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Study of the Curative Activity of Lemon Juice and *Boswellia Carterii* Infusion on the Urinary Crystallization of Calcium Salts in Rabbit Models

A Research Submitted to The Council of The College Of Veterinary Medicine / University of Al-Qadisiyah In Partial Fulfillment of The Requirement For The Degree of Bachelor of Science In Veterinary Medicine.

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بسم الله الرحمن الرحيم

﴿إِنَّ فِي خَلْقِ السَّمَاوَاتِ وَالْأَرْضِ وَاخْتِلَافِ اللَّيْلِ وَالنَّهَارِ لَآيَاتٍ لِأُولِي الْأَلْبَابِ(190) الَّذِينَ يَذْكُرُونَ اللَّهَ قِيَامًا وَقُعُودًا وَعَلَى جُنُوبِهِمْ وَيَتَفَكَّرُونَ فِي خَلْقِ السَّمَاوَاتِ وَالْأَرْضِ رَبَّنَا مَا خَلَقْتَ هَذَا بَاطِلًا سُبْحَانَكَ فَقِنَا عَذَابَ النَّارِ(191)﴾

صدق الله العلي العظيم

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Certificate of Supervisor

I certify that the research entitled "**Study of the Curative Activity of Lemon Juice and *Boswellia Carterii* Infusion on the Urinary Crystallization of Calcium Salts in Rabbit Models"** was prepared under my supervision at the College of Veterinary Medicine / University of Al-Qadisiyah.

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Acknowledgment

In the Name of Allah, the Most Merciful, the Most Compassionate all praise be to Allah, the Lord of the worlds; and prayers and peace be upon the messenger Mohamed and his family.

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DEDICATION

This research is dedicated to:

- The sake of Allah, my Creator and my Master,

My great teacher and messenger, Prophet Mohammed (May Allah bless and grant him), who taught us the purpose of life.

- My homeland Iraq, the warmest womb.
- The great Iraqi Popular Mobilization Forces and martyrs, the symbol of sacrifice.
- The College of Veterinary Medicine; my second magnificent home.
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List of abbreviations

Abbreviations	Full form
µL	Microliter
µm	Micrometer
B.W.	Body weight
BUN	Blood urea nitrogen
Ca^{+2}	Calcium
CaOx	Calcium oxalate
CaP	Calcium phosphate
D.W.	Distilled water
dl	Deciliter
gm	Gram
H&E	Hematoxylin and eosin
h.	hour
HCBD	hexachloro-1,3-butadiene
k.g.	kilogram
KOx	Potassium oxalate
KSD	kidney stone disease
Lab.	laboratory
Ltd	limited
M.W.	Molecular weight
MDCK	Madin-Darby canine kidney
mg	milligram
Mg^{+2}	magnesium
ml	milliliter
m/Eq.	milli-equivalent
ºC	centigrade
rpm	Round per minute
s.creatinine	Serum creatinine
SPSS	Statistical Package for the Social Sciences

ABSTRACT

Abstract

This study was aimed to evaluate the effect of *Boswellia carterii* (olibanum) infusion and fresh lemon juice in comparison to oxalic acid administered group. Twelve rabbits of both sexes were used in the present study. They were divided into three groups (four rabbits in each group). Oxalic acid was administered as a single dose (333 mg/kg B.W. orally) to induce renal calcium crystallization in all groups two hours after giving of distilled water (at a dose of 3 ml/ kg B.W) for the first control group, fresh lemon juice (at a dose of 6 ml/Kg B.W orally) for the second group (T1-group). *Boswellia Carterii* infusion (at a dose of 1 gm/10 ml/ rabbit orally) for the third group (T2-group), All the above tested agents were administered daily with the aid of gastric gavage all over the duration of the experiment (ten days).

Therapeutic effects of the tested agents were detected through assessment of renal function by measuring blood urea nitrogen (mg/dl) and serum creatinine (mg/dl) levels that were done by taking blood specimens from each animal of the studied groups at the third and tenth (last) days of the study duration. Urine samples also could be collected from the tested rabbits at the third and last days to be used later on as indicators of kidney improvement via microscopic examination of urine sediments. In addition to histo-pathological examination of kidney sections of the tested animals which were sacrificed at the last day of experiment.

