation (El-Shall *et al.,* 2004). In this regard, the citrate binds competitively with free calcium ions in the urine and reduce interactions with oxalate ions (El-Shall *et al.,* 2004), which prevents hypocitraturia in CaOx stone patients (Qiu *et al.,* 2004). Similarly, *A. pulcherrima* gel extract inhibited CaOx nucleation (25.5%) more than that of K-Cit (21.9%) and cystone (14.1%). This suggests that *G. fruticosus* and *A. pulcherrima* aqueous extracts may contain CaOx crystallization inhibitors. Previous studies suggested that plant extracts may possess negatively charged groups, which strongly chelate Ca^{2+} ions (Kavanagh *et al.,* 2000), and decrease CaOx dihydrate formations (Das *et al.,* 2005; Zhang *et al.,* 2012). The synergistic or combined effects of compounds present in the medicinal plant crude extracts often claim that they are more effective than that of purified compounds due to additive interactions (Rasoanaivo *et al.,* 2011; Caesar and Cech, 2019). In line with this, the synergistic effects of *G. fruticosus* EtOAc fraction was higher (56.9%; 3200 μg/ml) compared to its fractionated components (37%) on nucleation inhibition as indicated by *in vitro* studies.

The presence of flavonoids and saponins in *G. fruticosus* and *S. punctata* aqueous extracts may be attributed to CaOx anti-crystallization effects. In line with the present study, the previous studies on herbal extracts reported that phytochemical constituents like flavonoids, saponins and terpenoids are responsible for anti-urolithiatic effects (Pareta *et al.,* 2011b; Patel *et al.,* 2012b). These have been shown to inhibit CaOx crystallizations *in vitro* human urine as well as in animal urine studies by disaggregating the suspension of mucoproteins of the stone matrix (Sharma *et al.,* 2017a; Sikarwar *et al.,* 2017). Similarly, other researchers reported that the presence of flavonoids (Pourmorad *et al.,* 2006; Ahmed *et al.,* 2013), saponins and triterpenoids (Dinnimath and Jalalpure, 2012; Dinnimath and Jalalpure, 2018) may contribute for the antiurolithiatic activities. The latter authors suggest that these phytochemical constituents may interfere with urine supersaturation and reduce the activities of oxalate oxidase.

Flavonoids inhibit CaOx crystal deposition (Noorafshan *et al.,* 2013), and possess calcium channel blocking effects, CaOx dissolution potencies and antidiuretic activities (Pietta, 2000). Plant extract constituents such as tannins and polyphenols inhibit CaOx crystal formations as well as dissolve preformed CaOx crystals (Doddola *et al.,* 2008). Moreover, the presence of phenols and tannins in *G. fruticosus, S. punctata* and *A. pulcherrima* aqueous extracts should not be discounted for anticrystallization activities as proposed by Saha and Verma (2013). Furthermore, the administrations of *G. fruticosus* EtOAc fraction II reduced the size of calculi, which might be due to the presence of concentrated phytochemicals responsible for antiurolithiatic effects. Furthermore, it was also reported that steroidal constituents, which were found in *S. punctata* and *G. fruticosus* aqueous extracts

possess antiurolithiatic activities, suggesting that macromolecules inhibit CaOx nucleations in human urine (Chen *et al.,* 2007).

Aggregation of crystals is the most critical step, which produces renal tubular obstruction, and promotes stone formation (Masao, 2008). The previous *in vitro* studies showed that citrate prevents CaOx aggregations (Kok *et al.,* 1986; Kato *et al.,* 2004). In the present study, *G. fruticosus* and *A. pulcherrima* aqueous extracts showed a significant aggregation inhibitory effects. The CaOx crystal aggregation inhibition of the aqueous extract of *G. fruticosus* (33.3%) was greater than that of cystone (22.3%) ($p<0.05$), but lower than that of K-Cit (41.2%) ($P<0.05$) at 200 μg/ml. The EtOAc fraction of *G. fruticosus* has shown that aggregation inhibitions (16%) at 100 μg/ml similar to the effects of cystone (19.9%), but lower than that of K-Cit (38.7%) ($p<0.001$), which was less than its aqueous extract. Furthermore, the aggregation inhibitory effect of *G. fruticosus* fraction II was 58.9%, which was significantly higher than cystone (33.7%) and K-Cit (43.2%) at 3200 μg/ml ($p<0.05$). Microscopic observation of *in vitro* test showed that *G. fruticosus* aqueous extract reduced the number and size of CaOx crystals indicating the action of its phytochemicals (Appendix 3.1)**.** If the extract keeps CaOx particles dispersed in the solution, they are more easily eliminated. Similarly, *S. punctata, A. pulcherrima* and *G. fruticosus* aqueous extracts prevented lithogenic inducing factors, verifying their *in vitro* anti-nucleation and anti-aggregation effects. Furthermore, *A. pulcherrima* aqueous extract inhibited crystal aggregation (33.1%) far better compared to cystone (19.9%) ($p<0.01$) at 100 μg/ml.

The microscopic observation of *G. fruticosus, S. punctata* and *A. pulcherrima* aqueous extracts revealed the formation of more calcium oxalate monohydrate (COM) crystals

instead of calcium oxalate dihydrate (COD), which was contrary to the report of Saha and Verma (2013). It is known that COM is the predominant form of kidney stones, which is thermodynamically stable with higher tendencies to adhere to renal epithelial cells than COD crystals (Wesson *et al*., 1998; Atmani and Khan, 2000). The previous studies suggest that plant extracts might transform crystals from a pointy edged COM to smooth edged COD, which increases the chance of spontaneous passage of crystals in urine (Sheng *et al.*, 2005; Bawari *et al.,* 2018). However, the need to use a polarized light microscope to differentiate between COM and COD crystals was not possible in the present study. Lack of evidence for decreasing kidney stone weight following the *in vitro* test of *G. fruticosus* extract was indication that the test system was not an appropriate approach.

The successful induction of CaOx crystals in the experimental rats has enabled the evaluation of various parameters including calcium, oxalate, phosphate, magnesium, and citrate in the urine, serum, kidney homogenate and kidney histopathology. This was possible by the fact that EG is readily absorbed in the intestine and metabolized to oxalate in the liver, leading to hyperoxaluria (Fan *et al.,* 1999; Katzung and Trevor, 2004). EG increases the availability of substrates for synthesizing oxalate enzymes (Ratkalkar and Kleinman, 2011) as a result of which the urinary oxalic acid forms soluble salts in the presence of magnesium (Marshall and Robertson, 1976; Jahan *et al.,* 2019). In this process, acidic metabolites produce renal calcium leak, which is associated with absorption of calcium in the gut and calcium release from the bone, leads to hypercalciuria (calcium in 24 hr urine) and hypercalcemia (calcium in serum) (Moochhala *et al.,* 2008). The precipitation of oxalate in the kidneys, induce heterogeneous crystal nucleation and aggregation (Scheid *et al*., 2004; Ghelani *et al.,* 2016). Furthermore, oxalate promotes oxidative stress that

enhances the risk of nephrolithiasis, which is substantially retarded by antioxidants (Scheid *et al.,* 1996).

Hyperoxaluria has higher main risk for kidney stone formation than hypercalciuria (Robertson and Peacock, 1980). Similarly, it has also been reported that urinary oxalate contributes 15-fold more to kidney stone formation than urinary calcium (Borghi *et al.,* 1993; Doddola *et al.,* 2008). Therefore, measuring oxalate levels than calcium would be an accurate marker for CaOx stone detection since calcium could be excreted at high concentrations in the normal urine (Robertson and Peacock, 1980).

The present findings were in agreement with previous reports on anti-urolithiatic studies of plant extracts revealed by biochemical and histopathological determinations (Ahmed *et al.,* 2013; Dinnimath and Jalalpure, 2018). In the prevention study, the reduced number and size of urinary crystals were observed through microscopic examination of urine following *S. punctata* and *G. fruticosus* aqueous extract administration. *G. fruticosus* extract reduced urinary super-saturation, and minimized the level of CaOx excretions. Previous studies suggested that reductions in the size of urinary stones by herbal extracts could be due to their lithotriptic, and aggregation preventive effects (Dinnimath and Jalalpure; 2018; Jahan *et al.,* 2019).

The preventive study showed a significant reduction in the levels of urinary sodium, uric acid ($p<0.01$), potassium and creatinine ($p<0.05$) by *G. fruticosus* aqueous extract similar to K-Cit, whereas *G. fruticosus* EtOAc fraction, *A. pulcherrima* and the mixed aqueous extracts decreased the concentrations of sodium compared to lithiatic control ($p<0.001$). In curative study, sodium level was reduced significantly by *G. fruticosus* EtOAc fraction, *A.*

pulcherrima and the mixed aqueous extracts ($p<0.001$). In contrast, the previous studies reported that excessive excretions of sodium and chloride in the urine indicate the diuretic activity of the test extract (Pawar *et al.,* 2012; Jahan *et al.,* 2019), with increasing urinary output, which inhibit stone developments (Sasikala *et al.,* 2013). Diuretic activities might be attributed to increased salty excretions, and potentially prevent new stone formation (Pareta *et al*., 2011b).

In lithiatic control**,** the urinary concentrations of oxalate, calcium and phosphorus were elevated significantly ($p<0.05$), whereas the levels of magnesium and citrate decreased compared to the normal (healthy) control ($p<0.01$). This was supported by studies conducted by Dinnimath and Jalalpure (2018)**.** The aqueous extracts of *G. fruticosus*, *S. punctata* and *A. pulcherrima* ($p<0.05$) prevented hyperoxaluria in rats by lowering urinary excretion of oxalate and by increasing urinary excretions of citrate and magnesium levels compared to the untreated lithiatic group. The lower level of magnesium is a favorable condition for CaOx stone formation, which was noted among stone forming patients and stone induced rats (Rushton *et al.,* 1980; Jahan *et al.,* 2019). Magnesium binds with oxalate ions to form soluble oxalate complexes in the urine, and decreases the oxalate availability, which binds with calcium leading to CaOx formation (Touhami *et al.,* 2007; Divakar *et al*., 2010). Hence, it could be suggested that CaOx stones are mostly formed among people who are magnesium deficient. This was supported by the fact that *G. fruticosus* EtOAc fraction and mixed aqueous extract elevated the levels of magnesium and citrate ($p<0.01$) similar to K-Cit in the curative study. According to the previous studies, the possible mechanisms underlying the effects of *G. fruticosus* fraction appear to be mediated through *in vitro* nucleation and aggregation inhibitions, which help prevent stone recurrences. *S. punctata*

extract possesses strong antioxidant activities, suggesting its preventive effects against urolithiasis.

Other investigators reported similar results that CaOx stone formation can be reduced by increasing urinary citrate levels (Mi *et al*., 2012; Siddiqui *et al.,* 2018). Thus, an increased excretion of citrate levels by *G. fruticosus* and the mixed aqueous extracts might be due to increasing citrate metabolism. Additionally, the presence of phenols and flavonoids in *G. fruticosus* aqueous extract was also associated with increasing urinary citrate levels (Touhami *et al.,* 2007). In preventive and curative studies, *G. fruticosus* aqueous extract demonstrated reduction of uric acid level in urine ($p<0.05$) compared to lithiatic control with low CaOx formation.

Furthermore, administration of *S. punctata***,** *G. fruticosus* and *A. pulcherrima* aqueous extracts reduced the urinary excretions of oxalate, calcium, phosphorus and total proteins in hyperoxaluric rats, suggesting their protective effect against urolithiasis. In the preventive study, *S. punctata* extracts had exhibited reductions in oxalate urinary excretions similar to K-Cit ($p<0.05$), which came close to the normal control. Similarly, EtOAc fraction of *G. fruticosus* decreased the levels of oxalate in the urine ($p<0.001$) compared to lithiatic control in the curative study. In the therapeutic test, urinary levels of calcium and phosphate were significantly ($p<0.01$) reduced following *G. fruticosus* BuOH fraction and the mixed aqueous extracts administration. This indicates the presence of CaOx inhibitory constituents in the extract, which interferes with crystal nucleation and aggregation processes. Moreover, an increase in calcium concentration creates a favorable environment for nucleation, and precipitation of calcium oxalate or calcium phosphate with subsequent crystal growth (Bahuguna *et al.,* 2009b; Sathish *et al.,* 2010). Similarly, increased urinary phosphate and

oxalate excretions provide a favorable environment for the formation of calcium phosphate, in turn, leading to calcium oxalate crystal depositions in the renal tubules (Ratkalkar and Kleinman 2011; Siddiqui *et al.,* 2018).

The accumulation or elevation of nitrogenous waste products such as urea, creatinine, and uric acid in the serum lithiatic induced rats could be due to stone injury or obstruction of the urinary system (Ghelani *et al.,* 2016), which are markers for kidney function impairment (Patel *et al.,* 2012a). In the prevention study, *A. pulcherrima* aqueous extract improved glomerular filtration rate (GFR) as noted in reductions of serum creatinine level ($p<0.001$) compared to the untreated lithiatic group, at which creatinine and urea levels markedly elevated. Similarly, *S. punctata* reduced the levels of creatinine ($p<0.05$), and uric acids ($p<0.01$) in the urine compared to lithiatic control during curative test. The remarkable reduction in serum creatinine level clearly showed the curative effect of the present test extracts, which were similar to a study reported by Siddiqui *et al.* (2018). This might be attributed to reducing renal inflammation as a result of which there is improvement in GFR.

In the preventive study, the serum level of potassium was reduced by aqueous extracts of *S. punctata*, and *A. pulcherrima* ($p<0.01$), compared to lithiatic control. Similarly, *G. fruticosus* EtOAc extract ($p<0.01$) reduced serum level potassium in curative study. In addition, the effects of *G. fruticosus* BuOH fraction on the level of chloride was found to be close to the normal control during the curative study. The serum level of sodium significantly increased with *A. pulcherrima* aqueous extract ($p<0.01$) administration, whereas *G. fruticosus* BuOH fraction reduced sodium level significantly ($p<0.05$) compared to lithiatic control in the curative study. Treatment with *A. pulcherrima* extract significantly ($p<0.05$) reduced oxalate excretions in the serum in the preventive study; whereas the

aqueous, EtOAc and BuOH fractions of *G. fruticosus*, and the mixed extracts significantly ($p<0.01$) reduced oxalate concentrations in the serum in the post-treatment study compared to lithiatic control. This might be either due to the inhibition of oxalate formation, or interference with oxalate metabolism. Thus, treatment with *A. pulcherrima* extract markedly reduced the excretion of calcium in both preventive ($p<0.05$) and curative ($p<0.01$) test compared to the respective control. Similarly, the mixed extracts ($p<0.01$), and *G. fruticosus* EtOAc and BuOH fractions ($p<0.05$) decreased calcium levels compared to lithiatic control in curative test. The ability of these extracts to alter calcium and oxalate excretions may be due to the disintegration of mucoproteins, which are promoters of crystallization as reported by Doddola *et al.* (2008).

In the present study, the level of citrate in the serum increased by *S. punctata*, the mixed extracts and *G. fruticosus* BuOH fraction ($p<0.01$), suggesting a possible curative effect close to K-Cit treated group ($p<0.001$). The extract of *A. pulcherrima* reduced citrate excretions ($p<0.05$) in preventive study. The rationale of mixing extracts (*A. aspera*, *S. punctata* and *R. abyssinicus*) was due to traditional claims, which was proposed to be effective against urolithiasis (Abate, 1989), which remarkably decreased stone forming constituents. This was similar to cystone, which is a mixture of different plant extracts (Erickson *et al.*, 2011; Dulanjali and Srikaran, 2020). The presence of tannins and flavonoids can lead to the relaxation of smooth muscles of the urinary tract, which could facilitate the expulsion of kidney stones as studied in rats (Ghelani *et al.*, 2016), which were confirmed for their presence in the phytochemical analysis of *S. punctata*, *A. pulcherrima* and *G. fruticosus* extracts in the present study.

In the present preventive study, a reduced serum ALT levels were observed in the treatment of the of *G. fruticosus* and *A. pulcherrima* aqueous extracts significantly similar to K-Cit ($p<0.01$), and it also reduced significantly by *A. pulcherrima* extract ($p<0.001$) compared to lithiatic control in curative study. In addition, it was also evidently proved that the aqueous extracts of *S. punctata*, and *G. fruticosus* as well as *G. fruticosus* BuOH fraction lowered AST levels very significantly ($p<0.001$), which was close to K-Cit compared to the lithiatic control in the curative studies. In contrast, *G. fruticosus* and the mixed aqueous extracts increased serum AST levels significantly ($p<0.001$) in the curative test. In the prevention study, *A. pulcherrima* treatment reduced AST concentrations closer to the effects of cystone. This can be attributed to the fact that abnormal levels of liver enzymes, particularly aminotransferases including alanine aminotransferase (ALT), and aspartate aminotransferase (AST) are prognostic indicators of damages of liver cells, and impacts on cellular integrity of kidneys (Ghale-Salimi *et al.,* 2017; Zhao *et al.,* 2019). Normally, these enzymes are involved in the breakdown of amino acid metabolisms, which are located within the cytoplasm. However, the inflamed liver cells leak out or release ALT and AST enzymes into the blood circulation and get elevated in serum, which may also indicate the damage of organelle membranes (Ghale-Salimi *et al.,* 2017). Moreover, oxidative stress could be a major cause of cellular damage leading to release of ALT and AST into the blood and urine, which were supported by kidney histological findings (Khan, 2013b; Zhao *et al.,* 2019).

In the kidney homogenate analysis, the lowering effect on calcium level by *A. pulcherrima* aqueous extract ($p<0.05$) compared to the lithiatic control was similar to a related previous kidney homogenate analysis (Jahan *et al.,* 2019). The increased level of calcium in lithiatic

control may be due to lowering of GFR, which promotes deposition of CaOx in the renal tubules (Lakshmi *et al.*, 2014). In the present study, *S. punctata, A. pulcherrima*, and *G. fruticosus* aqueous extracts showed antioxidant activities, which might play a role in kidney stone treatment. A decrease in the antioxidant potentials of extracts in rats results in renal cell damage by reacting with polyunsaturated fatty acids in the membrane of cells (Bahuguna *et al*., 2009a). Thus, the mechanisms underlying the protective or curative effects of extracts would be mediated via antioxidant activities, which prevent urinary super-saturation and anti-mineralization effects (Kifayatullah *et al.*, 2015).

Exposure of renal epithelial cells to CaOx crystals and high oxalate level in nephrons can give rise to the generation of ROS, mitochondrial collapse and increased lipid peroxidation, which induces the cell death in cultured renal epithelial cells (Zhai *et al.,* 2013). This induces heterogeneous nucleation and causes crystal aggregation (Ghale-Salimi *et al.*, 2017). Accumulation of COM crystals has recently been linked closely with evidence of proximal tubular necrosis (Cruzan *et al.,* 2004; Pomara *et al.,* 2008). Glomerular and tubular damages following EG administrations, marked by rising in serum creatinine, urea, BUN, and uric acid in rats. Both *S. punctata* ($p<0.001$) and *G. fruticosus* ($p< 0.01$) aqueous extracts treated groups showed reductions in crystal depositions suggesting their preventive effects. This might be possible by preventing renal tubular distal obstructions caused by CaOx crystal accumulations. This was supported by Mcmartin (2009) reporting that the toxic nephropathy occurs only when high oxalate concentrations accumulate as COM crystals in the kidneys.

Experimentally induced CaOx crystal deposition in the kidneys is also associated with localized inflammation as evidenced by infiltration of monocyte and macrophages to the site

(Zhai *et al.,* 2013). In the present study, it has been noted that the renal tubules were markedly dilated; inflammation and congestion of blood vessels have been in the entire kidneys of untreated rats, similar to the reports of Zhao *et al.* (2019). This might be due to the fact that renal tubular cell injury is induced by the oxidative stress, which is produced during the attachment of crystals to renal tubular cells (Zhai *et al.,* 2013). However, in *G. fruticosus* EtOAc fraction ($p<0.05$) and its BuOH fraction ($p<0.05$) treated groups, the severity of CaOx crystal depositions reduced the kidneys compared to lithiatic control, which was similar to a study on the other herbal extract (Ahmed *et al.,* 2013).

In curative study, the aqueous extracts of *S. punctata* and *G. fruticosus,* and EtOAc fraction of *G. fruticosus* reduced the level of phosphate significantly ($p<0.05$) compared to the lithiatic control. Tissue injury could be caused by exposures to phosphate and calcium phosphate crystals, leading to the generation of oxidative stress, lipid peroxidation and depletion of antioxidant enzymes (Mcmartin, 2009). Consequently, the renal epithelial injury promotes crystal retention, as epithelial injury exposes a variety of crystal adhesion molecules on epithelial surfaces and promotes stone formation (Zhai *et al.,* 2013; Sun *et al.,* 2017). COM accumulates in the kidneys by attaching to tubular cell membranes, followed by internalization by endocytosis, leading to cell death. However, the mechanism by which COM induces cell death is not clearly known, although it is suggested that loss of membrane structures, induction of oxidative stress, and mitochondrial dysfunction would be contributing factors (Mcmartin, 2009). The precipitation of oxalate as COM in the tubular lumen has been linked with renal toxicity and inflammation, damaging the structures of mitochondria, and inhibiting mitochondrial respiratory functions in proximal tubular cells, alter cellular permeability leading to renal cell death (Cao *et al.,* 2001).