In conclusions, all of the tested plants resulted in significant therapeutic effect by reducing the levels of blood urea nitrogen, serum creatinine, and urinary excretion of calcium oxalate crystals. Histo-pathological examination of kidney sections also revealed an obvious improvement in the kidney samples came from attenuating calcium deposition and other nephron-pathological changes caused by oxalic acid.

CHAPTER ONE

INTRODUCTION

1.1. Introduction

Calcinosis of kidney was a term given by Albright in 1934 to describe the deposition of calcium salts in the renal parenchyma and it is used to describe diffuse, fine, renal parenchymal calcification (Khan and MacDonald, 2008; Louis, 2009). Conditions that can cause accumulation of calcium crystals in renal tissues include chronic glomerulonephritis, primary hyperoxalurias, renal tubular acidosis, renal cortical necrosis, ethylene glycol toxicity, hypercalcemia, use of certain medication such as acetazolamide, and vitamin D toxicity (Feehally *et al*., 2007; Monk and Bushinsky, 2008; Al-Haggar *et al*., 2009).

The excessive deposition of calcium crystals may lead to chronic tubule-interstitial disease and renal insufficiency or failure. Calcified cellular debris results in obstruction of the tubules, leading to occlusive atrophy of the nephrons, interstitial fibrosis and defect of tubular functions such as tubular acidosis and salt-losing nephritis. The histologic findings include calcium oxalate or calcium phosphate crystal deposits that mainly appear in the renal interstitium, but deposits may also be seen within the renal tubules (Khan and MacDonald, 2008).

Excessive ingestion of oxalates-containing food can result in a similar appearance and this encourage many researchers to use oxalic acid and calcium oxalate in an experimental model of renal calcium crystallization. This is due to its powerful necrotic effect on the glomerulus tissues, edematous effect on stroma and scarring that is usually confined to the cortex, in addition to the deposition of calcium oxalate crystals in and around the dead tissue (Khan and MacDonald, 2008; Feehally *et al*., 2007).

Renal calcification can be treated by many diuretic drugs and medicinal plants in order to reduce symptoms and prevent more calcium crystals from being deposited in the renal interstitium.

This paper described the ability of *Boswellia carterii* extract which prepared by infusion and fresh lemon juice to overcome the chemically-induced crystallization of calcium in renal parenchymal tissues that may represent an alternative to pharmacological therapies such as potassium citrate. In this study, we concurrently compared the calcium deposition rate in rabbits who received olibanum infusion and lemon juice therapy with that of control rabbits who received no medical prophylaxis.

CHAPTER TWO

REVIEW OF LITERATURES

2.1. Urolithiasis:

Urolithiasis or kidney stone disease (KSD) is a health condition that is rarely life-threatening, but has severe morbidity with a potential lifetime risk for up to 13 % of the general population. In most cases, kidney stones [mostly calcium (Ca^{+2}) salts] start to build up in adults when the skeleton is fully formed and the amount of excreted Ca^{+2} rises, e.g. when a large amount of $Ca^{+2}$and/or other metabolites is excreted in a small volume of urine because of the physiological need to conserve water (Bushinsky *et al*., 2008).

The onset of urolithiasis usually follows the ingestion and/or production of high amounts of main stone-forming compounds, such as oxalate. However, while the ingestion of oxalate-rich food is easily preventable, other urolithiasis-associated factors are more or less veiled and include various genetic mutations that alter general metabolism and homeostasis (Watts, 2005).In addition, some well-known systemic disorders, such as obesity or type 2 diabetes mellitus, significantly increase the incidence of urolithiasis (Sakhaee, 2008).

2.1.1.Pathophysiology:

The crucial moment in the pathophysiology of kidney stones is the formation of crystals in the tubular fluid or urine. Crystallization occurs when the crystal-forming materials, such as calcium oxalate (CaOx), calcium phosphate (CaP) or uric acid reach their upper metastable limits, which result in first solid phase precipitations, then grow, aggregate, and finally form stones (Coe and Parks, 1997).

All people tend to develop these crystals. However, populations prone to disease have larger and coarser crystals, leading to haematuria, pain, and, if not diagnosed on time, formation of sand-like material, gravel,

and stones. These stones are basically crystals embedded in protein complexes.