The inhibitory effects of herbal extracts on CaC_2O_4 crystal retention in renal tubules could be attributed to its antioxidant activities (Bashir and Gilani, 2009). The study conducted by Thamilselvan *et al.* (1997) demonstrated that exposure to high oxalate levels result in the production of super-oxide and hydroxyl free radicals, leading to redox imbalance, lipid peroxidation and protein oxidation, which facilitate changes in membrane integrity and cell death (Thamilselvan and Menon, 2005). These changes accelerate adherence and retention of CaC_2O_4 in renal tubules (Khan, 1995). Moreover, the antioxidant effects of flavonoids from herbs were shown to reduce oxidative injury and crystal depositions in the renal tubular cells (Rushton *et al.,* 1980; Jeong *et al.,* 2006).

Treatment with *G. fruticosus* and *A. pulcherrima* aqueous extracts decreased the amount of CaOx deposits in the renal tubules, which may be due to maintaining the balance between stone promoters and inhibitors, reducing super-saturation of urine, and CaOx crystal excretions in the urinary system (Patel *et al.,* 2016b; Kifayatullah *et al.,* 2019). The previous histopathological studies reported the inhibitory effects of herbal extracts against CaOx deposition in kidney tissues (Tsai *et al.,* 2008). Similar studies also reported that the prevention of glomerular atrophy and CaOx crystal depositions in the epithelial cells of the kidneys were found when treated with herbal extracts (Atmani and Khan, 2000; Jahan *et al.,* 2019). The antiurolithiatic agents containing various phytoconstituents may exert effects through multiple mechanisms. Some of these could be by increasing urine volume, inhibition of crystallization processes, improving renal function and the antioxidant effects against renal tissues, and anti-inflammatory activities (Nagal and Singla, 2013). An exposure to high levels of CaOx crystals produce cellular injury mediated through intracellular reactive oxygen species generation and membrane lipid peroxidations (Bashir

and Gilani, 2009). The test extracts containing flavonoids, saponins and alkaloids exert antioxidant effects (Kumar *et al.,* 2012a), which makes smooth muscle relaxant and would facilitate expulsion of renal stones (Rathod *et al.,* 2012a).

Although the initial qualitative analysis revealed that phenols, flavonoides, tannins, steroides, alkaloides, saponnins, and glycosides are present in *G. fruticosus* aqueous extract, the IR analysis revealed the presence of phenolic compounds, which would be responsible for antiurolithiatic effects as reported by Youn *et al.* (2017). In further study, GC-MS analysis of *G. fruticosus* phytoconstituents revealed 29 compounds, of which around 20 compounds possess good abundance showing the pharmaceutical values of this plant. Similarly, the various secondary metabolites or mixture of small molecules (mainly organic compounds) identified from medicinal plants using Gas chromatography attached to the Mass Spectrometer (GC-MS) (Komansilan *et al.,* 2012). Natural extracts are usually composed of many secondary metabolites, whereby the bioactivity of natural extracts can be represented by synergism between metabolites (Cragg and Newman, 2001; Newman and Cragg, 2016).

The major compounds identified from *G. fruticosus* fraction II were di-isooctyl phthalate, n-hexadecanoic acid, 3-(1-Methylhept-1-enyl)-5-methyl-2,5-dihydrofuran-2-one, and benzoic acid. However, isolating every single compound from a natural extract is not always possible due to the complex chemistry and presence of most secondary metabolites at very low levels (Salem *et al.,* 2020). GC-MS methods proved to be very effective and sensitive for the separation and detection of complex mixtures of phytoconstituents. GC-MS analysis is the primary step towards understanding the nature of bioactive compounds of plants and this type of analysis will be helpful for further elaborated study (Jain and Rijhwani, 2018).

The identified compounds would have various biological activities. Diisooctyl phthalate (1,2-benzenedicarboxylic acid) was the first constituent with peak area of 17.44%. It is a plasticizer compound known to have antimicrobial properties (Sathish *et al.,* 2012). Diisooctyl phthalate isolates from *Ulva lactuca* and *Eichhornia crassipes* showed antimicrobial activities. Similarly, diisooctyl phthalate isolates from *Nigella glandulifera* possessed antimelanogenetic activities (Roy, 2020). Palmitic acid or n-hexadecanoic acid is a saturated fatty acid compound are known to have anti-inflammatory (Aparna *et al.,* 2012), antioxidant (Yakubu *et al.,* 2017), and anticancer activities (Ravi and Krishnan, 2017). Moreover, it has also nematicide, pesticide, lubricant, antiandrogenic, flavor, hemolytic 5-alpha reductase inhibitor and hypo-cholesterolemic properties (Venkata-Raman *et al.,* 2012). They have antibacterial and antifungal properties. The anti-inflammatory effects might be via decreasing the production of the inflammatory mediators such as prostaglandin E_2, and nitric oxides (Aparna *et al.,* 2012).

It has been reported that isoborneol acrylate [3-(1-Methylhept-1-enyl)-5-methyl-2,5-dihydrofuran-2-one] showed antimicrobial activities (Asressu and Tesema, 2014). Similarly, benzoic acids also prevent bacterial infections (Parka *et al.,* 2001; Alvesalo *et al.,* 2006), and commonly used as antimicrobial preservative in food and beverages, especially in carbonated beverages, as it presents its strongest antibacterial activity (Lin and Huang, 2008). Benzoic acid is used to treat skin irritations and inflammations caused by burns, insect bites, and fungal infections in combination with salicylic acid. Therefore, the presence of various bioactive compounds justifies the use of *G. fruticosus* leaf extract for various ailments in traditional medicine. However, further isolations of individual

constituents and detailed investigations of the pharmacological importance of *G. fruticosus* will be required in our future study.

Limitations of the study

The major limitation of the study is that the retrospective study sample was not a fair representative of the general Ethiopian population since it was based on patients that came to the hospitals seeking treatment; as a result, it may overestimate the prevalence of stone diseases. Furthermore, patient clinical records were taken only from surgically operated patients, and none of relevant data from patients with clinical episodes admitted to the hospital. Also, it is possible that some erroneous and incomplete coding of patients' medical information could have limited the efforts to capture all stone diseases from hospital admitted cases. In addition, since the ESWL treatment performed on outpatients was not included under surgical treatment records, this may have led to under reporting of stone diseases.

There was no observed mortality at dose 2000 mg/kg during acute toxicity studies, although sub-acute toxicity studies were not conducted for some studied plants to ensure safety. DPPH assay was used to test antioxidant activities, although different substances may involve different mechanisms. Data quality may have been compromised by lack of access to the polarized light microscope that magnifies and better resolve the *in vitro* and the *in vivo* crystallizations to differentiate between COM and COD crystals. The effects of test extracts on urinary diuretic activities were not assessed. Moreover, the GC-MS identification of compounds from extracts was based only by retention time and % similarity in NIST (2014) database.

5. Conclusions and Recommendations

5.1. Conclusions

The findings of the retrospective study showed that urinary stone occurrence was increasing and the disease remains a public health problem in Ethiopia. The study demonstrated that hydro-ethanolic extracts of *A. aspera*, *S. punctata*, *R. abyssinicus*, *C. murale*, *A. pulcherrima*, *C. myrrha*, *G. fruticosus*, *C. ambrosioides* and *I. confertiflora* did not cause mortality in experimental rats at 2000 mg/kg in acute and sub-acute toxicity tests. However, the extracts of *C. murale* and *R. abyssinicus* reduced platelet concentrations in acute toxicity. Furthermore, the extracts of *R. abyssinicus*, *A. aspera* and *S. punctata* induced mild injuries to the liver. The information obtained from the present toxicity studies can serve as a baseline for further pharmacological studies on the extracts of the respective traditional medicinal plants.

S. punctata extract exhibited significant antioxidant activities equivalent to ascorbic acid, a drug currently in use. The *in vitro* studies showed that the aqueous extracts of *G. fruticosus*, *S. punctata* and *A. pulcherrima* inhibited CaOx crystallization showing anti-nucleation and anti-aggregation potencies, are relatively higher than cystone and K-Cit, the drugs currently in use. By providing experimental evidences for protective and curative effects of *G. fruticosus*, *S. punctata* and *A. pulcherrima* aqueous extracts against CaOx induced urolithiasis, the study has shown the potential of these traditional medicinal plants in the treatment of urolithiasis.

The *in vivo* findings suggest that the aqueous extract of *S. punctata*, and *G. fruticosus* prevent and treat urolithiasis, respectively. Their extracts significantly reduced the elevated levels of stone promoters in urine, serum, and kidney homogenates. *G. fruticosus* EtOAc

extract was effective against preformed CaOx stones in adult rats, and this was substantiated by the *in vitro* effect of *G. fruticosus* fraction II. The study has provided empirical evidences that substantiates the traditional use of *G. fruticosus* extract for the treatment of urolithiasis.

5.2. Recommendations

1. Further studies on chronic toxicity profiles and determination of the active compounds responsible for the observed antiurolithiatic effects of *G. fruticosus* extracts would be necessary to establish their pharmacological activities. A rigorous chemical and pharmacological study of the active components of *G. fruticosus* will be required to develop it as herbal treatment option to manage kidney stones.

2. Assessment of the effect of *G. fruticosus* extract on their diuretic effects that would facilitate stone passage is essential.

3. It is also vital to investigate the detailed mechanisms underlying the litholytic effects of pure compounds of *G. fruticosus* and elucidate the chemical structures to detect possible novel bioactive molecules that could be used as lead compounds to develop new candidate drugs for the treatment of urolithiasis.

4. Further validation of the anti-urolithiatic efficacy of *G. fruticosus* fraction II will be required through pre-clinical studies.

5. Further research is necessary to identify and purify the active compounds responsible for urolithiatic therapeutic activities.

References

Abate, G. (1989). *Etse Debdabe: Ethiopian traditional medicine* [Amharic version]. 1st`ed. Demise, S., Department of Biology, Science Faculty, Addis Ababa University Press, Addis Ababa, pp.33-183.

Abdel-Mogib, M. and Raghib, H. (2013). Two new pregnane glycoside diesters from *Caralluma russeliana*. *Nat. Prod. Res.* **27**:1287–1292.

Abdissa, D., Geleta, G., Bacha, K. and Abdissa, N. (2017). Phytochemical investigation of *Aloe pulcherrima* roots and evaluation for its antibacterial and antiplasmodial activities. *PLoS One.* **12** (3): e0173882.

Abraham, P.A. and Smith, C.L. (1984). Medical evaluation and management of calcium nephrolithiasis. *Med. Clin. North. Am*. **68** (2):281-99.

Afsar, B., Kiremit, M. C., Sag, A. A.,Tarim, K., Acar, O., Esen, T., *et al.* (2016). The role of sodium intake in nephrolithiasis: epidemiology, pathogenesis, and future directions. *Eur. J. Intern. Med.* **35**:16-19.

Agarwal, K. and Varma, R. (2013). Evaluation of *in vitro* anti-urolithiatic activity of *Portulaca oleracea L.* Pharmanest. *Int. J. Adv. Pharm. Sci.* **4**(6):1284-1293.

Agarwal, A.K., Baerson, S.R., Rogers, P.D., Jacob, M.R., Barker, K.S., Cleary, J.D., *et al.* (2003). Genome-wide expression profiling of the response to polyene, pyrimidine, azole, and echinocandin antifunal agents in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **278**:34998-35015.

Agarwal, K. and Varma, R. (2014a). *In vitro* calcium oxalate crystallization inhibition by *Achyranthes aspera* L. and Bryophyllum pinnatum Lam. *J. Pharm. Res. Int.* **5**(2):146-152.

Agarwal, K. and Varma, R. (2014b). *Ocimum gratissimum L.* A medicinal plant with promising antiurolithiatic activity. *Int. J.Pharm. Sci. Drug Res.* **6:**78-81.

Aggarwal, A., Gupta, S. and Sharma, R.K. (2005). Role of oxidative stress in female reproduction. *Reprod. Biol. Endocrinol*. **3**:28-48.

Aggarwal, A., Tandon, S., Singla, S.K. and Tandon, C.(2010). Reduction of oxalate-induced renal tubular epithelial (NRK-52E) cell injury and inhibition of calcium oxalate crystallization *in vitro* by aqueous extract of *Achyranthes aspera. Int. J. Green Pharm.* **4**(3):159-164.

Aggarwal, K. P., Narula, S., Kakkar, M. and Tandon, C. (2013). Nephrolithiasis: Molecular mechanism of renal stone formation and the critical role played by modulators. *Biomed Res. Int*. Article ID 292953, 21 pages.

Ahmad, B., Jan, Q., Choudhary, M.I. and Nisar, M. (2003). Phytochemical evaluation of *Chenopodium murale* L. *Asian J. Plant Sci.* **2**:1072-1078.

Ahmad, F., Nada, M.O, Farid, A. B., Haleem, M. A. and Razack, S. M. A. (2015). Epidemiology of urolithiasis with emphasis on ultrasound detection: A retrospective analysis of 5371 cases in Saudi Arabia. *Saudi J. Kidney Dis. Transpl*. **26**(2):386-391.

Ahmed, A., Wadud, A., Jahan, N., Bilal, A. and Hajera, S. (2013). Efficacy of *Adiantum capillus veneris* Linn. in chemically induced urolithiasis in rats. *J. Ethnopharmacol.* 1**46**:411-416.

Ahmed, K., Dasgupta, P. and Khan, M. S. (2006). Cystine calculi: challenging group of stones. *Postgrad. Med. J.* **82**(974):799-801.

Ahmed, S., Hasan, M. M. and Alam, Z. M. (2016). *In vitro* urolithiasis models: An evaluation of prophylactic management against kidney stones. *J. Pharmacognosy Phytother.* **5**(3):28-35.

Akanae, W., Tsujihata, M., Yoshioka, I., Nonomura, N. and Okuyama, A. (2010). *Orthosiphon grandiflorum* has a protective effect in a calcium oxalate stone forming rat model. *Urol. Res.* **38**:89-96.

Alam, M.N., Bristi, N.J. and Rafiquzzaman, M.(2012). Review on *in vivo* and *in vitro* methods evaluation of antioxidant activity. *Saudi Pharm. J.* **21:**143-52.

Alatab, S., Pourmand, G., Howairis, M.F.E.L., Buchholz, N., Najafi, I., Pourmand, M.R., *et al.* (2016). National profiles of urinary calculi. A comparison between developing and developed worlds. *Iran J. Kidney Dis.* **10**(2):51-61.

Alaya, A., Belgith, A., Hammadi, S., Nouri, A. and Najjar, M.F. (2012). Kidney stones in children and teenagers in the central coast region of Tunisia. *Iran J. Pedia*. **22**(3): 290-296.

Alaya, A., Nouri, A. and Najjar, M.F. (2009). Urolithiasis in Tunisian children: A study of 100 Cases. *Saudi J. Kidney Dis. Transpl*. **20**(6):1096-1100.

Al-Eisa, A.A., Al-Hunayyan, A. and Gupta, R. (2002). Pediatric urolithisis in Kuwait. *Int. Urol. Nephrol.* **33**(1):3-6.

Alelign, T. and Petros, B.(2018). Kidney stone disease: An update on current concepts. *Adv. Urol.* Volume 2018, Article ID 3068365, 12 pages.

Al-Risi, A.O.Z, Ali, N.M. and Ahuja, A. (2014). Study on prevalence and management of renal stones among Omani in patients at Sohar Hospital. *Sch. J. App. Med. Sci.* **2**(1):22-33.

Alvesalo, J., Vuorela, H., Tammela, P., Leinonen, M., Saikku, P. and Vuorela, P. (2006). Inhibitory effect of dietary phenolic compounds on *Chlamydia pneumoniae* in cell cultures. *Biochem. Pharmacol.* **71**(6):735–741.

Amna, O.F., Nooraain, H., Noriham, A., Azizah, A. and Husna, R.N. (2013). Acute and oral subacute toxicity study of ethanolic extract of *Cosmos caudatus* leaf in Sprague Dawley rats. *Int. J. Biosci. Biochem. Bioinforma*. **3**(4):301-305.

Andrews , F.W. (2009). The flowering plants of Anglo-Egyptian Sudan (1950-1956), 1st ed., Volume 1-III, Arbroath, T. Buncle and Co. Ltd., Arbroath, Scotland, for the Sudan Government.

Ankur, C., Amarchand, P., Aadarsh, C., Deepa, I., Pawar, R.S. and Patil, U. K. (2010). Potential of medicinal plants in kidney, gall and urinary stones. *Int. J. Drug Dev. Res*. **2**(2):431-447.

Aparna, V., Dileep, K. V., Mandal, P. K., Karthe, P., Sadasivan, C. and Haridas, M. (2012). Anti-inflammatory property of n-hexadecanoic acid: Structural evidence and kinetic assessment. *Chem. Biol. Drug Des.* **80**:434-439.

Arsad, S.S., Esa, N.M., Hamzah, H. and Othman, F. (2013). Evaluation of acute, subacute and subchronic oral toxicity of *Rhaphidophora decursiva* (Roxb.) Schott extract in male Sprague Dawley rats. *J. Med. Plant Res.* **7**:3030-3040.

Ashok, P., Coti, B.C. and Vishwanathswamy, A.H.M.(2010). Antiurolithiatic and antioxidant activity of *Mimuscops elengi* on Ethylene glycol induced urolithiasis in rats. *Indian J. Pharmacol*. **42**(6):380-383.

Asressu, K. H. and Tesema, T.K. (2014). Chemical and antimicrobial investigations on essential oil of *Rosmarinus officinalis* leaves grown in Ethiopia and comparison with other countries. *J. App. Pharm.* **6**(2):132-142.

Asselman, M., Verhulst, A., De Broe, M. E. and Verkoelen, C. F. (2003). Calcium oxalate crystal adherence to hyaluronan, osteopontin, and CD44 expressing injured/

regenerating tubular epithelial cells in rat kidneys. *J. Am. Soc. Nephrol.* **14**(12):3155–3166.

Atmani, F. and Khan, S.R. (2000). Effects of an extract from *Herniaria hirsuta* on calcium oxalate crystallization *in vitro*. *BJU International.* **85**:621–625.

Atmani, F. and Khan, S.R. (1999). Role of urinary bikunin in the inhibition of calcium oxalate crystallization. *J. Am. Soc. Nephrol.* **1**:385-8.

Atmani, F., Slimani, Y., Mimouni, M. and Hacht, B. (2003). Prophylaxis of calcium oxalate stones by *Herniaria hirsuta* on experimentally induced nephrolithiasis in rats. *BJU Int.* **92**:137-140.

Atmani, F., Slimani, Y., Mimouni, M., Aziz, M., Hacht, B. and Ziyyat, A. (2004). Effect of aqueous extract from *Herniaria hirsuta* L. on experimentally nephrolithiasic rats. *J. Ethnopharmacol.* **95**:87-93.

Atodariya, U., Barad, R., Upadhyay, S. and Upadhyay, U. (2013). Anti-urolithiatic activity of *Dolichos biflorus* seeds. *J. Pharm. Phytochem.* **2**(2):209-213.

Baheti, D.G. and Kadam, S. S. (2013a). Antiurolithiatic activity of a polyherbal formulation against calcium oxalate induced urolithiasis in rats. *J. Adv. Pharm. Educ. Res.* **3**(1): 61-71.

Baheti, D.G. and Kadam, S.S. (2013b). Antiurolithiatic activity of some traditional medicinal plants against calcium oxalate induced urolithiasis in rats. *Int. J. Pharm. Chem. Biol. Sci.* **3**(4):1276-1285.

Bahuguna, Y., Rawat, M., Juyal, V. and Gnanarajan, G. (2009a). Antilithiatic effect of grains of *Eleusine coracana. Saudi Pharm J.* **17**(2):182-188.

Bahuguna, Y., Rawat, M.S., Juyal, V. and Gupta, V. (2009b). Antilithiatic effect of flowers of *Jasminum auriculatum* Vahl. *Int. J. Green Pharm.* **3**(2):155-158.

Barbasa, C., Garciaa, A., Saavedraa, L. and Muros, M. (2002). Urinary analysis of nephrolithiasis markers. *J Chromatogr. B.* **781**(1-2):433-455.

Barros, M. E., Schor, N. and Biom, M. A. (2003). Effects of an aqueous extract from *Phyllanthus niruri* on calcium oxalate crystallization. *J. Urol.* **30**: 374-379.

Basavaraj, D. R., Biyani, C. S., Browning, A. J. and Cartledge, J. J. (2007). The role of urinary kidney stone inhibitors and promoters in the pathogenesis of calcium containing renal stones. *EAU-EBU Update Series*. **5**(3):126–136.