The most abundant are stones made of CaOx monohydrate or dehydrate, which are more or less mixed with apatite (phosphate-based minerals), brushite (CaP dihydrate),or urate (uric acid). Rare and uncommon stones are predominantly constituted of CaP, cystine, struvite(ammonium magnesium phosphate), ammonium salts, and sometimes therapeutic drugs (Coe and Parks, 2000).

Over 70 compounds were identified in a study on >10,500 stones; CaOx was the predominant compound present in ~87 % of cases, followed by CaP (~80 %) and purines (~19 %) (Daudon *et al*., 1995). In another study (Yoshida and Okada, 1990), approx. 70,000 stones were analyzed during a 10-year period and nearly 80 % were built from CaOx and/or brushite, ~7 % mainly from carbonate and various apatites, ~5 % from urate, and ~1 % from cystine. These data indicate that elevating the concentrations of certain compounds in urine can trigger the onset of KSD.

Although it has been proposed that hyperoxaluria could be significantly more important for the formation of CaOx stones than hypercalciuria (Robertson and Peacock, 1980), others have concluded that oxalate is most likely a less important contributor under physiological conditions, because oxalate secretion is relatively small compared to the secretion of calcium (Coe and Parks, 2000). However, it has been established that rises in urine oxalate concentrations elevate the potential for stone formation (Coe and Parks, 2000).

2.1.2.Sources of oxalate in the organism

Oxalate in the mammalian body originates from two sources. Most of body oxalate is a metabolic end-product generated largely in the liver and represents 85 % to 90 % of the total oxalate circulating in blood

(endogenous oxalate). An unknown proportion of the liver-produced oxalate is removed via bile secretion. The remainder (10 % to 15 %) of blood oxalate (exogenous oxalate) originates from the absorption of food in the gastrointestinal tract (Chen *et al*., 2003). The ratio between liver-generated and absorption-related sources depends on oxalate content in ingested food (Williams and Wilson, 1990). Both sources have a potentially important role in increasing oxalate concentrations in plasma and urine.

The bulk (90 % to 95 %) of circulating oxalate is ultimately excreted by the kidneys, whereas some 5 % to 10 % of blood oxalate is excreted in the terminal parts of the small intestine and colon. Only a small part of the total amount of oxalate in food (2 % to 10 %) is absorbed (which, however, contributes to 10 % to 15 % of the total blood oxalate), whereas the bulk (90 % to 98 %) is retained in the intestine.

To determine the food-related oxalate intake, various foodstuffs have been tested for oxalate content. Oxalate is present in different plant parts and in different quantities, the highest content being 15 % to 20 % of plant total dry weight (Pak *et al*., 2004). Spinach and other leafy vegetables are among the leading plants in this respect, with varying concentrations of oxalate as the plant matures. Chocolate, tea, vegetable juice, as well as cranberry and orange juices, were determined among the other notable oxalate sources. However, only spinach and other leafy vegetables are capable of actually inducing hyperoxaluria and, potentially, KSD (Caliskan, 2000).

Certain spices can also be a high source of oxalate, particularly cinnamon and turmeric, both of which are often recommended as healthy supplements that benefit the organism. Although both are rich in oxalate, due to different ratios of soluble and non-soluble oxalate, only turmeric

significantly increases urine oxalate and thus represents a potential risk factor for urolithiasis (Tang *et al*., 2008).

When comparing food consumption with regard to stone formers and non-formers, it has been established that stone formers consume significantly higher amounts of proteins (of both plant and animal origin) and purines (Trinchieri *et al*., 1991). The bulk of oxalate absorption, which can be modulated by Ca^{+2}, Mg^{+2}, and fibers in ingested food, occurs in proximal parts of the digestive tract 1 h to 8 h after ingestion (Holmes and Assimos, 2004), with two distinct peaks at 40 min and 120 min (Chen *et al*., 2003).

In humans, the daily oxalate intake reportedly ranges between 44 mg per day and352 mg per day, with an average of 130 mg to 152 mg per day (Holmes *et al*., 2001). However, the intestinal absorption of oxalate does not follow the ingested amount of this compound linearly; the highest ingestion/absorption ratio takes place at an ingested dose of 50 mg per day, while at higher doses (up to 250 mg per day), the overall absorption is higher but the ingestion/absorption ratio significantly lower (Siener *et al*., 2001). Exogenous oxalate has long been assumed to significantly contribute to hyperoxaluria. However, the current opinion is that hyperoxaluria is the result of a combination of increases in both the endogenous production and intestinal absorption of oxalate (Holmes *et al*., 1995).

Some studies have indicated that exogenous oxalate does not play any significant role in stone formation. Patients with ileo-cecal resection and jejuno-ileal bypass showed higher intestinal absorption and urine excretion of oxalate, indicating that oxalate absorption does not dependent solely on the amount ingested. These differences in oxalate absorption could be due to the action of the oxalate degrading gram-negative bacteria

Oxalobacter formigenes (O. formigenes), originally discovered in the rumen of cattle (Dawson *et al*., 1980).

2.1.3.Formation of oxalate crystals and onset of KSD

When the levels of oxalate and other factors in the urinary system reach the upper metastable limits that enable the formation of crystals, additional prerequisites seem to be needed before stones start to form. Damage to the cell membranes of renal tubules is one of the most important factors initiating stone formation.

Given the fact that the growth time for a crystal to reach an obstructing diameter of ~200 μm is at least1.5 h (Selvam, 2002), it is highly unlikely that a crystal would attach to a healthy tubule with unobstructed urine flow(Verkoelen *et al*., 1998). A crystal is more likely to attach and obstruct the urine flow if the primary injury is located in the anatomically narrow part of the tubule (Selvam, 2002).

Crystaluria also appeared dose-dependent after injecting hexachloro-1,3-butadiene(HCBD) alone and then abated with time, indicating that, in addition to oxalate, the formation of urinary crystals also requires a tubular damaging agent (Gambaro *et al*., 2006).Oxalate crystals, composed of KOx or CaOx monohydrate, can directly damage Madin-Darby canine kidney(MDCK) cells in a culture (Hackett *et al*., 1997).

2.1.4.oxalate concentration and urolithiasis

Various compounds, pathophysiological conditions, and even intestinal bacterial flora are known to either promote or inhibit the onset and severity of oxalate urolithiasis. They are summarized in Table 2.1:

Table (2.1): Compounds and conditions that affect the onset and severity of oxalate urolithiasis (Brizka *et al*., 2013).

Compounds or conditions	Promoters of urolithiasis	Inhibitors of urolithiasis
Oxalate precursors from food	glyoxylate, galactose, lactose, tryptophan, phenylalanine, tyrosine, creatinine, purines	
Vitamins	vitamin C (oxalate precursor)	vitamins A, C and E (scavengers of free radicals), vitamin B6
Various compounds		heparin, chondroitin sulfate, heparan sulfate, hyaluronic acid, polyapartic acid, polyglutamic acid, dextran sulfate, citrate
Specific proteins		crystal adhesion inhibitor protein, osteopontin, nephrocalcin, Tamm-Horsfall protein, prothrombin fragment 1
Mediators of the inflammatory response		monocyte chemo-attractant protein (a response to free radicals)
Compounds affecting free radicals	potassium oxalate monohydrate, calcium oxalate monohydrate (promoters of free radicals)	catalase, superoxide dismutase, manitol (free radical scavengers)
Physical influences	trauma and membrane injury	
Microorganisms		*Oxalobacter formigenes*

2.2. Tested agents:

2.2.1.Lemon

2.2.1.1. Description:

The lemon, ***Citrus limonum*** (L.) Osbeck, is a species of small evergreen tree in the flowering plant family Rutaceae, native to Asia. The juice of the lemon is about 5% to 6% citric acid, with a pH of around 2.2, giving it a sour taste. The distinctive sour taste of lemon juice makes it a key ingredient in drinks and foods such as lemonade and lemon meringue pie. (Wikipedia, Lemon**)**

Figure (2.1): lemon fruits

2.2.1.2. Chemical constituents

The presence of glycosides, tannins, flavonoids, alkaloids, saponins, carbohydrates, proteins and water-soluble vitamins besides citric acid in the reconstituted fruit juice extract was examined by standard phytochemical methods (Harbone, 1973).