Bashir, S. and Gilani, A.H. (2009). Antiurolithic effect of *Bergenia ligulata* rhizome: An explanation of the underlying mechanisms. *J. Ethnopharmacol.* **122**:106-116.

Bashir, S. and Gilani, A.H. (2011). Antiurolithic effect of berberine is mediated through multiple pathways. *Eur. J. Pharmacol*. **651**:168–175.

Bashir, S., Gilani, A.H., Siddiqui, A.A., Pervez, S., Khan, S.R., Sarfaraz, N.J., *et al*. (2010). *Berberis vulgaris* root bark extract prevents hyperoxaluria induced urolithiasis in rats. *Phytother. Res.* **24**:1250–1255.

Bawari, S., Sah, A.N. and Tewari, D. (2018). Antiurolithiatic activity of *Daucus carota*: An *in vitro* study. *Pharmacog. J.* **10**(5):880-884.

Begun, F.P. and Knoll, C.E. (2004). Effect of aqueous extract from *Herniaria hirsuta L.* on experimentally nephrolithiasic rats. *J. Ethnopharmacol.* **95**(1):87-93.

Bejar, E., Betz, J.M. and Barrett, M. (2004). Evaluating safety of herbal preparations. ***In***: Barrett, M. The handbook of clinically tested herbal remedies. V1. The Haworth Press, Inc., New York, London, Oxford, pp.1-1503.

Bekele, E. (2007). Study on actual situation of medicinal plants in Ethiopia. *Japan Assoc. Int. Coll. Agric. Fores*, http://www.jaicaf.or.jp/publications/ethiopia ac.pdf.

Belay, M.A. (2014). *Metsafe Adhnote,Tikmu Mulu Tena Lehulu* [Amharic]. Artistic P.E.B/11256 2014. Book of Ethiopian traditional medicine, Addis Ababa, pp.1-35.

Bello, I., Bakkouri, A. S., Tabana, Y. M., Al-Hindi, B., Al-Mansoub, M. A., Mahmud, *et al*. (2016). Acute and sub-acute toxicity evaluation of the methanolic extract of *Alstonia scholaris* stem bark. *Med. Sci.* **4**:4.

Bensatal, M.A. and Ouahrani, M. R. (2008). Inhibition of crystallization of calcium oxalate by the extraction of *Tamarix gallica* L. *Urol. Res.* **36** (6):283–287.

Bhardwaj, S. and Gupta, D. (2012). Study of acute, subacute and chronic toxicity test. *Int. J. Adv. Res. Pharmaceu. BioSci.* **2**:103-129.

Bhaskar, V.H. and Shelke, T.T. (2012). Lithiasis: Causes, evaluation and treatment. *Int. J. Res. Pharm. Biomed.Sci.* **3**(2):468-474.

Binu, T. and Vijayakumari, B.(2013). *Scoparia dulcis* L. as a remedy for urolithiasis. *Int. J. Plant Sci.* **8**(1):163-165.

Boelsterli, U.A. (2003). *Mechanistic Toxicology. The molecular basis of how chemicals disrupt biological targets*. 1st ed. Taylor and Francis Inc., London and New York, pp.1-314.

Borghi, L., Meschi, T., Guerra, A. and Novanni, A. (1993). Randomized prospective study of a non-thiazide diuretic, indapamide, in preventing calcium stones recurrences. *J Cardiovasc. Pharmacol.* **22**:78–86.

Borisov, V.V. and Dzeranov, N.K. (2011). Urolithiasis: therapy of patients with kidney and ureter stones. Moscow: Publishing house of the Russian society of Urologists, pp. 88.

Bortolomeazzi, R., Sebastianutto, N., Toniolo, R. and Pizzariello, A. (2007). Comparative evaluation of the antioxidant capacity of smoke flavoring phenols by crossing bleaching inhibition, DPPH radical scavenging and oxidation potential. *Food Chem.* **100**:1481–1489.

Brand-Williams, W., Cuvelier, M.E. and Berset, C. (1995). Use of a free radical method to evaluate anti-oxidant activity. *Food Sci. Technol.* **28:** 25-30.

Buchholz, N.P., Abbas, F., Afzal, M., Khan, R., Rizvi, I. and Talati, J. (2003). The prevalence of silent kidney stones: An ultrasonographic screening study. *J. Pak. Med. Assoc.* **53**:24-25.

Burkill, H. (1985). The useful Plants of west tropical Africa, Volume 1, families A-D. 2nd ed. Kew: Royal Bot Gardens.

Butterweck, V. and Khan, S. R. (2009). Herbal medicines in the management of urolithiasis: alternative or complementary? *Planta Medica.* **75(**10):1095-1103.

Caesar, L.K. and Cech, N.B. (2019). Synergy and antagonism in natural product extracts: when 1 + 1 does not equal 2. *Nat. Prod. Rep.* **36**: 869.

Cakıroglu, B., Eyyupoglu, E., Hazar, A. I., Uyanik, B. S. and Nuhoglu, B. (2016). Metabolic assessment of recurrent and first renal calcium oxalate stone formers. *Arch. Ital. Urol. Androl.* **88**: 101-105.

Cao, L.C., Jonassen, J., Honeyman, T.W. and Scheid, C. (2001). Oxalate-induced redistribution of phosphatidylserine in renal epithelial cells. Implications for kidney stone disease. *Am. J. Nephrol.* **21**:69 -71.

Casuga, F.P, Castillo, A.L. and Corpuz, M.J-A.T. (2016). GC-MS analysis of bioactive compounds present in different extracts of an endemic plant *Broussonetia luzonica* (Blanco) (Moraceae) leaves, *Asian Pac. J. Trop. Biomed.* **6**(11): 957–961.

Cerini, C., Geider, S., Dussol, B., Hennequin, C., Daudon, M., Veesler, S., *et al.* (1999). Nucleation of calcium oxalate crystals by albumin: involvement in the prevention of stone formation. *Kidney Int.* **55**:1776-1786.

Ch, U., Murthy, P.P.V., Lakshmi, P.S., Mounika, V., Jyothi, N.N. and Anitha, P. (2017). Evaluation and antiurolithiatic activity of *Raphanus sativus* extract by *in vivo* on experimentally induced urolithiasis in rats. *Int. J. Pharm. Pharm. Anal*. **01**(02):71-78.

Chanchal, D.K., Niranjan, P., Alok, S., Kulshreshtha, S., Dongray, A. and Dwivedi, S. (2016). A brief review of medicinal plant and screening method of antilithiatic activity. *Int. J. Pharmacogn.* **3**(1):1-9.

Channa, N.A., Ghangro, A. B., Soomro, A. M. and Noorani, L. (2007). Analysis of kidney stones by FTIR spectroscopy. *J. Liaquat Univ. Med. Health Sci.* 66 -73.

Chaturvedi, L. S., Koul, S., Sekhon, A., Bhandari, A., Menon, M. and Koul, H. K. (2002). Oxalate selectively activates p38 mitogenactivated protein kinase and C-Jun N-terminal kinase signal transduction pathways in renal epithelial cells. *J. Biol. Chem.* **277**(15):13321–13330.

Chaudhary, A., Singla, S. K. and Tandon, C. (2010). *In vitro* evaluation of *Terminalia arjuna* on calcium phosphate and calcium oxalate crystallization. *Indian J. Pharm. Sci.* **72**(3):340-345.

Chauhan, C.K., Joshi, M. J. and Vaidya, A. D. B. (2008). Growth inhibition of struvite crystals in the presence of herbal extract *Commiphora wightii*. *J. Mater. Sci*. **20**(1): 85-92.

Chen, Y.C., Ho, C.Y., Chen, L.D., Hsu, S.F. and Chen, W.C. (2007). Wu-Ling San formula inhibits the crystallization of calcium oxalate *in vitro*. *Am. J. Chin. Med.* **35**:533-541.

Chhiber, N., Sharma, M., Kaur, T. and Singla, S. (2014). Mineralization in health and mechanism of kidney stone formation. *Int. J. Pharm. Sci. Invent.* **3**:25-31.

Chow, K., Dixon, J., Gilpin, S., Kavanagh, J. P. and Rao, P. N. (2004). Citrate inhibits growth of residual fragments in an *in vitro* model of calcium oxalate renal stones. *Kidney Int*. **65**:1724-1730.

Christmas, K.G. (2001). Factors affecting crystallization, dispersion, and aggregation of calcium oxalate monohydrate in various urinary environments, ***In***: *Materials science and engineering, Doctor of Philosophy*, University of Florida, Gainesville, pp.165.

Coe, F. L., Evan, A. and Worcester, E. (2005). Kidney stone disease. *J. Clin. Invest.* **115** (10):2598-2608.

Coe, F. L., Parks, J. H. and Asplin, J. R. (1992). The pathogenesis and treatment of kidney stones. *N. Engl. J. Med*. **327**(16):1141–1152.

Courbebaisse, M., Prot-Bertoye, C., Bertocchio, J., Barona, S., Maruani, G., Briandd, S., *et al.* (2017). Nephrolithiasis of adult: from mechanisms of preventive medical treatment. *Rev. Med. Intern.* **38**(1):44-52.

Cragg, G.M. and Newman, D.J. (2001). Natural product drug discovery in the next millennium. *Pharm. Biol.* **39**:8-17.

Cruzan, C., Corley, R.A., Hard, G.C., Mertens, J.J.W.M., McMartin, K.E., Snellings, W.M., *et al.* (2004). Subchronic toxicity of ethylene glycol in Wistar and F344 rats related to metabolism and clearance of metabolites. *Toxicol Sci*. **81**:502-511.

Cunningham, P., Noble, H., Al-Modhefer, A.-K. and Walsh, I.(2016). Kidney stones: pathophysiology, diagnosis and management. *Br. J. Community Nurs.* **25**(20): 1112-1116.

Curhan, G.C. (2007). Epidemiology of stone disease. *Urol. Clin. North Am.* **34:**287–293.

da Silva, M.G.C., Amorim, R.N.L., Câmara, C.C., Fontenele-Neto, J.D. and Soto-Blanco, B. (2014). Acute and sub-chronic toxicity of aqueous extracts of *Chenopodium ambrosioides* leaves in rats. *J. Med. Food.* **17**: 979-984.

Dal-Moro, F., Mancini, M., Tavolini, I. M., De Marco, V. and Bassi, P. (2005). Cellular and molecular gateways to urolithiasis: A new insight. *Urol. Int.* **74**:193–197.

Das, I., Gupta, S.K., Ansari, S.A., Pandey, V.N. and Rastogi, R.P. (2005). *In vitro* inhibition and dissolution of calcium oxalate by edible plant *Trianthema monogyna* and pulse *Macrotyloma uniflorum* extracts. *J. Cryst. Growth.* **273**:546-554.

Daudon, M., Bazin, D. and Letavernier, E. (2015). Randall's plaque as the origin of calcium oxalate kidney stones. *J. Urol.* **43**:5–11.

Dawson, C. H. and Thomson, C.R.V. (2012). Kidney stone disease: pathophysiology, investigation and medical treatment. *J. Clin. Med*. **12**(5): 467-471.

de-Bruijn, W..C, Boeve, E.R., van Run, P.R., van Miert, P.P., Romijn, J.C., Verkoelen, C.F., Cao, L.C. and Schröder, F.H. (1994). Etiology of experimental calcium oxalate monohydrate nephrolithiasis in rats. *Scanning Microsc*. **8**:541-9.

Deganello, S. (1986). Phase transitions of calcium oxalate trihydrate and epitaxy in the weddellite-whewellite system. *Scanning Electron Microsc.* **4**:1721-1728.

Demissew, S., Friis, I., Awas, T., Wilkin, P., Weber, O., Bachman, S., *et al.* (2011). Four new species of *Aloe (*Aloaceae*)* from Ethiopia, with notes on the ethics of describing new taxa from foreign countries. *Kew Bull.* **66**:111-121.

Deo, R., Jain, Y.S.K., Alok, S., Mahor, A., Bharti, J.P. and Jaiswal, M. (2011). Herbal plants used in the treatment of urolithiasis. *Int. J. Pharm. Sci. Res.* **2**(6):1412-1420.

de-Water, R., Boeve, E.R., van Miert, P.P., Deng, G., Cao, L.C., Stijnen, T., *et al.* (1996). Experimental nephrolithiasis in rats: the effect of ethylene glycol and vitamin D_3 on the induction of renal calcium oxalate crystals. *Scanning Microsc.* **10**:591–601.

Diallo, D., Paulsen, B.S. and Hvemm, B. (1996). Production of traditional medicine: preparations accepted as medicines in Mali. ***In:*** Hostettmann, K., Chinyanganya, F., Maillard, M. and Wolfender, J.-L. (ed.) *Chemistry, biological and pharmacological properties of African medicinal plants*: *Proceedings of the first International IOCD-Symposium,* Victoria falls, Zimbabwe, February 25-28 Harare: UZ Publications, pp. 235-243.

Dinnimath, B.M. and Jalalpure, S.S. (2012). Spectral analysis of fractions of *Aerva lanata* (L). *Indian J. Nat. Prod*. **28(**2):14-18.

Dinnimath, B.M. and Jalalpure, S.S. (2018). Antioxidant and antiurolithiatic efficacy of *Aerva lanata* (L) fractions by *in vitro* and *in vivo* screening techniques. *Indian J. Pharm. Educ. Res.* **52**(3):426-436.

Divakar, K., Pawar., A.T., Chandrasekhar, S.B., Dighe, S.B. and Divakar, G.(2010). Protective effect of the hydro-alcoholic extract of *Rubia cordifolia* roots against ethylene glycol induced urolithiasis in rats. *Food Chem. Toxicol.* **48**:1013-8.

Doddola, S., Pasupulati, H., Koganti, B. and Prasad, K.V.S. R. G. (2008). Evaluation of *Sesbania grandiflora* for antiurolithiatic and antioxidant properties. *J. Nat. Med*. **62**:300-307.

Dog, T.L. (2004). Determining efficacy of herbal preparations. ***In:*** Barrett, M. *The Handbook of clinically tested herbal remedies.* Volume 1. The Haworth press, Inc., New York, London, Oxford, pp.1-1503.

Dulanjali, S.S. and Srikaran, R. (2020). Anti-urolithiatic activity of extract of *Andrographis paniculata* plant on calcium oxalate crystals: An *in vitro* preliminary study. *J. Pharm. Innov*. https://doi.org/10.1007/s12247-020-09501-w.

Durkee, C.T. and Balcom, A. (2006). Surgical management of urolithiasis. *Pediatr. Clin. N. Am.* **53**: 465- 477.

Dursun, I., Poyrazoglu, H. M., Dusunsel, R., Gunduz, Z., Gurgoze, M. K., Demirci, D., *et al.* (2008). Pediatric urolithiasis: An 8-year experience of a single center. *Int. Urol. Nephrol.* **40**:3-9.

Dursun, M., Otunctemur, A. and Ozbek, E. (2015). Kidney stones and Ceftriaxone. *Eur. Med. J. Urol.* **3**(1): 68–74.

Dybing, E., Doe, J., Groten, J., Kleiner, J., O'brien, J., Renwick, *et al.* (2002). Hazard characterization of chemicals in food and diet: Dose response, mechanisms and extrapolation issues. *Food Chem. Toxicol.* **40**:37-282.

Edvardsson, V. O., Indridason, O. S., Haraldsson, G., Kjartansson, O. and Palsson, R. (2013). Temporal trends in the incidence of kidney stone disease. *Kidney Int*. **83**(1):146-152.

Edwards, S., Demissew, S. and Hedberg, I. (1997). Flora of Ethiopia and Eritrea.Volume 6: Hydrocharitaceae to Arecaceae. The national Herbarium, Addis Ababa University, Addis Ababa, Ethiopia and Department of Systematic Botany, Uppsala, Sweden. pp.128.

Edwards, S., Tadesse, M., Demissew, S. and Hedberg, I. (2000). Flora of Ethiopia and Eritrea. Volume 2, part 1. Magnoliaceae to Flacourtiaceae. The national Herbarium, Addis Ababa University, Addis Ababa, Ethiopia and Department of Systematic Botany, Uppsala, Sweden. pp.283-340.

Elsebai, M. and Mohamed, I. (2015). New pregnane glycoside derivative from *Caralluma retrospiciens* (Ehrenb). *Nat. Prod. Res.* **29**:1426-1431.

Elsewefy, D.A., Farweez, B.A. and Ibrahim, R.R. (2014). Platelet indices: Consideration in thrombocytopenia. *Egypt J. Haematol*. **3**(39):134-138.

El-Shall, H., Jeon, J.-h., Abdel-Aal, E. A., Khan, S. R., Gower, L. and Rabinovich, Y. I. (2004). A study of primary nucleation of calcium oxalate monohydrate-II. Effect of urinary species. *Cryst. Res. Technol.* **39**: 222-229.

Elzagallaai, A.A., Koren, G. and Rieder, M.J. (2013). The predictive value of the *in vitro* platelet toxicity assay (iPTA) for the diagnosis of hypersensitivity reactions to sulfonamides. *J. Clin. Pharmacol.* **53**(6): 626-32.

Elzbieta, S., Sulkowski, S., Koda, M., Zalewski, B., Kanczuga-Koda, L. and Sulkowska, M. (2005). Lipid peroxidation and antioxidant status in colorectal cancer. *World J. Gastroenterol.* **11**(3):403-406.

El-Zoghby, Z.M., Lieske, J.C., Foley, R.N., Bergstralh, E.J., Li, X., Melton 3rd, L.J., *et al.* (2012). Urolithiasis and the risk of ESRD. *Clin. J. Am. Soc. Nephrol.* **9**:1409-1415.

Erickson, S.B., Vrtiska, T.J. and Lieske, J.C.(2011). Effect of cystone on urinary composition and stone formation over a one year period. *Phytomed.* **18:**863-86.

Evan, A. P. (2010). Physiopathology and etiology of stone formation in the kidney and the urinary tract. *Pediatr. Nephrol*. **25**(5): 831–841.

Evan, A., Lingeman, J., Coe, F. and Worcester, E. (2006). Randall's plaque: pathogenesis and role in calcium oxalate nephrolithiasis. *Kidney Int.* **69**(8):1313-1318.

Evan, A.P., Lingeman, J.E., Coe, F.L., Parks, J.H., Bledsoe, S.B., Shao, Y., *et al*. (2003). Randall's plaque of patients with nephrolithiasis begins in basement membranes of thin loops of Henle. *J. Clin. Invest.* **111**(5):607-616.

Evan, A.P., Worcester, E.M., Coe, F.L., Williams, J. Jr. and Lingeman, J.E. (2015). Mechanisms of human kidney stone formation. *Urolith.* **43** (Suppl.1):19-32.

Fagali, N. and Catal, A. (2008). Antioxidant activity of conjugated linoleic acid isomers, linoleic acid and its methyl ester determined by photoemission and DPPH techniques. *Biophys. Chem*. **137**:56-62.

Fan, J., Micheal, A., Paramjit, G. and Chandhoke, S. (1999). Impact of ammonium chloride administration on rat ethylene glycol urolithiasis models. *Scanning Microscopy Int.* **13**:299-306.

Fasano, J.M. and Khan, S.R. (2001). Intra-tubular crystallization of calcium oxalate in the presence of membrane vesicles: An *in vitro* study. *Kidney Int.* **59**:169-178.

Ferreira, P., Cardoso, T., Ferreira, F., Fernandes-Ferreira, M., Piper, P. and Sousa, M.J. (2014). *Mentha piperita* essential oil induces apoptosis in yeast associated with both cytosolic and mitochondrial ROS-mediated damage. *FEMS Yeast Res.* **14**(**7**):1006-1014.

Fichtl, R. and Adi, A. (1994). Honey bee flora of Ethiopia, pp.1-122.

Finlayson, B. (1974). Symposium on renal lithiasis. Renal lithiasis in review. *Urol. Clin. North Am.* **1**:181–212.

FMoH. (2013). Federal Ministry of Health: Health and health related indicators, Ethiopian Fiscal year 2005 (Version 2): pp.1-68.

FMoH. (2015). Federal Ministry of Health: Health and Health Related Indicator, Ethiopian Fiscal Year 2008 (Version 1): pp.1-66.

Foti, M.C., Daquino, C. and Geraci, C. (2004). Electron-transfer reaction of cinnamic acids and their methyl esters with the DPPH radical in alcoholic solutions. *J. Org. Chem.* **69:**2309-2314.

Fouada, A., Yamina, S., Nait, M.A., Mohammed, B. and Abdlekrim, R. (2006). *In vitro* and *in vivo* antilithiasic effects of saponin rich fraction isolated from *Herniaria hirsute*. *J. Bras. Nefrol.* **28**(4):199-203.

Frassetto, L. and Kohlstadt, I. (2011). Treatment and prevention of kidney stones: An update. *Am Fam Physician*. **84**(11):1234-1242.

Freitas, A.M., Schor, N. and Boim, M.A.(2002).The effect of *Phyllanthus niruri* on urinary inhibitors of calcium oxalate crystallization and other factors associated with renal stone formation. *BJU Int*. **89**:829-834.

Gabow, P.A., Clay, K., Sullivan, J.B. and Lepoff, R.(1986). Organic acids in ethylene glycol intoxication. *Ann. Intern. Med*. **105**:16-20.

Gadanya, A., Sule, M. and Atiku, M. (2011). Acute toxicity study of "GADAGI" Tea on rats. *Bayero J. Pur. Appl. Sci*. **4:**147-149.

Gahlot, M., Bhatt, P. and Joshi, J. (2018). Study on yield of plant extracts using different solvents and methods. *Bull. Env. Pharmacol. Life Sci.* **7**(6):65-67.

Gandhare, B., Kavimani, S. and Rajkapoor, B.(2013). Acute and sub-acute toxicity study of methanolic extract of *Ceiba pentandra* (Linn.) Gaertn. on rats. *Int. J. Sci. Res.* **5**: 315-324.

Garimella, T.S., Jolly, C.I. and Narayanan, S. (2001). *In vitro* Studies on Antilithiatic Activity of Seeds of *Dolichos biflorus* Linn. and Rhizomes of *Bergenia ligulata* Wall. *Phytothera. Res.* **15**:351–355.

Gavamukulya, Y., Abou-Elella, F., Wamunyokoli, F., Hany A. and El-Shemy, H. (2015). GC-MS analysis of bioactive phytochemicals present in ethanolic extracts of leaves of *Annona muricata*: A further evidence for its medicinal diversity. *Pharmacogn. J*. **7**(5): 300-304.

Gecit, I., Kavak, S., Oguz, E.K., Pirincci, N., Gunes, M., Kara, M., *et al.* (2014). Tissue damage in kidney, adrenal glands and diaphragm following extracorporeal shock wave lithotripsy. *Toxicol. Ind. Health.* **30**:845.

Getie, M., Gebre-Mariam, T., Rietz, R., Hohne, C., Huschka, C., Schmidtke, M., *et.al.* (2003). Evaluation of the anti-microbial and antiinflammatory activities of the medicinal plants *Dodonaea viscosa*, *Rumex nervosus* and *Rumex abyssinicus*. *Fitoterapia*. **74** (1-2):139-143.

Ghadirkhomi, A., Safaeian, L., Zolfaghari, B., Aghaye-Ghazvini, M.R. and Rezaei, P.(2016). Evaluation of acute and sub-acute toxicity of *Pinus eldarica* bark extract in Wistar rats. *Avicenna J. Phytomed.* **6**(5):558-566.

Ghale-Salimi, M.Y., Eidi, M., Ghaemi, N. and Khavari-Nejad, R.A. (2017). Antiurolithiatic effect of the taraxasterol on ethylene glycol induced kidney calculi in male rats. *Urolith.* https://doi.org/10.1007/s00240-017-1023-9.

Ghelani, H., Chapala, M. and Jadav, P. (2016). Diuretic and antiurolithiatic activities of an ethanolic extract of *Acorus calamus L*. rhizome in experimental animal models. *J. Tradit. Complement Med.* **6:**431-436.

Giannossi, L. and Summa, V. (2012). A review of pathological biomineral analysis techniques and classification schemes. **In*:*** *An Introduction to the study of*

mineralogy, Aydinalp, C., ed., InTech Open, InTech, IMAA-CNR, Italy, http://www. intechopen.com/books/.

Giday, M. and Teklehaymanot, T. (2013). Ethnobotanical study of plants used in management of livestock health problems by the Afar people of Ada'ar district, Afar regional state, Ethiopia. *J. Ethnobiol. Ethnomed.* **9**:8.

Gilhotra, U. K. and Christina, A.J.M. (2011). Effect of *Rotula aquatica* Lour. on ethylene-glycol induced urolithiasis in rats. *Int. J. Drug Dev. Res.* **3**(1):273-280.

Gnessin, E., Lingeman, J.E., Andrew, P. and Evan, A.P. (2010). Pathogenesis of renal calculi. *Turk. J. Urol.* **36**:190-199.

Goyder, D.J. and Nicholas, A.(2001). A revision of Gomphocarpus (Apocynaceae: Asclepiadeae). *Kew Bulletin.* **56**:769-836.

Grases, F., Costa-Bauzá, A., Ramis, M., Montesinos, V. and Conte, A. (2002). Simple classification of renal calculi closely related to their micromorphology and etiology. *Clin. Chim. Acta.* **322**:29-36.

Green, M.L., Hatch, M. and Freel, R.W. (2005). Ethylene glycol induces hyperoxaluria without metabolic acidosis in rats. *Am. J. Physiol. Renal Physiol.* **289**:536–543.

Griffith, D. P. (1978). Struvite stones. *Kidney Int*. **13**(5):372–382.

Guengerich, F.P. (2011). Mechanisms of drug toxicity and relevance to pharmaceutical development. *Drug Metab. Pharmacokinet*. **26**(1):3-14.

Gurib-Fakim, A. and Mahomoodally, M. (2013). African flora as potential sources of medicinal plants: towards the chemotherapy of major parasitic and other infectious diseases-a review. *Jordan J. Biol. Sci.* **6**:77–84.

Gutteridge, J.M.C. (1994). Biological origin of free radicals, and mechanisms of anti-oxidant protection. *Chem. Biol. Interact.* **91**(2-3):133-140.

Habermann, T.M. (2006). Mayo clinic internal medicine review 7th ed. Mayo clinic scientific press and informa healthcare, USA, INC.2006-2007, pp. 684.

Hackett, R. L., Shevock, P. N. and Khan, S. R. (1995). Alterations in MDCK and LLC-PK1 cells exposed to oxalate and calcium oxalate monohydrate crystals. *Scanning Microsc*. **9**(2):587-596.

Hackett, R., Shevock, P. and Khan, S. (1994). Madin-Darby canine kidney cells are injured by exposure to oxalate and to calcium oxalate crystals. *Urol. Res.* **22**(4):197–203.

Halliwell, B. (2007). Dietary polyphenols: Good, bad, or indifferent for your health?". *Cardiovasc. Res.* **73**(2): 341–7.

Hammarsten, G. (1929). On calcium oxalate and its solubility in the presence of inorganic salts with special reference to the occurrence of oxaluria. *Carlsberg Lab Carls.* **17**:1-83.

Hassan, I. and Zietlow, S.P. (2002). Acute pancreatitis after extracorporeal shock wave lithotripsy for a renal calculus. *Urol.* **60**:1111–1113.

Havagiray, R., Shashi, A.C., Jain, S.K. and Monika, S. (2010). Herbal treatment for urinary stones. *Int. J. Pharm. Sci. Res.* **1**(2):1412-1420.

Hawkins, R.I. (1972). The importance of platelet function tests in toxicological screening using laboratory animals. *Lab. Anim.* **6:**155-167.

Heaney, R.P. (2008).Vitamin D, and calcium interactions: functional outcomes. *Am. J. Clin. Nutr.* **88**(Suppl):541-4.

Hedberg, I. and Edwards, S. (1989). Flora of Ethiopia. Volume 3. Pittosporaceae to Araliaceae. The national Herbarium, Addis Ababa University, Addis Ababa, Ethiopia and Department of Systematic Botany, Uppsala, Sweden. pp.452.

Hedberg, I., Edwards, S. and Nemomissa, S. (2003). Flora of Ethiopia and Eritrea. Volume 4, part 1. Apiaceae to Dipsacaceae. The national Herbarium, Addis Ababa, Ethiopia, and Department of Systematic Botany, Uppsala, Sweden.pp.123-124.

Hedberg, I., Friis, I. and Edwards, S. (2004). Flora of Ethiopia and Eritrea. Volume 4, part 2. Asteraceae (Compositae). The national Herbarium, Addis Ababa, Ethiopia, and Department of Systematic Botany, Uppsala, Sweden.pp.122.

Hedberg, I., Kelbessa E., Edwards, S., Demissew, S. and Persson E. (2006). Flora of Ethiopia and Eritrea.Volume 5. Gentianaceae to Cyclocheilaceae. The national Herbarium, Addis Ababa University, Addis Ababa,Ethiopia and Department of Systematic Botany, Uppsala, Sweden. pp.516-557.

Heilberg, I.P. and Schor, N. (2006). Renal stone disease: Causes, evaluation and medical treatment. *Arq. Bras. Endocrinol. Metabol.* **50**(4**)**:823-833.

Hennequin, C., Lalanne, V., Daudon, M., Lacour, B. and Drueke, T. (1993). A new approach to studying inhibitors of calcium oxalate crystal growth. *Urol. Res.* **21:**101–8.

Hennequin, C., Tardivel, S., Medetognon, J., Drueke, T., Daudon, M. and Lacour, B.(1998). A stable animal model of diet-induced calcium oxalate crystalluria. *Urol. Res.* **26**:57-63.

Hess, B., Meinhardt, U., Zipperle, L., Giovanoli, R. and Jaeger, P. (1995). Simultaneous measurements of calcium oxalate crystal nucleation and aggregation: Impact of various modifiers. *Urol. Res.* **23(**4):231–238.

Hess, B., Nakagawa, Y. and Coe, F.L. (1989). Inhibition of calcium oxalate monohydrate crystal aggregation of urine proteins. *Am. J. Physiol.* **257**:99-106.

Hess, R., Bartels, M.J. and Pottenger, L.H. (2004). Ethylene glycol: An estimate of tolerable levels of exposure based on a review of animal and human data. *Arch. Toxicol.* **78**: 671-680.

Hesse, A., Brandle, E., Wilbert, D., Kohrmann, K.U. and Alken, P. (2003). Study on the prevalence and incidence of urolithiasis in Germany comparing the years 1979 vs 2000. *Eur. Urol.* **44**(6):709-713.

Hirose, M., Yasui, T., Okada, A., Hamamoto, S., Shimizu, H., Itoh, Y., *et al.* (2010). Renal tubular epithelial cell injury and oxidative stress induce calcium oxalate crystal formation in mouse kidney. *Int. J. Urol.* **17**: 83-92.

Hoetelmans, R.M. (2017). IC_{50} versus EC_{50}. PK-PD relationships for anti-retroviral drugs. Amsterdam: Slotervaart Hospital. Archived from the original on 2020-12-12 via U.S. Food and Drug Administration. http://en.wikipedia.org/wiki/IC50.

Hossain, M.A., AL-Raqmi, K. A., AL-Mijizy, Z.H., Weli, A.M. and Al-Riyami, Q. (2013). Study of total phenol, flavonoids contents and phytochemical screening of various leaves crude extracts of locally grown *Thymus vulgaris*. *Asian Pac. J. Trop. Biomed*. **3**(9):705-10.

Hounnasso, P.P., Avakoudjo, J.D.G., Paré, A.K., Brahima, K., Ouattara, A., Agounkpé, M.M., *et al.* (2015). Symptomatic urinary lithiasis: Epidemiology and management at urology, Department of University Hospital of Cotonou. *J. Urol.* **5**:7-12.

Hu, F.L., Lu, R.L., Huang, B. and Ming, L.(2004). Free radical scavenging activity of extracts prepared from fresh leaves of selected Chinese medicinal plants. *Fitoterapia.* **75**:14-23.

Huang, D., Ou, B. and Prior, R. (2005). The chemistry behind antioxidant capacity assays. *J. Agric. Food Chem.* **53**:1841-1856.

Ibrahim, F. Y. and El-Khateeb, A.Y. (2013). Effect of herbal beverages of *Foeniculum vulgare* and *Cymbopogon proximus* on inhibition of calcium oxalate renal crystal formation in rats. *Ann. Agric. Sci.* **58**(2):221-229.

Ighodaro, O.M. and Akinloye, O.A.(2018). First line defense antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defense grid. *Alexandria J. Med*. **54:**287-293.

Iguchi, M., Takamura, C., Umekawa, T., Kurita, T. and Kohri, K. (1999). Inhibitory effects of female sex hormones on urinary stone formation in rats. *Kidney Int.* **56**:479-485.

Indridason, O.S., Birgisson, S., Edvardsson, V.O., Sigvaldason, H., Sigfusson, N. and Palsson, R. (2006). Epidemiology of kidney stones in Iceland: A population-based study. *Scand. J. Urol. Nephrol.* **40:**215-20.

Iwasaki, S. (1998). Natural organic compounds that affect to microtubule functions. Yakugaku Zasshi, **118** (4):112-26.

Jaganathan, R., Ravinayagam, V., Panchanadham, S. and Palanivelu, S. (2012). Toxicological, biochemical and histopathological evaluation of Tridham, a Siddha medicine in Wistar albino rats. *J. Biochem. Technol.* **4**(1):541-548.

Jahan, N., Bano, H., Makbul, S.A., Kumar, B.N. and Mushir, A. (2019). Effects of hydroalcoholic extract of *Cyperus rotundus* L. rhizome against ethylene glycol and ammonium chloride-induced urolithiasis in male Sprague-Dawley rats. *Urol. Sci.* **30**:99-106.

Jain, P,K, and Rijhwani, S. (2018). Comparative GC-MS analysis of *Cyamopsis tetragonoloba* fruit extracts. *Int. J. Pharm. Sci. Res.* **9**(10):4236-42.

Jan, G., Khan, M.A. and Jan, F. (2009). Medicinal value of the Asteraceae of Dir Kohistan valley, NWFP, Pakistan. *J. Ethnob. Leaflets.***13**:1205-1215.

Jayaramaiah, K.K., Anturlikar, S.D., Viswanatha, G.L., Agadihiremath, T., Patki, P.S. and Rafiq, M. (2013). Cystone, a well-known herbal formulation, inhibits struvite crystal growth formation in single diffusion gel growth technique. ***J. Exp. Integr. Med***. **3**(1):51-55.

Jeong, B.C., Kim, B.S., Kim, J.I. and Kim, H.H. (2006). Effects of green tea on urinary stone formation: An *in vivo* and *in vitro* study. *J. Endourol.* **20**:356-61.

John, J.A. and Shahidi, F. (2010). Phenolic compounds and antioxidant activity of Brazil nut (*Bertholletia excelsa*). *J. Funct. Foods.* **2**:196-209.

Johri, N., Cooper, B., Robertson, W., Choong, S., Rickards, D. and Unwin, R. (2010). An update and practical guide to renal stone management. *Nephron. Clin. Pract.* **116**:c159–c171.

Joseph, K. C., Bharat, B., Parek, H. and Joshi, M. J. (2005). Inhibition of growth of urinary type calcium hydrogen phosphate dihydrate crystals by tartaric acid and tamarind, *Curr. Sci.* **88**:1232-1238.

Kaito, K., Otsubo, H., Usui, N., Yoshida, M., Tanno, J., Kurihara, E., *et al.* (2005). Platelet size deviation width, platelet large cell ratio, and mean platelet volume have sufficient sensitivity and specificity in the diagnosis of immune thrombocytopenia. *Br. J. Haematol.* **128**:698-702.

Kajander, E.O. and Ciftcioglu, N. (1998). Nanobacteria: An alternative mechanism for pathogenic intra-and extracellular calcification and stone formation. *Proc. Natl. Acad. Sci.* **95:**8274-8279.

Kalaisezhiyen, P. and Sasikumar, V. (2012). GC- MS evaluation of chemical constituents from methanolic leaf extract of *Kedrostis foetidissima* (Jacq.) cogn, *Asian J. Pharm. Clin. Res.* **5**(4):77-81.

Kale, O.E., Awodele, O. and Akindele, A.J. (2019). Sub-acute and subchronic oral toxicity assessments of *Acridocarpus smeathmannii* (DC.) Guill. and Perr. Root in Wistar rats. *Toxicol. Rep.* **6**:161-175.

Kalpana, S., Nirmaladevi, R., Shrinidhi, Rai, T. and Karthika, P. (2013). Inhibition of calcium oxalate crystallization *in vitro* by extract of banana cultivar monthan. *Int. J. Pharm. Pharm. Sci.* **5**:649-53.

Kalyan, S., Betanabhatla, B., Christina, A.J.M. and Syama, B.M. (2009). Antilithiatic activity of *Hibiscus sabdariffa* linn. on ethylene glycol induced lithiasis in rats. *Nat. Prod. Rad.* **8**(1):43-47.

Karadi, R.V., Gadge, N.B., Alagawadi, K.R. and Savadi, R.V. (2006). Effect of *Moringa oleifera* Lam. root-wood on ethylene glycol induced urolithiasis in rats. *J. Ethno. pharmacol*. **105**:306-311.

Karamakar, D. and Patki, P. (2008). Evaluation of efficacy and safety of herbal formulation cystone in the management of urolithiasis: Meta-analysis of 50 clinical studies. *Internet J. Alternat. Med.* **8**(1):1-10.

Kato, Y., Yamaguchi, S., Yachiku, S., Nakazono, S., Hori, J., Wada, N. and Hou, K.(2004). Changes in urinary parameters after oral administration of potassium-sodium citrate and magnesium oxide to prevent urolithiasis. *Urol.* **63**(1):7-11.

Katzung, B.G., Masters, S.B. and Trevor, A.J. (2004). *Basic and Clinical Pharmacology.* 9th ed. New York: McGrawHill Publishing Co. pp.1202. ISBN 10: 0071219315.

Kaushik, J., Tandon, S., Gupta, V., Nayyar, J., Singla, S.K. and Tandon, C. (2017). Response surface methodology based extraction of *Tribulus terrestris* leads to an upsurge of antilithiatic potential by inhibition of calcium oxalate crystallization processes. *PLoS One*, **12**(8): e0183218.

Kavanagh, J.P., Jones, L. and Rao, P.N. (2000). Calcium oxalate crystallization kinetics studied by oxalate-induced turbidity in fresh human urine and artificial urine. *Clin. Sci.* (Lond.), **98**:151-158.

Keo, S., Meng, C., Oeung, S., Nov, V., Auon Lon, S., Vichet,T., Va, T., Sourn, M. and Chea, S. **(**2017). Preliminary phytochemical screening of selected medicinal plants of Cambodia. *Asian J. Pharm. Pharmacol.* **1**(4):16-23.

Khan, A. (2018). *In vitro* and *in vivo* models for the study of urolithiasis. *Urol. J.* 1-5.

Khan, A., Khan, S.R. and Gilani, A.H. (2012). Studies on the *in vitro* and *in vivo* antiurolithic activity of *Holarrhena antidysenteric. Urol. Res.* **40:**671-681.

Khan, N.I., Shinge, J.S. and Naikwade, N.S. (2010). Antilithiatic effect of *Helianthus Annuus* Linn. Leaf extract in ethylene glycol and ammonium chloride induced nephrolithiasis. *Int. J. Pharm. Pharm. Sci.* **2**(4):180-184.

Khan, S. and Thamilselvan, S.(1999). Nephrolithiasis: A consequence of renal epithelial cell exposure to oxalate and calcium oxalate crystals. *Mol. Urol.* **4**:305-312.

Khan, S. R. (1995a). Experimental calcium oxalate nephrolithiasis and the formation of human urinary stones. *Scanning Microsc.* **9**(1):89-101.

Khan, S. R. (2004). Crystal induced inflammation of the kidneys results from human studies, animal models and tissue culture studies. *J. Clin. Exp. Nephrol*. **8**(2):75-88.

Khan, S. R. and Canales, B.K. (2009). Genetic basis of renal cellular dysfunction and the formation of kidney stones. *Urol. Res.* **37**(4**)**:169-180.

Khan, S. R., Atmani, F., Glenton, P., Hou, Z.C., Talham, D. R. and Khurshid, M. (1996). Lipids and membranes in the organic matrix of urinary calcific crystals and stones. *Calcif. Tissue Int.* **59**:357-365.

Khan, S. R., Pearle, M. S., Robertson, W. G., Gambaro, G., Canales, B.K., Doizi, S., *et al.* (2016). Kidney stones. *Nat. Rev. Dis. Primers.* **2**:16008.

Khan, S. R., Shevock, P. N. and Hackett, R. L.(1992). Acute hyperoxaluria, renal injury and calcium-oxalate urolithiasis. *J. Urol.* **147**(1):226-230.

Khan, S.R. (1995b). Calcium oxalate crystal interaction with renal tubular epithelium, mechanism of crystal adhesion and its impact on stone formation. *Urol. Res.* **23**:71-79.

Khan, S.R. (2013a). Animal models of calcium oxalate kidney stone formation. ***In****:* Conn PM (ed.) Animal models for the study of human disease, Cambridge, MA: Academic Press, pp.483-498.

Khan, S.R. (2013b). Reactive oxygen species as the molecular modulators of calcium oxalate kidney stone formation: Evidence from clinical and experimental investigations. *J. Urol.* 803-811.

Khan, S.R. and Hackett, R.L. (1985). Calcium oxalate urolithiasis in the rat: Is it a model for human stone disease? A review of recent literature. *Scan Electron Microsc.* **2**:759-774.

Khan, S.R. and Kok, D.J. (2004). Modulators of urinary stone formation. *Front. Biosci*. **9**(1-3):1450-1482.