Physicochemical screening of lemon peel showed different chemicals including; protein (9.42 %), fat (4.98%), ash (6.26%), fiber

(15.18%), sodium (755.5 mg/100g), potassium (8600 mg/100g), calcium (8452.5 mg/100g), copper (4.94 mg/100g), iron (147.65 mg/100g), magnesium (1429.50 mg/100g), zinc (13.94 mg/100g) and phosphorus (6656 mg/100g) (Janati *et al*., 2012)

2.2.1.3. Medical uses

Lemon juice is believed to be effective against diphtheria and upper respiratory tract infections. In South Africa, lemon juice has been used in the treatment of oral thrush (Wright *et al.*, 2009). In many parts of the world, lemon juice is also used as sanitizers to remove food borne pathogens from fresh fruits, vegetables and fish. Studies have shown that concentrated or freshly squeezed lemon juice has antibacterial activity against *Vibrio species*, *Salmonella typhimurium.*

In Nigeria, herbalists use lemon juice in the treatment of diarrhea, dysentery, typhoid fever, wound infections, urinary tract infection and arthritis(Okeke *et al*., 2015).

2.2.2. *Boswellia carterii* (olibanum):

2.2.2.1. Description

Frankincense (also known as *olibanum*) is an aromatic resin used in incense and perfumes, obtained from trees of the genus *Boswellia* in the family Burseraceae.

Figure (2.2): Olibanum resin

2.2.2.2. Chemical composition

These are some of the chemical compounds present in frankincense:

- "acid resin (56 %), soluble in alcohol and having the formula $\mathbf{C_{20}H_{32}O_4}$"
- gum (similar to gum arabic) 30-36%
- 3-acetyl-beta-boswellic acid (*Boswellia sacra*)
- alpha-boswellic acid (*Boswellia sacra*)
- 4-O-methyl-glucuronic acid (*Boswellia sacra*)
- incensoleacetate, $C_{21}H_{34}O_3$
- phellandrene
- (+)-cis- and (+)-trans-olibanic acids(Wikipedia, *Boswellia carterii*).

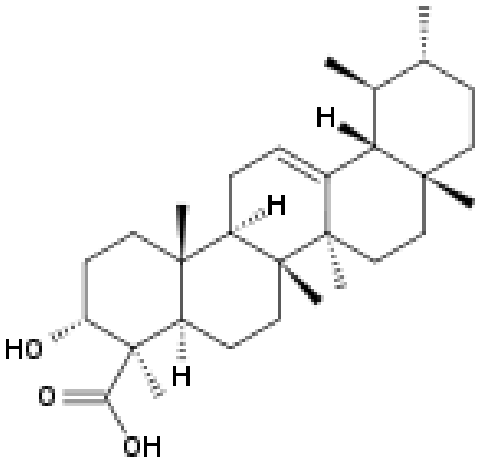

Figure (2.3): Structure of β-boswellic acid, one of the main active components of frankincense

2.2.2.3. Uses

It has been mainly used in traditional Chinese medicine to alleviate pain and inflammation (Al-Jawad *et al*., 2012). The extract of *B. carterii* contains potentially active triterpene acids such as boswellic acids and incensole acetate (Frank *et al*., 2009). The plant resin has been used for treating ulcerative colitis, chronic colitis,

Crohn's disease and osteoarthritis due to its anti-inflammatory effects. In the folk medicine, *B. carterii* resin is prescribed either alone or in combination with other plants for diabetic patients (Helal *et al*., 2005). Historically, it has been used as incense in religious and cultural ceremonies, and it is now widely used as an adhesive agent and as an ingredient in cosmetic preparations (Zhang *et al*., 2013; Al-Mehdar and Albattah, 2016).

2.3. Aims of the study

The present study aimed to:

1- Evaluate the activity of oxalic acid to deposit calcium crystals in renal parenchymal tissue during a period of 10 days to be studied later on as a model of calcium oxalate urolithiasis.

2- Demonstrate the efficacy of oral dosing of lemon juice and *Boswellia carterii* infusion in attenuation of calcium crystal deposition and urinary calcium oxalate excretion.

CHAPTER THREE

MATRERIALS AND METHODS

Materials and Methods

3.1. Materials:

3.1.1. The laboratory apparatuses , Instruments , chemicals agents and kits used in this study with their remarks: are summarized in table (3.1),(3.2),(3.3) and (3.4).