Khan, S.R.(1997). Interactions between stone forming calcific crystals and macromolecules. *Urol. Int.* **59**(2):59-71.

Khan, S.R.(2014). Reactive oxygen species, inflammation and calcium oxalate nephrolithiasis. *Transl. Androl. Urol.* **3**:256-276.

Khan, S.R., Glenton, P.A. and Byer, K.J. (2006). Modeling of hyperoxaluric calcium oxalate nephrolithiasis: Experimental induction of hyperoxaluria by hydroxy-L-proline. *Kidney Int.* **70**: 914-923.

Khan, S.R., Johnson, J.M., Peck, A.B., Cornelius, J.G. and Glenton, P.A. (2002). Expression of osteopontin in rat kidneys: Induction during ethylene glycol induced calcium oxalate nephrolithiasis. *J. Urol.* **168**: 1173-81.

Khan, Z.A., Assiri, A.M., Al-Afghani, H.M. and Maghrbi, T.M. (2001). Inhibition of oxalate nephrolithiasis with *Ammi visnaga* (Alkhillah). *Int. Urol. Nephrol.* **33**(4): 605-608.

Khare, P, Mishra, VK, Arun, K, Bais, N. and Singh, R. (2014). Study on *in vitro* anti-lithiatic activity of *phyllanthus niruri* Linn. Leaves by homogenous precipitation and turbiditory method. *Int. J. Pharma. Pharmaceut. Sci.* **6**(4):124-127.

Kifayatullah, M., Rahim, H., Jan, N., Abbas, S., Khan, M.S. and Ikram, M. (2019). Anti-urolithiatic effect of *Pericampylus glaucus* against Ethylene glycol induced urolithiasis in male Sprague Dawley rats. *Sains Malays.* **48**(5):1075-1081.

Kifayatullah, M., Senguptha, P., Mustafa, M.S., Das, S.K. and Sisugoswomi, M. (2015). Evaluation of ethanolic extract of *Pericampylus glaucus* (Lamk.) Merr for total phenolic, total flavonoids contents and *in vitro* anti-oxidant activity. *Int. J. Pharmacogn. Phytochem. Res.* **7**: 677-683.

Kishore, D.V., Moosavi, F. and Varma, D.R.K. (2013). Effect of Ethanolic extract of *Portulaca oleracea* linn. on ethylene glycol and ammonium chloride induced urolithiasis. *Int. J. Pharm. Pharm. Sci.* **5**(2):134-140.

Knoll, T. (2007). Stone disease. *Eur. Urol. Suppl.* **6**:717-722.

Knoll, T. (2010). Epidemiology, pathogenesis and pathophysiology of urolithiasis. *Eur. Urol. Suppl.* **9**(12):802-806.

Kok, D. J. and Khan, S. R. (1994). Calcium oxalate nephrolithiasis, a free or fixed particle disease. *Kidney Int.* **46**(3):847–854.

Kok, D. J., Papapoulos, S. E. and Bijvoet, O. L. (1990). Crystal agglomeration is a major element in calcium oxalate urinary stone formation. *Kidney Int.* **37**(1):51-56.

Kok, D. J., Papapoulos, S. E. and Bijvoet, O. L. M. (1986). Excessive crystal agglomeration with low citrate excretion in recurrent stone-formers. *Lancet.* **327**(8489):1056-1058.

Komansilan, A., Abadi, A.L., Yanuwiadi, B. and Kaligis, D.A.(2012). Isolation and identification of biolarvicide from Soursop (*Annona muricata* Linn) seeds to mosquito (*Aedes aegypti*) Larvae. *Int. J. Eng. Technol.* **12**(03):28-32.

Kore , C., Tadesse, A., Teshome, B., Daniel, K., Kassa, A. and Ayalew, D. (2018). The magnitude of chronic kidney disease and its risk factors at Zewditu Memorial Hospital, Addis Ababa, Ethiopia. *J. Nephrol. Ther.* **8**:3.

Krambeck, A. E., Handa, S. E., Evan, A. P. and Lingeman, J. E.(2010a). Profile of the brushite stone former. *J. Urol.* **184**(4):1367-1371.

Krambeck, A. E., Handa, S. E., Evan, A.P. and Lingeman, J. E. (2010b). Brushite stone disease as a consequence of lithotripsy? *Urol. Res.* **38**(4):293-299.

Krambeck, A.E., Gettman, M.T., Rohlinger, A.L., Lohse, C.M., Patterson, D.E. and Segura, J.W. (2006). Diabetes mellitus and hypertension associated with shock wave lithotripsy of renal and proximal ureteral stones at 19 years of follow up. *J. Urol.* **175**:1742-1747.

Kumar, S. B. N., Kumar, K. G., Srinivasa, V. and Bilal, S. (2012a). A review on urolithiasis. *Int. J. Pharm. Life Sci.* **2**(2):269-280.

Kumar, S., Sigmon, D., Miller, T., Carpenter, B., Khan, S., Malhotra, R., Scheid, C. and Menon, M. (1991). A new model of nephrolithiasis involving tubular dysfunction injury. *J. Urol.* **146**(5):1384-1389.

Kumar, S.V.S., Chandrika, G., Mahesh, K. and Meghanath, P.V.S. (2012b). Hepatoprotective activity of *Achyranthes aspera* linn. against paracetamol induced toxicity. *Int. J. Pharm. Sci.* **4**(5):299-302.

Kumaran, M.G. and Patki, P.S. (2011). Evaluation of an Ayurvedic formulation (cystone), in urolithiasis: A double blind, placebo-controlled study. *Eur. J. Integr. Med.* **3**:23-28.

Kwak, C., Kim, H. K., Kim, E. C., Choi, M. S. and Kim, H. H. (2003). Urinary oxalate levels and the enteric bacterium *Oxalobacter formigenes* in patients with calcium oxalate urolithiasis. *Eur. Urol.* **44**(4):475-481.

Lakshmi, K.S., Prabhakaran, V., Mallikarjuna, G. and Gowthami, A. (2014). Antilithiatic activity of *Trianthema portulacastrum* L. and *Gymnema sylvestre* R. Br against ethylene glycol induced urolithiasis. *Int. J. Pharm. Sci. Rev. Res.* **25**(1):16-22.

Larkins, N.J. (1999). Free radical biology and pathology. *J. Equine Vet. Sci.* **19**: 84-89.

Lee, Y.H., Chang, L.S. and Chen, M.T.(1991). Characterization of Ethylene glycol induced urolithiasis model in rats. *J. Urol.* **2**:518-524.

Lee, Y.H., Huang, W.C., Huang, J.K. and Chang, L.S. (1996). Testosterone enhances, whereas estrogen inhibits calcium oxalate stone formation in ethylene glycol treated rats. *J. Urol.* **156**:502-505.

Leonard, B. and Takayuki, S. (2009). *Introduction to Food Toxicology*. 2nd ed. Burlington: Elsevier. pp.124. ISBN 9780080921532.

Leth, P.M. and Gregersen, M. (2005). Ethylene glycol poisoning. *Forensic. Sci. Int.* **155**:179-184.

Li, X., Wang, W., Su, Y., Yue, Z. and Bao, J. (2017). Inhibitory effect of an aqueous extract of *Radix Paeoniae* Alba on calcium oxalate nephrolithiasis in a rat model. *Ren. Fail*. **39**(1):120-129.

Lieske, J.C., Huang, E. and Toback, F.G.(2000). Regulation of renal epithelial cell affinity for calcium oxalate monohydrate crystals. *Am. J. Physiol. Renal. Physiol.* **278**:130-137.

Lieske, J.C., Peña, de la Vega, L.S., Slezak, J.M., Bergstralh, E.J., Leibson, C.L., *et al.* (2006). Renal stone epidemiology in Rochester, Minnesota: An update. *Kidney Int.*. **69**:760-764.

Lin, J. and Huang, W. (2008). Simultaneous determination of benzoylperoxide and benzoic acid content in flour by ultraviolet spectrophotometry [J]. *Guangdong Trace Elem. Sci.* **4**:014.

Liu, C., Liao,Z., Liu, S,, Sun, J., Yao, G. and Wang, H. (2014). A new pregnane glycoside from *Rubus phoenicolasius* and its antiproliferative activity. *Nat. Prod. Res*. **28**:1843-1846.

Ljunghall, S. and Danielson, B.G. (1984). A prospective study of renal stone recurrences. *Br. J. Urol.* **56**:122-124.

Lobo, V., Patil, A., Phatak, A. and Chandra, N. (2010). Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacogn. Rev.* **4**(8):118-126.

Lopez, M. and Hoppe, B. (2008). History, epidemiology and regional diversities of urolithiasis. *Pediatr. Nephrol.* **25**(1):49-59.

Lulekal, E., Asfaw, Z., Kelbessa, E. and Van Damme, P. (2014). Ethnoveterinary plants of Ankober district, North Shewa Zone, Amhara region, Ethiopia. *J. Ethnobiol. Ethnomed.* **10**:21.

MacDonald-Wicks, L.K., Wood, L.G. and Garg, M.L. (2006). Review Methodology for the determination of biological antioxidant capacity *in vitro*: A review. *J. Sci. Food Agric.* **86**:2046-2056.

Mahmoud, A.M. (2013). Hematological alterations in diabetic rats: Role of adipocytokines and effect of citrus flavonoids. *Exp. Clin. Sci. Int. J.* **12**:647-657.

Malhotra, K. K. (2008). Medical aspects of renal stones: review. *J. Indian Acad. Clin. Med.* **9**(4): 282-286.

Marengo, S. R. and Romani, A. M. (2008). Oxalate in renal stone disease: the terminal metabolite that just won't go away. *Nat. Clin. Pract. Nephrol.* **4**(**7**):368-377.

Marshall, R.W. and Robertson, W.G. (1976). Nonograms for the estimation of the saturation of urine with calcium oxalate, calcium phosphate, magnesium ammonium phosphate, uric acid, sodium acid urate. *Clin. Chim. Acta.* **72**:253-257.

Marzouk, A.M., Osman, S.M. and Gohar, A.A. (2016). A new pregnane glycoside from *Gomphocarpus fruticosus* growing in Egypt. *Nat. Prod. Res.* **30**(9):1060-1067.

Masao, T. (2008). Mechanism of calcium oxalate renal stone formation and renal tubular cell injury. *Int. J. Urol.* **15**:115-20.

Masarani, M. and Dinneen, M. (2007). Ureteric colic: new trends in diagnosis and treatment. *Postgrad. Med. J.* **83**:469-472.

Masterson, J.H., Phillips, C.J., Crum-Cianflone, N.F., Krause, R.J., Sur, R.L. and L'Esperance, J.O. (2017). A 10-year retrospective review of nephrolithiasis in the Navy and Navy Pilots. *J. Urol*. **02**:074.

Mattle, D. and Hess, B. (2005). Preventive treatment of nephrolithiasis with alkali citrate: A critical review. *Urol. Res.* **33**(2):73-79.

Mcmartin, K. (2009). Are calcium oxalate crystals involved in the mechanism of acute renal failure in ethylene glycol poisoning? *Clin. Toxicol.* **47**:859-869.

McMartin, K.E. and Wallace, K.B. (2005). Calcium oxalate monohydrate, a metabolite of ethylene glycol, is toxic for rat renal mitochondrial function. *Toxicol. Sci.* **84:**195-200.

Mekonnen, T., Urga, K. and Engidawork, E. (2010). Evaluation of the diuretic and analgesic activities of the rhizomes of *Rumex abyssinicus* Jacq. in mice. *J. Ethnopharmacol.* **127**(2):433-439.

Menon, M. and Koul, H. (1992). Calcium oxalate nephrolithiasis. *J. Clin. Endocrinol. Metab*.**74**:703-707.

Mi, J., Duan, J., Zhang, J., Lu, J., Wang, H. and Wang, Z. (2012). Evaluation of antiurolithic effects and the possible mechanisms of *Desmodium styracifolium* and *Pyrrosiae petiolosa* in rats. *Urol Res.* **40**:151-161.

Middleton, E. Jr., Kandaswami, C. and Theoharides, T.C. (2000). The effects of plant flavonoids on mammalian cells: Implications for inflammation, heart disease and cancer. *Pharmacol. Rev.* **52**(4): 673-751.

Mikawlrawng, K., Kumar, S., and Vandana, R. (2014). Current scenario of urolithiasis and the use of medicinal plants as antiurolithiatic agents in Manipur (North East India): A review. *Int. J. Herb. Med.* **2**(1):1-12.

Mittal, A.K., Kaler, A. and Banerjee, U.C. (2012). Free radical scavenging and antioxidant activity of silver nanoparticles synthesized from flower extract of *Rhododendron dauricum*. *Nano Biomed. Eng.* **4**(3):118-124.

Moe, O.W. (2006). Kidney stones: Pathophysiology and medical management. *Lancet.* **367** (9507): 333-344.

Momtaz, S. and Abdollahi, M. (2010). An update on pharmacology of Satureja species; from antioxidant, antimicrobial, anti-diabetes and anti-hyperlipidemic to reproductive stimulation. *Int. J. Pharmacol.* **6**:346-353.

Moochhala, S.H., Sayer, J.A., Carr, G. and Simmons, N.L. (2008). Renal calcium stones: Insights from the control of bone mineralization. *Exp. Physiol.* **93**:43-49.

Moreira, S. S., Tamashiro, L. K., Jorge, B.C., Balin, P.S., Heredia-Vieira, S.C., Almeida, G.L., *et al.* (2018). Toxicological safety evaluation in acute and 28 day studies of

aqueous extract from *Serjania marginata* Casar. (Sapindaceae) levels in rats. *J. Ethnopharmacol*. **231**:197-204.

Moryama, M. T., Domiki, C., Miyazawa, K., Tanaka, T. and Suzuki, K. (2005). Effects of oxalate exposure on Madin-Darby canine kidney cells in culture: renal prothrombin fragment-1 mRNA expression. *Urol. Res.* **33**(6):470-475.

Mothana, R., Al-Musayeib, N., Al-Ajmi, M., Cos, P. and Maes, L. (2014). Evaluation of the *in vitro* antiplasmodial activity of medicinal plants used in Saudi and Yemeni traditional medicine. *Evid. Based complement Altern. Med.* 1-7.

Mulay, S.R., Kulkarni, O.P., Rupanagudi, K.V., Migliorini, A., Darisipudi, M.N., Vilaysane A., *et al.* (2013). Calcium oxalate crystals induce renal inflammation by NLRP3-mediated IL-1beta secretion. *J. Clin. Invest.* **123**:236-246.

Murugan, V.M. and Satishkumar, A.S. (2001). Effect of *Aerva lanata* leaf extract and *Vediuppu chunnam* on the urinary risk factors of calcium oxalate urolithiasis during experimental hyperoxaluria. *Pharmacol. Res.* **43**:89-93.

Nagal, A. and Singla, R.K. (2013). Herbal resources with antiurolithiatic effects: A review. *Indo. Glob. J. Pharm. Sci.* **3**:6-14.

Naghii, M.R., Babaei, M. and Hedayati, M. (2014). Androgens involvement in the pathogenesis of renal stones formation. *PLoS ONE.* **9**(4): e93790.

Newman, D.J. and Cragg, G.M. (2016). Natural products as sources of new drugs from 1981 to 2014. *J. Nat. Prod.* **79:** 629-661.

Ngo, T. C. and Assimos, D. G. (2007). Uric acid nephrolithiasis: Recent progress and future directions. *Rev. Urol.* **9**:17–27.

NIH. (2005). Kidney Disease. NIH, Bethesda, Maryland, USA. http://kidney.niddk.nih.gov/kudis'eases /pubs/stonesadults/index.htm.

Niki, E., Shimaski, H. and Mino, M. (1994). Antioxidantism-free radical and biological defense. *Gakkai. Syuppn. Center. Tokyo.* 3-16.

Noorafshan, A., Doust, S.K. and Karimi, F. (2013). Diosmin reduces calcium oxalate deposition and tissue degeneration in nephrolithiasis in rats: A stereological study. *Korean J. Urol.* **54**:252–7.

Norman, A.W. (1990). Intestinal calcium absorption: A vitamin D-hormone mediated adaptive response. *Am. J. Clin. Nutr.* **51**:290-300.

Noshad, H., Ahmadpour, F., Soltanpour, B. and Ghojazadeh, M. (2014). Study of renal stones complications in 200 patients in Tabriz, Iran. *J. Anal. Res. Clin. Med.* **2**(4): 187-92.

Notten, N. (2010). *Gomphocarpus physocarpus* E. Mey. plant the Africa. SANBI. http://pza.sanbi.org/gomphocarpusphysocarpus. [Accessed 24 Sep 2018].

Obligado, S. H. and Goldfarb, D. S. (2008). The association of nephrolithiasis with hypertension and obesity: A review. *Am. J. Hypertens.* **21**(3):257-264.

Odvina, C.V., Preminger, G.M., Lindberg, J.S., Moe, O.W. and Pak, C.Y.C. (2003). Long-term combined treatment with thiazide and potassium citrate in nephrolithiasis does not lead to hypokalemia or hypochloremic metabolic alkalosis. *Kidney Int.* **63**:240-247.

OECD. (2001). Guidelines for the testing of chemicals. Acute oral toxicity-Acute toxic class method: No. 423: pp.1-14.

Ogbuehi, I.H., Ebong, O.O. and Obianime, A.W. (2015). Oral acute toxicity (LD50) study of different solvent extracts of *Abrus precatorius* Linn. leaves in Wistar rats. *Eur. J. Exp. Biol*. **5**:18-25.

Okon, B.E., Essien, E.E., Poh, C.F. and Orji, M.H. (2015). An evaluation of the subacute toxicity and haemostatic effects of leaves extract of *Achyranthes aspera* in mice and albino rats. *Eur. J. Med. Plant*. **7**(1):16-25.

Ouyang, J.M., Zheng, H. and Deng, S.P. (2006). Simultaneous formation of calcium oxalate (mono-, di- and trihydrate) induced by potassium tartrate in gelatinous system. *J. Crys. Growth.* **293**:118-123.

Pak, C.Y. (1998). Kidney stones. *Lancet.* **351**:1797-1801.

Pak, CY. (1989). Prevention and treatment of kidney stones. Role of medical prevention. *J. Urol.* **141**(3:2):798-801.

Palaniyamma, D. and Jeyaraman, R. (2017). Evaluation of efficacy and safety of a herbal formulation cystone forte in the management of urolithiasis. *J. Urol. Res.* **4**(4):1093.

Panigrahy, S.K., Kumar, A. and Bhatt, R. (2017). Antioxidant potentials of successive solvent extracts from the unexplored *Hedhychium coronarium* rhizome. *J. Food Sci. Technol.* **54**(10):3297–3306.

Pareta, S. K., Patra, K.C. and Harwansh, R. (2011a). *In vitro* calcium oxalate crystallization inhibition by *Achyranthes indica* Linn. Hydroalcoholic extract: An approach to antilithiasis. *Int. J. Pharma. Bio. Sci*. **2**(1):432-437.

Pareta, S.K., Patra, K.C., Mazumder, P.M. and Sasmal, D. (2011b). Establishing the principle of herbal therapy for antiurolithiatic activity: A review. *J. Pharmacol. Toxicol.* **6**:321-332.

Park, S. and Pearle, M. S. (2007). Pathophysiology and management of calcium stones. *Urol. Clin. North Am.* **34**(3):323-334.

Parka, E-S., Moonb, W-S., Songc, M-J., Kimc, M-N., Chungb, K-H. and Yoona, J-S. (2001). Antimicrobial activity of phenol and benzoic acid derivatives. *Int. Biodeterior. Biodegradation*. **47**:209-214.

Parmar, M. S.(2004). Kidney stones. *Br. Med. J.* **328** (7453):1420-1424.

Parshuram, N. S. and Sateesh, B. (2019). Acute and 28-day oral toxicity studies of methanolic extract of *Lagenaria siceraria* (Cucurbitaceae) fruit in rats. *Drug Chem. Toxicol.* 1-9.

Patel, P., Patel, M., Saralai, M. and Gandhi, T. (2012a). Antiurolithiatic effects of *Solanum xanthocarpum* fruit extract on ethylene-glycol-induced nephrolithiasis in rats. *J. Young Pharm.* **4**:164-170.

Patel, P.K., Patel, M.A., Vyas, B.A., Shah, D.R. and Gandhi, T.R. (2012b). Antiurolithiatic activity of saponin rich fraction from the fruits of *Solanum xanthocarpum* Schrad. and Wendl. (Solanaceae) against ethylene glycol induced urolithiasis in rats. *J. Ethnopharmacol.* **144**(1):160-70.

Pawar, A.T., Gaikwad, G.D., Metkari, K.S., Tijore, K.A., Ghodasara, J.V. and Kuchekar, B.S. (2012). Effect of *Terminalia chebula* fruit extract on ethylene glycol induced urolithiasis in rats. *Biomed. Aging Pathol.* **2**:99-103.

Penniston, K.L., Jones, A.N., Nakada, S.Y. and Hansen, K.E. (2009). Vitamin D repletion does not alter urinary clacium excretion in healthy postmenopausal women. *BJU Int*. **104**:1512-1516.

Phatak, R.S. and Hendre, A. S. (2015). *In vitro* antiurolithiatic activity of *Kalanchoe pinnata* extract. *Int. J. Pharmacogn. Phytochem. Res.* **7**(2):275-279.

Phillips, R., Hanchanale, V.S., Myatt, A., Somani, B., Nabi, G. and Biyani, C.S. (2015). Citrate salts for preventing and treating calcium containing kidney stones in adults. *Cochrane Database Syst. Rev*. **10**.1-41.

Pietta, P.G. (2000). Flavonoids as antioxidants. *J. Nat. Prod.* **63**(7):1035-1042.

Polat, S. (2019). An *in vitro* evaluation of the effects of *Urtica dioica* and *Fructus urtica* Piluliferae extracts on the crystallization of calcium oxalate. *J. Cryst. Growth.* **522:** 92–102.

Polterat, O. (1997). Antioxidants and free radical scavengers of natural origin. *Current Org. Chem.* **1**:415-440.

Pomara, C., Fiore, C., D'Errico, S., Riezzo, I. and Fineschi, V.(2008). Calcium oxalate crystals in acute ethylene glycol poisoning: A confocal laser scanning microscope study in a fatal case. *Clin. Toxicol.* **46**:322-324.

Pourmorad, F., Hosseinimehr, S.J. and Shahabimajd, N. (2006). Antioxidant activity, phenol and flavonoid contents of some selected Iranian medicinal plants. *Afr. J. Biotechnol.* **5:**1142–1145.

Prasad, K.V., Bharathi, K. and Srinivasan, K.K. (1993). Evaluation of *Musa Parasidica* Linn (Cultivar)-"Puttubale" stem juice for antilithiatic activity in albino rats. *Indian J. Physiol. Pharmacol.* **37**:337-341.

Prasanth, K.M., Suba, V., Ramireddy, B. and Srinivasa, B.P. (2015). Acute and subchronic oral toxicity assessment of the ethanolic extract of the root of *Oncoba spinosa* (Flacourtiaceae) in rodents. *J. Biochem. Technol.* **14**:1849-1855.

Prezioso, D., Strazzullo, P., Lotti, T., Bianchi, G., Borghi, L., Caione, P., *et al.* (2015). Dietary treatment of urinary risk factors for renal stone formation. A review of CLU Working Group. *Arch. Ital. Urol. Androl.* **87**(2):105-20.

Pucci, N. D., Marchini, G.S., Mazzucchi, E., Reis, S. T., Srougi, M., Evazian, D., *et al.* (2018). Effect of *Phyllanthus niruri* on metabolic parameters of patients with kidney stone: A perspective for disease prevention. *Int. Braz. J. Urol.* **44**(4):758-764**.**

Qiu, S. R., Wierzbicki, A., Orme, C. A., Cody, A. M., Hoyer, J. R., Nancollas, G. H., *et al.* (2004). *Proc. Natl. Acad. Sci. USA.* **101**:1811-1815.

Rabinovich, Y. I., Esayanur, M., Daosukho, S., Byer, K. J., El-Shall, H. E. and Khan, S. R. (2006). Adhesion force between calcium oxalate monohydrate crystal and kidney

epithelial cells and possible relevance to kidney stone formation. *J. Colloid. Interface Sci.* **300**(1):131-140.

Raheem, O.A., Khandwala, Y.S., Sur, R.L., Ghani, K.R. and Denstedt, J.D. (2017). Burden of urolithiasis: Trends in prevalence, treatments and costs. *Eur. Urol.* **3**:18-26.

Rahman, K. (2007). Studies on free radicals, antioxidants and co-factors. *Clin. Interv. Aging.* **2**(2):219-36.

Rajesh, R.K., Chitra, K. and Paarakh, P.M. (2011). *Aerva lanata* (Linn.) Juss. Ex Schult. An Overview. *Asian Pac. J. Trop. Biomed.* **2**:36-40.

Rao, R.M., Khan, Z.A. and Shah, A.H. (2001). Toxicity studies in mice of *Commiphora molmol* oleogum-resin. *J. Ethnopharmacol.* **76**:151-154.

Rao, R.R., Tiwari, A.K., Reddy, P.P., Babu, K.S., Ali, A.Z., Madhusudana, K., *et al.* (2009). New furanoflavanoids, intestinal α-glucosidase inhibitory and free-radical (DPPH) scavenging, the activity of antihyperglycemic root extract of *Derris indica* (Lam.). *Bioorg. Med. Chem.* **17**:5170-5.

Rashed, T., Menon, M. and Thamilselvan, S. (2004). Molecular mechanism of oxalate-induced free radical production and glutathione redox imbalance in renal epithelial cells: effect of antioxidants. *Am. J. Nephrol.* **24**(5):557-568.

Rasoanaivo, P., Wright, C.W., Willcox, M.L. and Gilbert, B.(2011). Whole plant extracts versus single compounds for the treatment of malaria: synergy and positive interactions. *Malar. J.* **10** (Suppl.1): S4.

Rates, S.M. (2001). Plants as source of drugs. *Toxicon*. **39**(5):603-613.

Rathod, N.R., Biswas, D., Chitme, H.R., Ratna, S., Muchandia, I.S. and Chandra, R. (2012a). Antiurolithiatic effects of *Punica granatum* in male rats. *J. Ethnopharmacol.* **140**:234-38.

Rathod, V. D., Fitwe, P., Sarnaik, D. and Kshirsagar, S. N. (2013b). *In vitro* anti-urolithiatic activity of Corn Silk of *Zea Mays*. *Int. J. Pharm. Sci. Rev. Res.* **21**(2):16-19.

Ratkalkar, V. N. and Kleinman, J. G. (2011). Mechanisms of stone formation. *Clin. Rev. Bone Miner. Metab.* **9**(3-4):187-197.

Ravi, L. and Krishnan, K. (2017). Cytotoxic potential of N-hexadecanoic acid extracted from *Kigelia pinnata* leaves. *Asian J. Cell Biol.* **12**:20-27.

Ravikumar, S. and Gnanadesigan, M. (2012). Hepatoprotective and antioxidant properties of *Rhizophora mucronata* Mangrove plant in CCl_4 intoxicated rats. *Int. J. Clin. Exp. Med.* **4**:66-72.

Reddy, C.V. and Kamble, A. K. (2014). Toxicity study of *Achyranthus aspera. Int. Lett. Nat. Sci*. **9**:85-96.

Renata, C., Fabio, V., Angela, B. and Sergio, S. (2003). Citrate and mineral metabolism: Kidney stones and bone disease. *Front. Biosci*. **8**:1084-1106.

Rhiouani, H., El-Hilaly, J., Israili, Z.H. and Lyoussi, B. (2008). Acute and sub-chronic toxicity of an aqueous extract of the leaves of *Herniaria glabra* in rodents. *J. Ethnopharmacol.* **118**:378-386.

Riese, R.J., Riese, J.W., Kleinman, J.G., Wiessner, J.H., Mandel, G.S. and Mandel, N.S.(1988). Specificity in calcium oxalate adherence to papillary epithelial cells in culture. *Am. J. Physiol.* **255**:1025-1032.

Rivers, K., Shetty, S. and Menon, M. (2000). When and how to evaluate a patient with nephrolithiasis. *Urol. Clin. N. Am.* **27**(2):203-13.

Robak, J. and Marcinkiewicz, E. (1995). Scavenging of reactive oxygen species as the mechanism of drug action. *Pol. J. Pharmacol. Pharm.* **47**: 89-98.

Roberts, D.M.., Gallapatthy, G., Dunuwille, A. and Chan, B.S. (2015). Pharmacological treatment of cardiac glycoside poisoning. *Br. J. Clin. Pharmacol.* **18**(3):488-495.

Robertson, W. G., Heyburn, P. J., Peacock, M., Hanes, F. A. and Swaminathan, R. (1979). The effect of high animal protein intake on the risk of calcium stone-formation in the urinary tract. *Clin. Sci.* **57**(3):285-288.

Robertson, W.G. and Peacock, M. (1980). The course of idiopathic calcium disease: hypercalciuria or hyperoxaluaria? *Nephron.* **26**:105-110.

Robinson, M.R., Leitao, V.A., Haleblian, G.E., Scales, C.D., Chandrashekar, Jr. A., Pierre, S. A., *et al.* (2009). Impact of long-term potassium citrate therapy on urinary profiles and recurrent stone formation. *J.Uurol.* **181**(3):1145-50.

Romero, V., Akpinar, H. and Assimos, D.G. (2010). Kidney stones: A global picture of prevalence, incidence, and associated risk factors. *Rev Urol.* **12**(2/3): e86–e96.

Roy, R.N. (2020). Bioactive natural derivatives of phthalate ester. *Crit. Rev. Biotechnol*. 1-17.

Rule, A.D., Bergstralh, E.J., Melton, L.J., Li, X., Weaver, A.L. and Lieske, J.C. (2009). Kidney stones and the risk of chronic kidney disease. *Clin. J. Am. Soc. Nephrol.* **4**(4):804-811.

Rule, A.D., Roger, V.L., Melton, 3rd L.J., Bergstralh, E.J., Li, X., Peyser, P.A., *et al.* (2010). Kidney stones associate with increased risk for myocardial infarction. *J. Am. Soc. Nephrol.* **21**(10):1641-1644.

Rushton, H.G., Spector, M., Rodgers, A.L. and Magura, C.E. (1980). Crystal deposition in the renal tubules of hyperoxaluric and hypomagnesemic rats. *Scan Electron Microsc.* **3**:387-394.

Saad, B., Azaizeh, H., Abu-Hijleh, G. and Said, O. (2006). Safety of traditional Arab herbal medicine. *Evid. Based Compl. Altern. Med.* **3**:433-439.

Safarinejad, M.R. (2007). Adult urolithiasis in a population-based study in Iran: Prevalence, incidence, and associated risk factors. *Urol. Res.* **35**:73-82.

Saha, S. and Verma, R.J. (2013). Inhibition of calcium oxalate crystallization *in vitro* by an extract of *Bergenia ciliata. Arab J. Urol.* **11**:187–192.

Saha, S. and Verma, R.J. (2012). Efficacy of *Dolichos biflorus* in the management of nephrotoxicity. *Asian Pac. J. Trop. Biomed.* 1471-1476.

Sakhaee, K. (2008). Nephrolithiasis as a systemic disorder. *Curr. Opin. Nephrol. Hypertens.* **17**(3):304-9.

Salem, M.A., de Souza, L.P., Serag, A., Fernie, A.R., Farag, M.A., Ezzat, S.M., *et al.* (2020). Metabolomics in the context of plant natural products research: From sample preparation to metabolite analysis. *Metabolites.* **10**:37.

Samal, L., Pattanaik, A.K., Mishra, C., Maharana, B.R., Sarangi, L. N. and Baithalu, R. K. (2011). Nutritional strategies to prevent urolithiasis in animals. *Vet. World.* **4**(3):142-144.

Samuel, A.J.S.J., Mohan, S., Chellappan, D.K., Kalusalingam, A. and Ariamuthu, S. (2012). *Hibiscus vitifolius* (Linn.) root extracts shows potent protective action against anti-tubercular drug induced hepatotoxicity. *J. Ethnopharmacol.* **141**(1):396-402.

Sanchez-Mareno, C. (2002). Review: Methods used to evaluate the free radical scavenging activity in foods and biological systems. *Food Sci. Technol. Int.* **8**(3):121-137.

Santhoshi, B., Chidrawar, V.R. and Rao, V.M. (2015). A brief review of the potential medicinal plants and screening models of urolithiasis. *Int. J. Appl. Biol. Pharm.Technol*. **6**(1):37-45.

Sanyacharernkul, S., Itghiarbha, A., Kongtawelert, P., Meepowpan, P., Nuntasaen, N. and Pompimon, W. (2009). A new polyoxypregnane glycoside from the roots of *Dregea volubilis* (L.f) Benth. ex Hook. f and its chondroprotective effect. *Am. J. Biochem. Biotechnol.* **5**:202-209.

Saranya, R. and Geetha, N. (2014). Inhibition of calcium oxalate (CaOx) crystallization *in vitro* by the extract of beet root (*Beta vulgaris* L.). *Int. J. Pharm. Pharm. Sci*. **6:**361-365.

Sasidharan, H., Mallya, S.V., Suchitra, P. and Kumar, K.N.S. (2018). *In vitro* evaluation of *Scoparia dulcis* Linn. for antiurolithiatic activity. *J. Phytopharmacol*. **7**(3):284-286.

Sasikala, V., Radha, S.R. and Vijayakumari, B. (2013). *In vitro* evaluation of *Rotula aquatica* Lour. for antiurolithiatic activity. *J. Pharm. Res.* **6**:378-382.

Sathish, R., Natarajan, K. and Nikhad, M.M. (2010). Effect of *Hygrophila spinosa* T. Anders on ethylene glycol induced urolithiasis in rats. *Asian J. Pharm. Clin. Res.* **3**(4):61-63.

Sathish, S.S., Janakiraman, N. and Johnson, M. (2012). Phytochemical Analysis of *Vitex altissima* L. using UV-VIS, FTIR and GC-MS. *Int. J. Pharm. Sci. Drug Res.* **4**(1):56-62.

Satish, H., Dang, R., Devi, K., Shivananda, B. G. and Shridhar, K. A. (2010). *In-vitro* anti-lithiatic activity study of *Tribulus terrrestris* fruits and *Boerhaavia diffusa* roots. *Der. Pharmacia. Lettre.* **2**(3):12-20.

Sayer, J. A. (2008). The Genetics of nephrolithiasis. *Nephron Exp. Nephrol*. **110** (2):37-43.

Scales, J.C.D., Smith, A.C., Hanley, J.M. and Saigal, C. S. (2012). Prevalence of kidney stones in the United States. *Eur. Urol.* **62**(1):160–165.

Schaeffer, A.J., Feng, Z., Trock, B.J., Mathews, R.I., Neu, A.M., Gearhart, J.P. and Matlaga, B.R. (2011). Medical comorbidities associated with pediatric kidney stone disease. *Urol.* **77**(1):195–199.

Scheid, C., Koul, H., Hill, W.A., Narod, L., Kennington, L., Honeyman, T., Jonassen, J. and Mani, M. (1996). Oxalate toxicity in LLC-PK1 cells: Role of free radicals. *Kidney Int.* **49**:413–417.

Scheid, C.R., Cao, L.C., Honeyman, T. and Jonassen, J.A. (2004). How elevated oxalate can promote kidney stone disease: changes at the surface and in the cytosol of renal cells that promote crystal adherence and growth. *Front Biosci.* **9**:797-808.

Schepers, M. S., Vander, B. G., Romijn, J. C., Schrooderand, F. H. and Verkoelen, C. F. (2002). Urinary crystallization inhibitors do not prevent crystal binding. *J. Urol.* **167**(4): 1844-1847.

Serhat, G. and Kupeli, B. (2006). Consumption of historical and current phytotherapeutic agents for urolithiasis: A critical review. *J. Urol.* **176**:450-455.

Sethi, A., Bhatia, A., Maurya, A., Panday, A., Bhatia, G., Shrivastava, A., *et al.* (2013). Proficient synthesis of biologically active pregnane derivatives and its glycoside - experimental and theoretical approach. *J. Mol. Struct.* **1052**:112–124.

Sharif, H., Mukhtar, M., Mustapha, Y., Baba ,G. and Lawal, A. (2015). Acute and subchronic toxicity profile of *Euphorbia pulcherrima* methanol extract on Wistar albino rats. *Adv. Pharm. J.* Article ID 539646, 9 pages.

Sharma, I., Khan, W. and Ahmad, S. (2017a). *In vitro* and *ex vivo* approach for anti-urolithiatic potential of bioactive fractions of gokhru with simultaneous HPLC analysis of six major metabolites and their exploration in rat plasma. *Pharm. Biol.* **55**(1):701-711.

Sharma, I., Khan, W., Parveen, R., Alam, M.J., Ahmad, I., Ansari, M.H.R., *et al*. (2017b). Antiurolithiasis activity of bioactivity guided fraction of *Bergenia ligulata* against Ethylene glycol induced renal calculi in rat. *Biomed. Res. Int*. Volume 2017, Article ID 1969525, 11 pages.

Sharma, O.P. and Bhat, T.K. (2009). DPPH antioxidant assay revisited. *Food Chem*. **113** (5): 1202-1205.

Sheng, X., Ward, M.D. and Wesson, J.A. (2005). Crystal surface adhesion explains the pathological activity of calcium oxalate hydrates in kidney stone formation. *J. Am. Soc. Nephrol.* **16**(**7)**:1904-8.

Siddiqui, W.A., Shahzad, M., Shabbir, A. and Ahmad, A. (2018). Evaluation of anti-urolithiatic and diuretic activities of watermelon (*Citrullus lanatus*) using *in vivo* and *in vitro* experiments. *Biomed. Pharmacother.* **97**:1212-1221.

Sigurjonsdottir, V. K., Runolfsdottir, H. L., Indridason, O. S., Palsson, R. and Edvardsson, V.O. (2015). Impact of nephrolithiasis on kidney function. *BMC Nephrol.* **16**(1): 149.

Sikarwar, I., Dey, Y.N., Wanjari, M.M., Sharma, A., Gaidhani, S.N. and Jadhav, A.D. (2017). *Chenopodium album* Linn. leaves prevent ethylene glycol-induced urolithiasis in rats. *J. Ethnopharmacol.* **195**:275-82.

Silberstein, J., Lakin, C.M. and Parsons, J.K. (2008). Shock wave lithotripsy and renal hemorrhage. *Rev. Urol.* **10**:236-241.

Singh, A.K., Attrey, D.P., Deep, P., Dubey, S., Naved, T. and Roy, B. (2014). Acute and subacute toxicity studies of pharmacologically active Seabuckthorn leaf extract. *Int. J. Pharm. Sci.* **6**:415-419.

Singh, K.B. and Sailo, S. (2013).Understanding epidemiology and etiologic factors of urolithiasis: An overview. *Sci. Vis.* **13**(4):169-174.

Singhto, N., Sintiprungrat, K., Sinchaikul, S., Chen, S.T. and Thongboonkerd, V. (2010). Proteome changes in human monocytes upon interaction with calcium oxalate monohydrate crystals. *J. Proteome Res.* **9**(8):3980-3988.

Sivakumar, R. and Dhivya, A. (2015). GC-MS analysis of bioactive compounds on ethyl acetate extract of *Cordia monica* Roxb. leaves. *Int. J.Res. Dev.* *Pharm life Sci.* **4**(1):1328-1333.

Skolarikos, A., Straub, M., Knoll, T., Sarica, K., Seitz, C., Petřík, A., *et al.* (2015). Metabolic evaluation and recurrence prevention for urinary stone patients: EAU guidelines. *Eur. Urol.* **67**(4):750-763.

Słojewski, M. (2011). Major and trace elements in lithogenesis. *Cent European J. Urol.* **64**:561.

Sofia, N. H., Manickavasakam, K. and Walter, T.M. (2016). Prevalence and risk factors of kidney stone. *Glob. J. Res. Anal.* **5**(3):183-187.

Sousa, Z.L., de Oliveira, F.F., da Conceição, A.O., Silva, L.A.M. , Rossi, M.H., Santos, J.da-S., *et al.* (2012). Biological activities of extracts from *Chenopodium*

ambrosioides Lineu and *Kielmeyera neglecta* saddi. *Ann. Clin. Microbiol. Antimicrob*. **11**:20.

Spivacow, F. R., Negri, A. L., Polonsky, A. and Valle, E.E.D. (2010). Long-term treatment of renal lithiasis with potassium citrate. *Urol.* **76**(6):1346-1349.

Srisubat, A,, Potisat, S, Lojanapiwat, B, Setthawong, V. and Laopaiboon, M. (2009). Extracorporeal shock wave lithotripsy (ESWL) versus percutaneous nephrolithotomy (PCNL) or retrograde intrarenal surgery (RIRS) for kidney stones. *Cochrane Database Syst. Rev*. **4**:CD007044.

Stamatelou, K.K., Francis, M.E., Jones, C.A., Nyberg, L.M. and Curhan, G.C. (2003). Time trends in reported prevalence of kidney stones in the United States: 1976-1994. *Kidney Int.* **63**(5):1817–1823.

Straub, M. and Hautmann, R. E. (2005). Developments in stone prevention. *Curr. Opin. Urol.* **15**(2):119-126.

Sujatha, D., Singh, K., Vohra, M., Kumar, K.V. and Sunitha, S. (2015). Antilithiatic activity of phlorotannin rich extract of *Sarghassum wightii* on calcium oxalate urolithiais: *In vitro* and *in vivo* evaluation. *Int. Braz. J. Urol*. **41**(3):511-520.

Sun, X-Y., Xu, M. and Ouyang, J-M. (2017). Effect of crystal shape and aggregation of calcium oxalate monohydrate on cellular toxicity in renal epithelial cells. *J. Am. Chem. Soc.Omega.* **2**: 6039-6052.

Sun, Y. (1990). Free radicals, antioxidant enzymes and carcinogenesis. *Fre. Radic. Biol. Med*. **8**:583-599.

Tarikua, Y., Hymeteb, A., Hailuc, A. and Rohlof, J. (2010). Essential oil composition, antileishmanial, and toxicity study of *Artemisia abyssinica* and *Satureja punctata* Sp. from Ethiopia. *Chem. Biodivers*. **7**:1009-1018.

Taylor, E. N., Stampfer, M. J. and Curhan, G.C. (2005). Obesity, weight gain and the risk of kidney stones. *J. Am. Med. Assoc*. **293**(4):455-462.

Tchey, D.U., Ha, Y.S., Kim, W.T., Yun, S.J., Lee, S.C. and Kim, W.J. (2011). Expectant management of ureter stones: outcome and clinical factors of spontaneous passage in a single institution's experience. *Korean J. Urol.* **52**:847-851.

Teichman, J. M. and Joel, M. H. (2004). Acute renal colic from ureteral calculus. *N. Engl. J. Med.* **350**(7):684-693.

Teklu, S., Alemayehu, G. and Abegaz, B.M. (1998). Chemical composition of the essential oil of *Satureja punctata*. *J. Essent. Oil Res.* **10**(3):339-341.

Teo, S.D., Stirling, S., Thomas, A., Kiorpes, A. and Vikram, K. (2002). A 90-day oral gavage toxicity study of D-methylphenidate and D, L-methylphenidate in Sprague-Dawley rats. *Toxicol.* **179**:183-196.

Tepe, B. and Cilkiz, M. (2015). A Pharmacological and phytochemical overview on Satureja. *Pharm. Biol.* **54**(3):375-412.

Thamilselvan, S. and Menon, M. (2005). Vitamin E therapy prevents hyperoxaluria induced calcium oxalate crystal deposition in the kidney by improving renal tissue antioxidant status. *BJU Int.* **96**:117-126.

Thamilselvan, S., Hackett, R.L. and Khan, S.R. (1997). Lipid peroxidation in Ethylene glycol induced hyperoxaluria and calcium oxalate nephrolithiasis. *J. Urol.* **157:**1059-1063.

Tiselius, H.G.(2000). Stone incidence and prevention. *J. Clin. Urol*. **26**:452-462.

Tiwari, A., Soni, V., Londhe, V., Bhandarkar, A., Bandawane, D. and Nipate, S. (2012). An overview of potent indigenous herbs for urinary tract infirmity: Urolithiasis. *Asian J. Pharm. Clin. Res.* **5**(1**)**:7-12.

Touhami, M., Laroubi, A., Elhabazi, K., Loubna, F., Zrara, I., Eljahiri, Y., *et al*. (2007). Lemon juice has protective activity in a rat urolithiasis model. *BMC Urol.* **7**:18.

Travison, T.G., Araujo, A.B., Hall, S.A. and McKinlay, J.B. (2009). Temporal trends in Testosterone levels and treatment in older men. *Curr. Opin. Endocrinol. Diabetes Obes.* **16**:211–217.

Trinchieri, A. (2013). Diet and renal stone formation. *Minerva. Med*. **104**(1):41–54.

Trinchieri, A., Ostini, F., Nespoli, R., Rovera, F., Montanarl, E. and Zanetti, G. (1999). A prospective study of recurrence rate and the risk factor for recurrence after a first renal stone. *J Urol.* **162**:27-30.

Tsai, C-H., Chen, Y-C., Chen, L-D., Pan, T-C., Ho, C-Y., Lai, M-T., *et al*. (2008). A traditional Chinese herbal antilithic formula, Wulingsan, effectively prevents the renal deposition of calcium oxalate crystal in ethylene glycol-fed rats. *Urol. Res.* **36**:17-24.

Tsujihata, M. (2008). Mechanism of calcium oxalate renal stone formation and renal tubular cell injury. *Int. J. Urol.* **15**(2**)**:115-120.

Tzou, D.T., Taguchi, K., Chi, T. and Stoller, M.L. (2016). Animal models of urinary stone disease. *Int. J. Surg.* **36**: 596-606.

Uniyal, S.K., Singh, K.N., Jamwal, P. and Lal, B.(2006). Traditional use of medicinal plants among the tribal communities of Chhota Bhangal, Western Himalaya. *J. Ethnobiol.* **2**:1-14.

Unuofin, J.O., Otunola, G.A. and Afolayan, A.J. (2018). Evaluation of acute and sub-acute toxicity of whole-plant aqueous extract of *Vernonia mespilifolia* Less. in Wistar rats. *J. Integr. Med.* 1-13.

Uyeturk, U., Dagıstan, E., Aktas, G., Ozyalvacli, M.E., Tekce, H. and Yilmaz, B. (2014). Association between urinary stone disease and peri-renal tissue thickness. *N. J. Med. Res.* **4**(3):193-196.

Vanachayangkul, P., Chow, N., Khan, S.R. and Butterweck, V. (2011). Prevention of renal crystal deposition by an extract of *Ammi visnaga* L. and its constituents khellin and visnagin in hyperoxaluric rats. *Urol. Res.* **39**(3):189-95.

Venkata-Raman, B., Samuel, L.A., Pardha, S.M., Narashimha, R.B., Naga, V.K.A., Sudhakar, M., *et al*. (2012). Antibacterial, antioxidant activity and GC-MS analysis of *Eupatorium odoratum. Asian J. Pharm. Clin. Res*. **5** (Suppl. 2): 99-106.

Vennila, V. and Mariyal, A. M. (2015). *In vitro* analysis of phytochemical and anti-urolithiatic activity of various extracts of *Melia Dubia* leaves. *World J. Pharm. Pharm. Sci.* **4**(4):1277-1289.

Verkoelen, C. F., vander Boom, B. G. and Romijn, J. C. (2000). Identification of hyaluronan as a crystal-binding molecule at the surface of migrating and proliferating MDCK cells. *Kidney Int.* **58**(3):1045-1054.

Vijaya, T., Rao, N.V.R., Babu, A.N., Kumar, M.S., Nirojini, P.S., Reddy, B.S., *et al*. (2013). Antiurolithiatic activity of methanolic extract of dried leaves of glochidion velutinium using ethylene glycol induced rats. *Int. J. Bio. Pharma. Res*. **4**(12):878-884.

Vinson, J.A., Yong, H., Xuchui, S. and Zubik, L. (1998). Phenol antioxidant quantity and quality in foods: vegetables. *J. Agric. Food Chem*. **46**:3630-3634.

Vyas, B., Vyas, R., Joshi, S. and Santani, D. (2011). Antiurolithiatic activity of whole-plant hydro-alcoholic extract of *Pergularia daemia* in Rats. *J. Young. Pharm.* **3**:36-40.

Wagstaff, D.J. (2008). International poisonous plants checklist. An evidence-based reference. *CRC Press, Boca Raton, Fla.* ISBN: 9781420062526.

Wang, Z., Wang, M., Mei, W., Han, Z. and Dai, H. (2008). A new cytotoxic pregnanone from *Calotropis gigantea*. *Molecules.* **13**:3033-3039.

Wesson, J.A., Worcester, E.M., Wiessner, J.H., Mandel, N.S. and Kleinman, J.G. (1998). Control of calcium oxalate crystal structure and cell adherence by urinary macromolecules. *Kidney Int.* **53**(4):952-7.

WHO. (1993). I.P.C.S Environmental Health Criteria, 155: Biomarkers and risk assessment: Concepts and principles. Geneva, World Health Organization, pp.11-17.

WHO. (2011). The world medicines situation, Traditional medicines: Global situation, issues and challenges, Geneva, pp. 1-3.

Wiessner, J.H., Hasegawa, A.T., Hung, L.Y., Mandel, G.S. and Mandel, N.S. (2001). Mechanisms of calcium oxalate crystal attachment of injured renal collecting duct cells. *Kidney Int.* **59**:637- 644.

Wiessner, J.H., Hung, L.Y. and Mandel, N.S. (2003). Crystal attachment to injured renal collecting duct cells: Influence of urine proteins and pH. *Kidney Int.* **63**:1313-20.

Wintola, O.A. and Afolayan, A.J. (2011). Phytochemical constituents and antioxidant activities of the whole leaf extract of *Aloe ferox* Mill. *Pharmacogn. Mag.***7**:325-33.

Wintrobe, M.M. and Greer, J.P. (2009). Wintrobe's clinical hematology. 11th ed. Philadelphia:Wolters Kluwer Lippincott Williams and Wilkins health. ISBN: 978-1-49-634742-8.

Worcester, E. M. and Coe, F. L. (2008). Nephrolithiasis. *Primary Care.* **35**(2):369-391.

Xi, Q., Ouyang, J., Pu, J., Hou, J. and Wang. S. (2015). High concentration of calcium stimulates calcium oxalate crystal attachment to rat tubular epithelial NRK cells through Osteopontin. *Urol.* **86**(4**)**:844e1-844e5.

Xu, H., Zisman, A. L., Coe, F.L. and Worcester, E.M. (2013). Kidney stones: An update on current pharmacological management and future directions. *Expert Opin. Pharmacother.***14**(4):435-447.

Yadav, R.D., Jain., S. K., Alok, S., Mahor, A., Bharti, J. P. and Jaiswal, M. (2011). Herbal plants used in the treatment of urolithiasis: A review. *Int. J. Pharm. Sci. Res.* **2**(6):1412-1420.

Yadav, R.N.S. and Agarwala, M. (2011). Phytochemical analysis of some medicinal plants. *J. Phytol.* **3**(12):10-14.

Yakubu, E**.**O**.**, Otitoju, O**.** and Onwuka, J**.** (2017). Gas Chromatography-Mass Spectrometry (GC-MS) analysis of aqueous extract of *Daniellia oliveri* stem bark. *Pharm. Anal. Acta.* **8**:568.

Yamaguchi, S., Wiessner, J. H., Hasegawa, A. T., Hung, L.Y., Mandel, G.S. and Mandel, N.S. (2005). Study of a rat model for calcium oxalate crystal formation without severe renal damage in selected conditions. *Int. J. Urol.* **12**:290-298.

Yasir, F. and Waqar, M.A. (2011). Effect of indigenous plant extracts on calcium oxalate crystallization having a role in urolithiasis. *Urol. Res.* **39**:345-350.

Yineger, H. and Yewhalaw, D. (2007). Traditional medicinal plant knowledge and use by local healers in Sekoru district, Jimma Zone, Southwestern Ethiopia. *J. Ethnobiol. Ethnomed.* **3**(24):3-24.

Yoshida, O. and Okada, Y. (1990). Epidemiology of urolithiasis in Japan: A chronological and geographical study. *Urol. Int.* **45**:104-11.

Youn, S.H., Kwon, J.H., Yin, J., Tam, L.T., Ahn, H.S., Myung, S.C., *et al.* (2017). Anti-inflammatory and anti-urolithiasis effects of polyphenolic compounds from *Quercus gilva* blume. *Mol.* **22**:1121.

Yuen, J. W., Gohel, M. D. I., Poon, N. W., Shum, D. K. Y., Tam, P.-C. and Au, D. W. T. (2010). The initial and subsequent inflammatory events during calcium oxalate lithiasis. *Clin. Chim. Acta.* **411**:1018–1026.

Zahid, I. H., Bawazir, A. S. and Naser, R. (2013). Plant based native therapy for the treatment of kidney stones in Aurangabad (MS). *J. Pharmacogn. Phytochem.* **1**(6):189-193.

Zerwekh, J.E., Odvina, C.V., Wuermser, L.A. and Pak, C.Y. (2007). Reduction of renal stone risk by potassium-magnesium citrate during 5 weeks of bed rest. *J. Urol.* **177**:2179-2184.

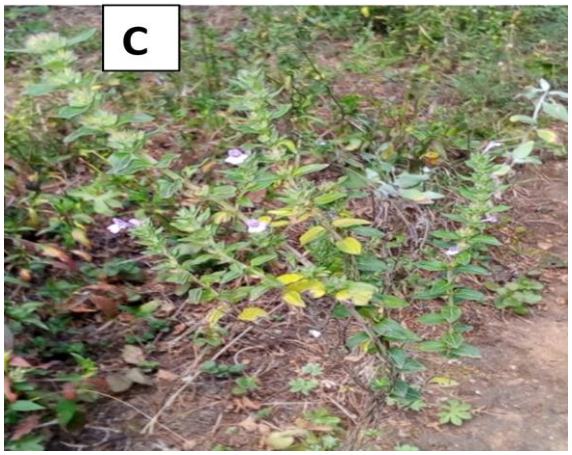
Zhai, W., Zheng, J., Yao, X., Peng, B., Liu., M., Huang, J., *et al.* (2013). Catechin prevents the calcium oxalate monohydrate induced renal calcium crystallization in NRK-52E cells and the ethylene glycol induced renal stone formation in rat. *BMC. Complement Altern. Med.* **13**(1):228.

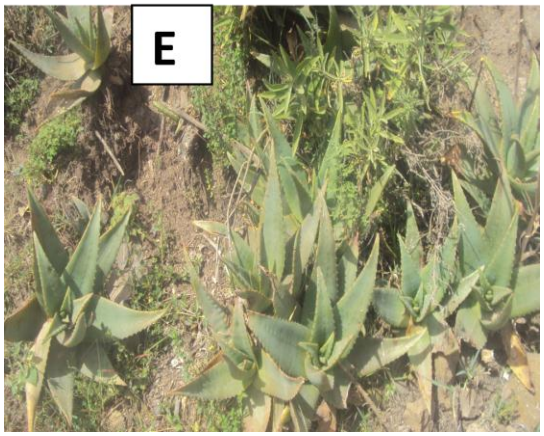
Zhang, C.-Y., Wu, W.-H., Wang, J. and Lan, M.-B. (2012). Antioxidant properties of polysaccharide from the brown sea-weed *Sargassum graminifolium* (Turn.), and its effects on calcium oxalate crystallization. *Mar. Drugs*. **10**:119-130.

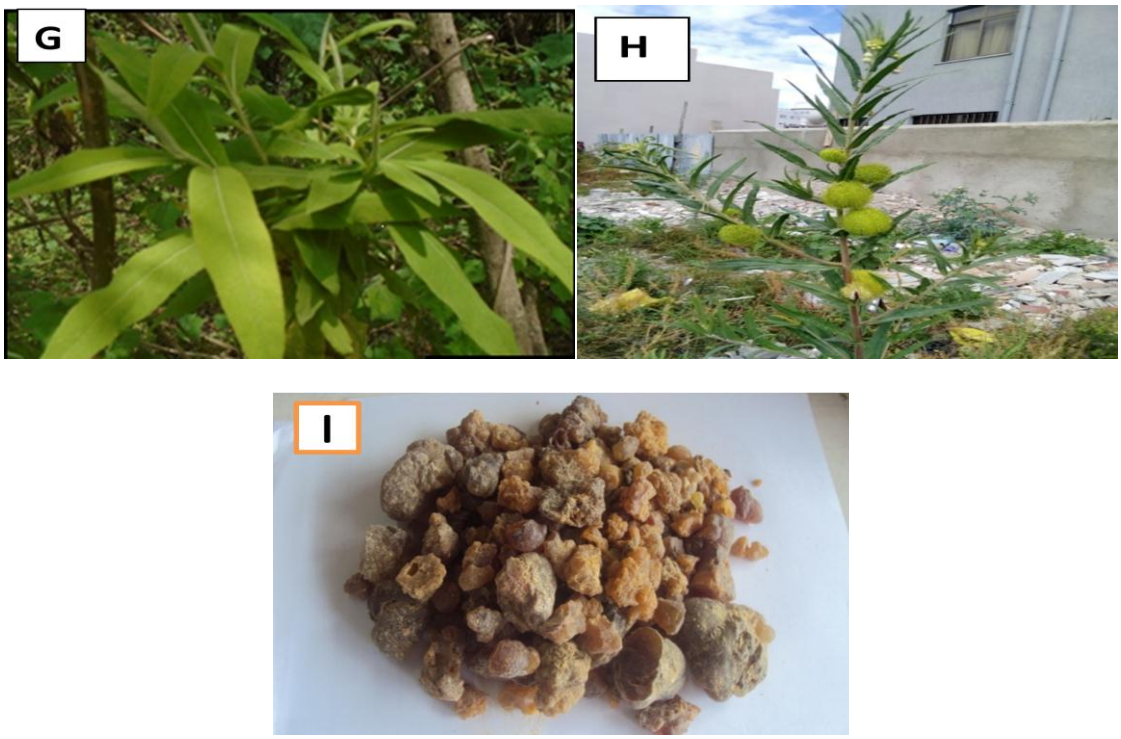
Zhao, B., Su, B., Zhang, H., Liu, W., Du, Q. and Li, Y. (2019). Antiurolithiatic effect of ferulic acid on ethylene glycol induced renal calculus in experimental rats. *Trop. J. Pharm. Res.* **18**(1):109-115.

Appendices

Appendix 1. Figures showing medicinal plants collected for the present study.







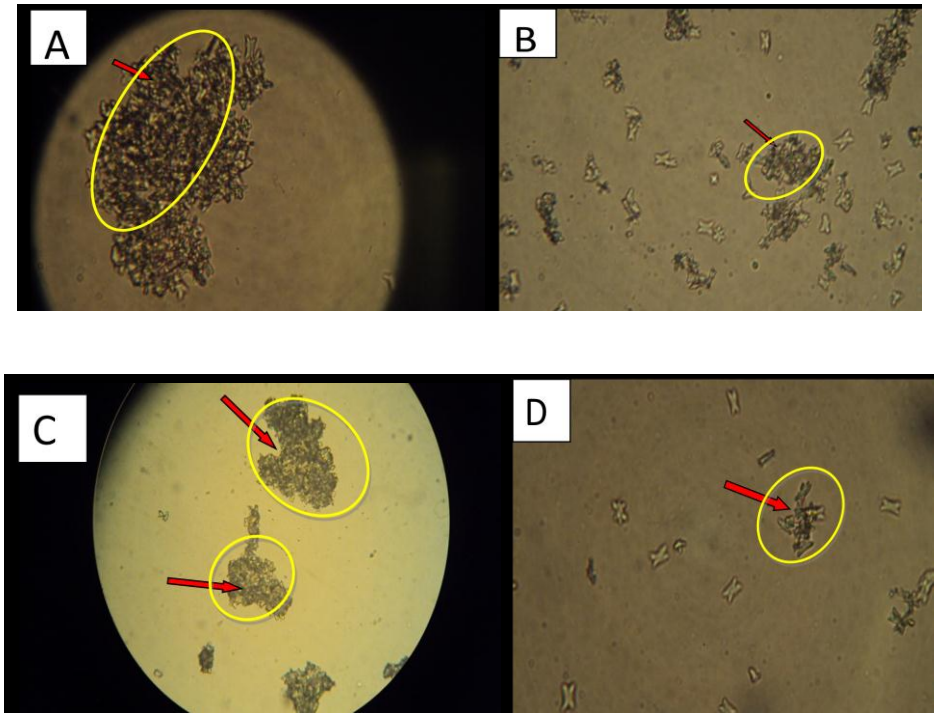
(A) *Achyranthes aspera;* (**B**) *Rumex abyssinicus;* (**C**) *Satureja punctata*; (**D**) *Chenopodium murale;* (**E**) *Aloe pulcherrima;* (**F**) *Chenopodium ambriosoides*; (**G**).*Inula confertiflora*; (**H**) *Gomphocarpus fruticosus*; and (**I**) *Commiphora myrrh*-resins. **Note:** *Inula confertiflora* (Photo taken by Dr. Minbale Gashu) (December 2017).

Appendix 2. Medicinal plants collected in the field and the extraction processes.

Appendix 2.1. Plant specimen collection at "Entoto" around Addis Ababa, washing, and extractions in Biomedical Sciences Laboratory, AAU.

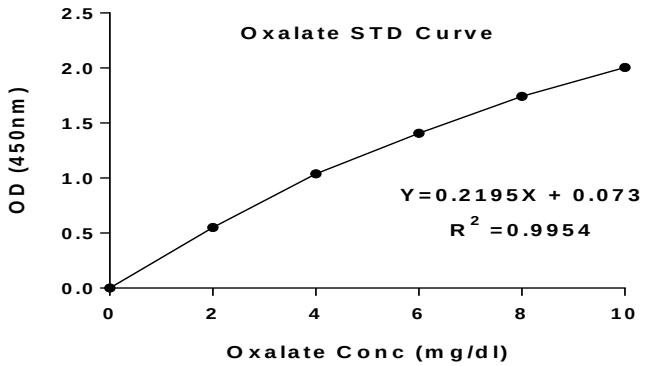
Appendix 3. Photographs showing laboratory activities.

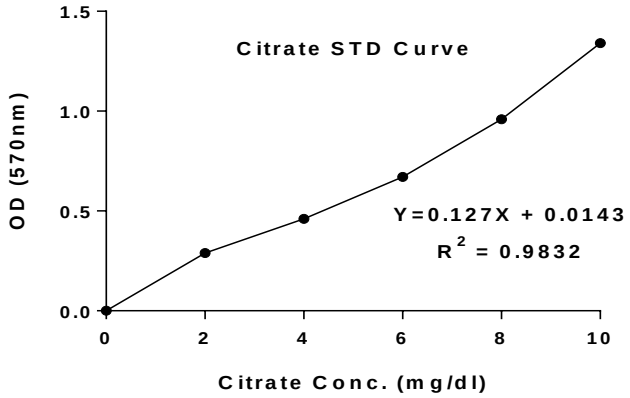
Appendix 3.1. CaOx crystal density variations in the presence of inhibitors (test extract/ drugs), and without inhibitors (the blank) during *in vitro* Calcium oxalate nucleation assay.



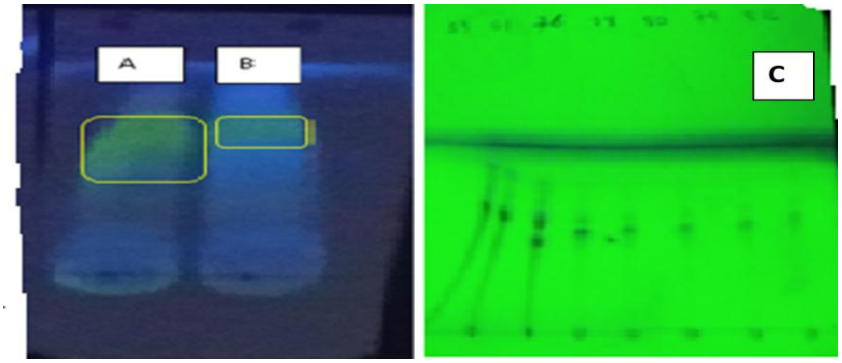
The photomicrographs observed under a light Microscope (40x) of calcium oxalate crystals in nucleation assay (in synthetic urine) at dose 200µg/ml after 30minutes incubation indicating: (A) the control group /blank, (B) K-Cit, (C) Cystone, and (D) *G. fruticosus* extracts [Arrows indicated CaOx Crystals].

Appendix 3.2. Standard Curves of (A) Oxalate, and (B) Citrate assays used to determine its concentrations in the biological samples (urine, serum and kidney homogenates).



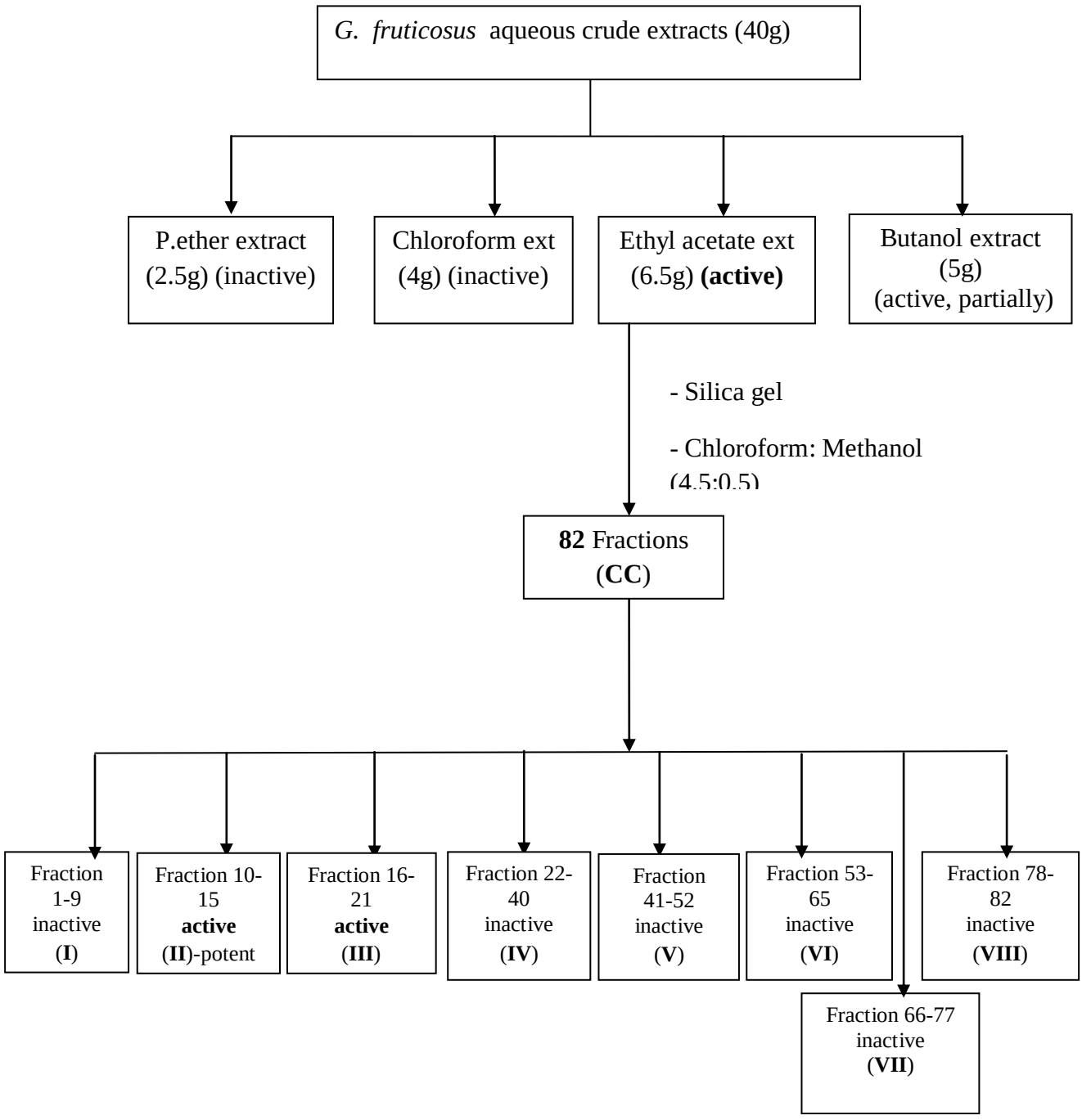


Appendix 3.3. Visualizing TLC Plates. Thin layer chromatography (TLC) for *Gomphocarpus fruticosus* leaf extracts (A) EtOAc fraction, and (B) BuOH fraction at 365nm.



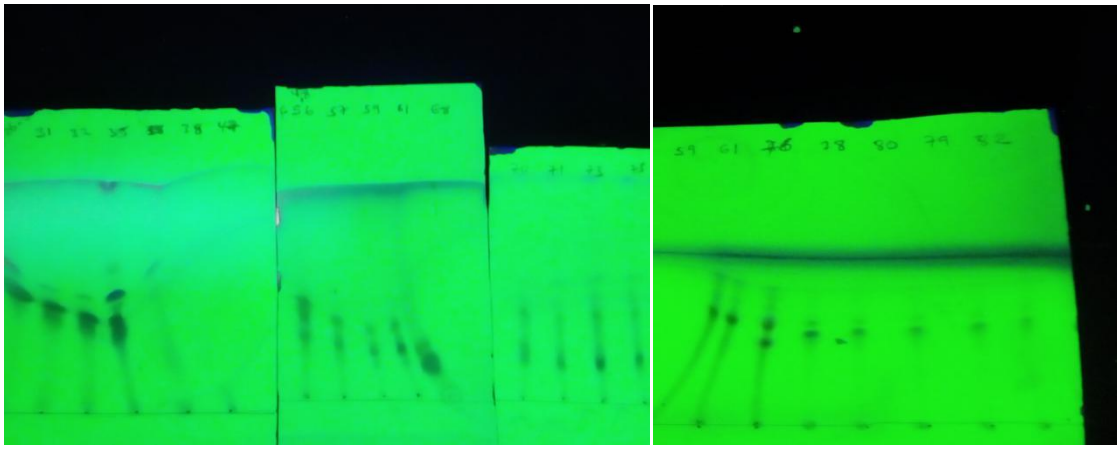
The solvent mixture/system: Dichromethane: Methanol (4.5: 0.5); (C) TLC for *G. fruticosus* EtOAc CC fractions at 254nm. The solvent System: Chloroform: Methanol (4.5:0.5), at Organic Chemistry Laboratory of AAU, August 2019.

Appendix 3.4. Schematic representations for Column Chromatography (CC) fractions of *G. fruticosus* EtOAc extracts .



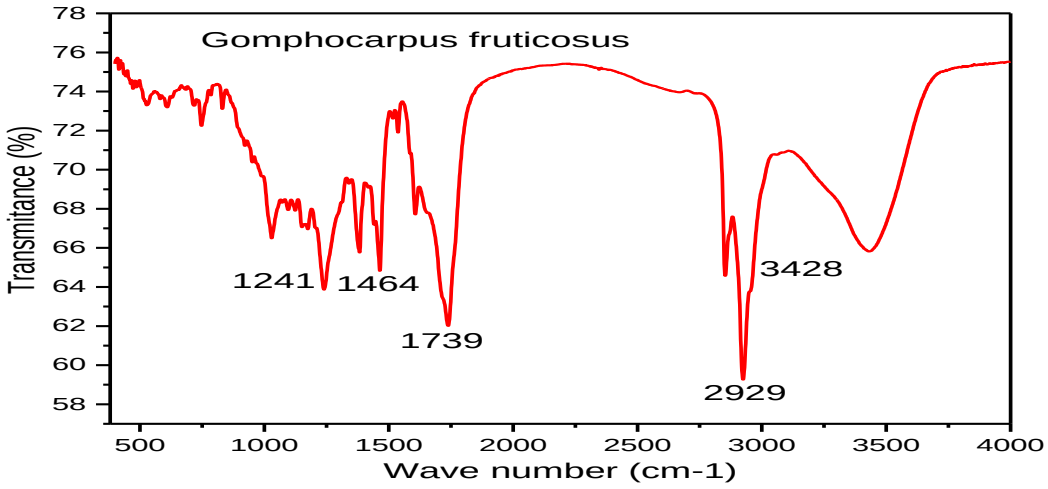
Note: ext = represents extract

Appendix 3.5. Visualizing TLC Plates.



Thin layer chromatography (TLC) for *Gomphocarpus fruticosus* EtOAc fractions. The Solvent System was Chloroform: Methanol (4.5:0.5), at Organic Chemistry Laboratory of AAU, August 2019.

Appendix 3.6. FTIR Spectrum analysis of *G. fruticosus* EtOAc active fraction indicating functional groups.



The FTIR characterization of the *G. fruticosus* EtOAc active fraction identified different frequencies of bending and stretching of functional groups. This showed characteristic bands at 3428 cm^{-1}, which could be related to stretching vibration of O-H groups or phenol OH, the bands at 2929 and about at 2880 cm^{-1} are due to alkane C-H stretching. A band found at 1734 cm^{-1} is due to the stretching vibration of C=O. The band about at 1590 cm^{-1} and at 1464 cm^{-1} could be related to the stretching vibration of aromatic ring or conjugated C=C. The bands about at 1391 cm^{-1} and at 1241 cm^{-1} would be related to C-H bending

vibration and to O-H bending vibration respectively. The band about at 1060 cm^{-1} would be due to C-O stretching vibration. Although IR spectra needs to be supported by GC-MS spectral data, this preliminary finding showed a phenol structure.

Appendix 4. Phytochemical screening methods.

Test for Alkaloids

About 2 mg of the ethanol solutions of the crude extract was mixed with 2 ml of 1% HCl and heated gently. Then, Mayer's reagent was added to the mixture. The formation of turbidity or precipitate was taken as evidence for the presence of alkaloids (Yadav and Agarwala, 2011). In addition, 1gm powder samples taken in a conical flask and added ammonia solution (3 ml). It was allowed to stand for a few minutes to evaluated free alkaloids. Chloroform (10 ml) was added to the conical flask shaken by hand and then filtered. The chloroform was evaporated from the crude extract by water bath, and added Mayer's reagent (3 ml). A cream colour precipitation was obtained immediately that showed the presence of alkaloids (Hossain *et al.,* 2013).

Test for Flavonoids

Alkaline reagent test: 2 mg of the crude extract was taken in a test tube and mixed with 2 ml of 2% solution of NaOH solution. An intense yellow colour was appeared in the test tube. This colour became colourless on the addition of a few drops of diluted acid that indicated the presence of flavonoids (Yadav and Agarwala, 2011; Hossain *et al.,* 2013).

Test for Glycosides

Molisch's test: 2 mg of the ethanol solution of the crude extract was mixed with 2 ml of Molisch's reagent and the mixture was shaken properly. After that, 2 ml of the concentrated H_2SO_4 was poured carefully along the side of the test tube. The appearance of a reddish-violet ring at the interphase indicated the presence of carbohydrate (Yadav and Agarwala, 2011).

Test for Terpenoids

About the 2 ml of aqueous crude extract solution was dissolved in 2 ml of chloroform and evaporated to dryness. To this, 2 ml of concentrated H_2SO_4 was added and heated for about 2 minutes. Formation of grayish colour indicated the presence of terpenoids (Yadav and Agarwala, 2011).

Test for Resins

Turbidity Test: About 10 ml of distilled water were added to 1 mg of dried plants, and ultrasonicated for 15 min at 30 ºC. The mixture was filtered. The occurrence of turbidity showed the presence of resins (Keo *et al.,* 2017). Another test was performed by mixing

1mg of the crude extract, which was dissolved in 2ml acetone and the solution was poured in distilled water. Turbidity indicates the presence of resins.

Test for Saponins

About 5 ml of distilled water crude extract was prepared in a test tube. Then, a drop of sodium bicarbonate solution was added, and it was shaken vigorously by hand for 5 min. It was left for 3 minutes. A stable foam layer on the top of the test tube indicated the presence of saponins (Yadav and Agarwala, 2011; Hossain *et al.,* 2013).

Test for Steroids

Salkowski's test: 2 mg crude extract was dissolved with 3 ml of chloroform. Then, 3 ml of concentrated H_2SO_4 was added to the test tube, sidewise and shaken gently. A red colour produced in the lower chloroform layer indicated the presence of steroids (Yadav and Agarwala, 2011; Hossain *et al.,* 2013). Another test was performed by mixing 2 mg of the crude extract with 2 ml of chloroform. Then, 2 ml of each of the concentrated H_2SO_4, and acetic acid were poured into the mixture. The development of a greenish coloration indicated the presence of steroids (Yadav and Agarwala, 2011).

Test for Tannins

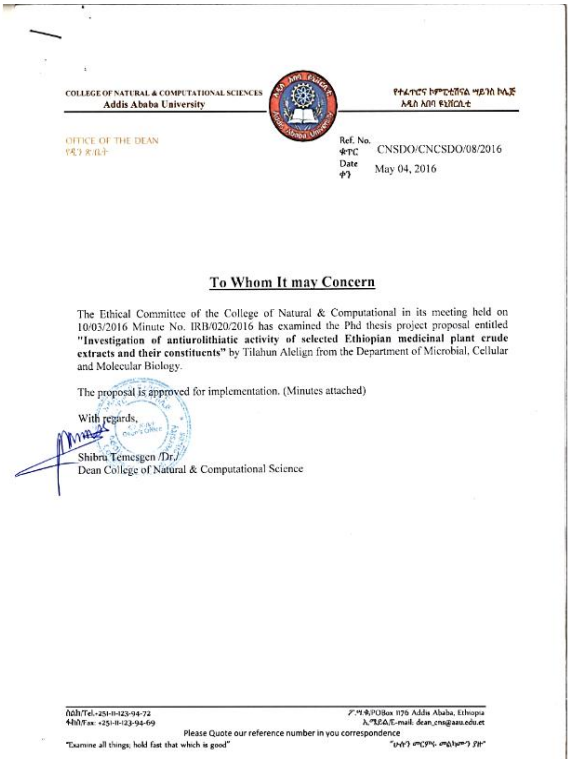
About 2 ml of the ethanol solution of the extract was taken in a test tube, and diluted with chloroform and added the acetic anhydride (1 ml). Finally, sulphuric acid (1 ml) was added carefully by the side of test tube to the solution. A green colour was formed, which showed the presence of tannins (Yadav and Agarwala; Hossain *et al.,* 2013). In addition, 2 ml of ethanol solutions of the crude extract was mixed with 2 ml of 2% solution of $FeCl_3$. A blue-green coloration indicated the presence of tannins (Yadav and Agarwala, 2011).

Test for Phenols

Ferric chloride test: One gram (1gm) of dried plant was added with 10 ml of ethanol and ultrasonicated for 15 min at 30 ºC. The mixture was filtered, and 2 ml of the filtrate was added with 5 ml of distilled water. The filtrate was treated with few drops of Iron III chloride (5% $FeCl_3$). Dark green color indicated the presence of phenolic compounds (Yadav and Agarwala, 2011; Keo *et al.,* 2017).

Appendix 5. Miscellaneous

Appendix 5.1. Certificate of Research Approval by Institutional Ethical Committee.



COLLEGE OF NATURAL & COMPUTATIONAL SCIENCES
Addis Ababa University

OFFICE OF THE DEAN

Ref. No. CNSDO/CNCSDO/08/2016
Date May 04, 2016

To Whom It may Concern

The Ethical Committee of the College of Natural & Computational in its meeting held on 10/03/2016 Minute No. IRB/020/2016 has examined the Phd thesis project proposal entitled **"Investigation of antiurolithiatic activity of selected Ethiopian medicinal plant crude extracts and their constituents"** by Tilahun Alelign from the Department of Microbial, Cellular and Molecular Biology.

The proposal is approved for implementation. (Minutes attached)

With regards,

Shibru Temesgen /Dr./
Dean College of Natural & Computational Science

Please Quote our reference number in you correspondence
"Examine all things; hold fast that which is good"

COLLEGE OF NATURAL & COMPUTATIONAL SCIENCES
Addis Ababa University

OFFICE OF THE DEAN

Ref. No. CNSDO/396/08/2016
Date April 22 2016

To Whom It may Concern

College of Natural Science Institutional Review Board (CNS-IRB) in its meeting held on 16/04/2016 Minute No. IRB/021/2016 has reviewed an Phd thesis project proposal entitled **"Respospective study on the prevalence or Renal stone, Gall stone and Their Co-occurrence among patients that attended Tikur Anbessa and St. Paulos Teritary Referral Hospitals (From 2006-2015),** Addis Ababa by Tilahun Alelign from the Department of Microbial, Cellular and Molecular Biology

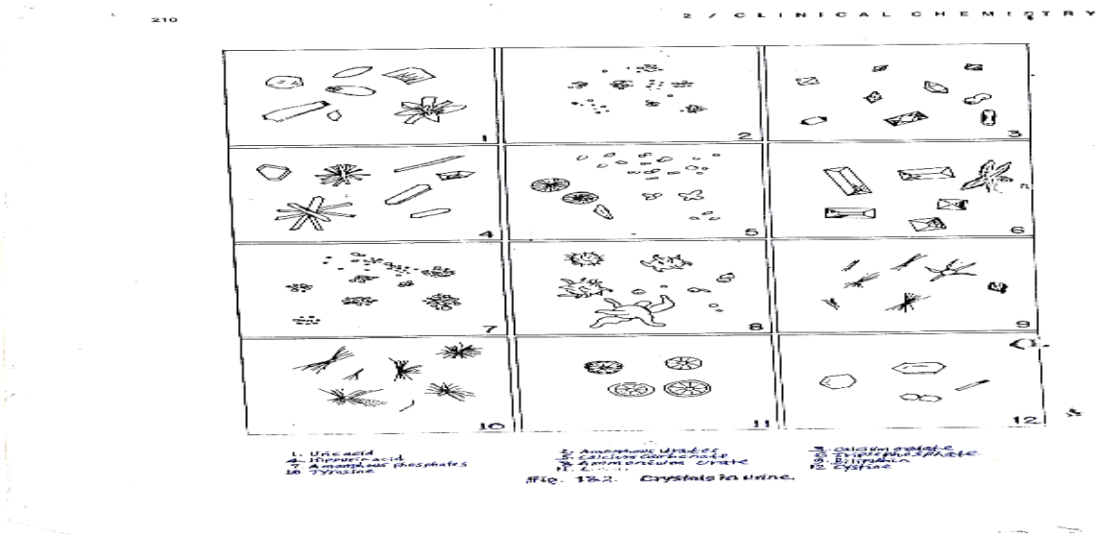
The proposal was approved for implementation.

With regards,

Shibru Temesgen /Dr./
Dean College of Natural & Computational Science

Please Quote our reference number in you correspondence
"Examine all things; hold fast that which is good"

Appendix 5.2. Crystals in the urine.



Appendix 6. **Declaration**

I, the undersigned, declare that the dissertation is my own original work and it has not been presented for degrees in any other university. All sources of the material used have been duly acknowledged.

Name: *Tilahun Alelign Wassie (ID. No. GSR/1856/06)*

Signature ----------

Date: *14 June 2021*