Table (3.1): The Laboratory apparatuses that used in the present study

No.	Laboratory apparatuses	Company	Country
1	Centrifuge	GEMMY	Taiwan
2	Electrical grinder	Shownic	China
3	Microscope	Olympus	Japan
4	Refrigerator	Marshal / Kyoto	Japan
5	Sensitive Balance	Kern	Germany
6	Spectrophotometer	Apple	Japan

Table (3.2): The laboratory Instruments that used in the present study with their remarks

No.	Laboratory Instruments	Company	Country
1	Beaker 150 ml	Jlassco	Germany
2	Beaker 400 ml	Marnfield	Germany
3	Disposable Syringes 3ml	Medeco	Belgium
4	Micropipette 5 - 50 µL and Tips	CYAN	Belgium
5	Sterile plain tubes	Proton	Malaysia
6	Eppendorf tubes	Sarstedt	Germany

Table (3.3): Chemical agents and solutions that used in the present study with their remarks

No.	Chemical agents	Company	Country
1	Chloroform	Al-Tharthar	Iraq
2	Distilled water	Lab. of microbiology	Iraq
3	Ethanol 96%	Samarra	Iraq
4	Formalin 10%	Labort	India
5	Oxalic acid	Hopkins and William	England

Table (3.4): kits that used in in the present study with their remarks

No.	kits	Company	Country
1	Blood urea nitrogen (BUN) kit	RANDOX (Randox Laboratories Ltd)	United Kingdom
2	Creatinine kit	BIOLABO Reagents (Biolabo SA)	France
3	Urine dipstick (CYBOW)	DFI Co., Ltd	Korea

3.1.2. Animals

Twelve healthy, local, domestic rabbits weighing (755-1259) gm of both sexes were needed in this study. They were provided by the local animal's market at Al-Diwaniya province. All rabbits were housed 3 per cage, which was provided with a wire-mesh floor. They were fed standard oxoid-pellets and given water *ad libitum.* .

3.1.3. Tested Medicinal plants

Resins of *Boswellia carterii* and lemon fruits were used in the present study. These plant materials were obtained from the local market.

3.1.4. Experimental design and animal grouping:

Rabbits were randomly divided into 3 groups (four animals in each group). Calcium crystallization of kidney was induced in each group of animals by using a single dose of oxalic acid (M.W.=126.07 gm/mol) 333 mg/kg B.W. orally by using gastric gavage to avoid aspiration of this agent (Al-Ebady, 2005). The experiment groups were as the following:

1- **The first group (control group):** it was received 3 ml of distilled water orally 2 hours before induction of renal calcium crystallization by oxalic acid and remained under the effect of oxalic acid all over the duration of experiment (10 days).
2- **The second group (T1-group):** it was treating with freshly squeezed lemon juice as a single daily dose of 6 µl/gm body weight by oral gavage (i.e., 6 ml/ kg B.W.) at the concentration of 100%(Touhami *et al*., 2007), started 2 hours before administration

of oxalic acid and continued for 10 consecutive days. (Pretreatment was to prevent or attenuate the severity of calcium ions deposition within renal glomeruli that could be later produced by oxalic acid).

3- **The third group (T2-group):** it was given *Boswellia carterii* infusion at a dose of 1 gm/ 10 ml of D.W/ rabbit as described above (Al-Jawad and Khalaf, 2005).*Boswellia carterii* infusion was used in a similar way to that done with the second group.

3.2. Methods:

3.2.1. Method of *Boswellia carterii* extraction by infusion:

The aqueous extract of *Boswellia Carterii* was prepared by dissolving one volume of well grinded-plant resins to ten volume of water (i.e. 1 gm of plant to 10 ml of D.W.) at 80 ^{0}C in a flask, after shaking well it was allowed to stand for 10 minutes, cooled and filtered. For dispensing purposes, aqueous extract by infusion should be used within 12 hour of its preparation (Al-Jawad and Khalaf, 2005).

Whereas Lemon juice was prepared by squeezing as a fresh drink without additives and given to rabbits once at the time of dosing

3.2.2. Blood sampling:

Blood samples were taken for biochemical analysis of renal functions before induction of renal calcium crystallization by administration of oxalic acid to determine the normal values of blood urea nitrogen (BUN) and serum creatinine of all tested animals and after 3,10

days of renal calcium crystallization induction by oxalic acid administration. Blood samples were obtained by the heart puncture; 3 ml could be aspirated in each occasion. They were collected in dry glass centrifuge tubes, allowed to coagulate at room temperature and centrifuged at 3500 rpm for 5 minutes for the purpose of serum separation. The clear, non-haemolysed supernatant sera were separated using clean, dry, disposable, plastic syringes and stored at -20°C for subsequent biochemical analysis.

3.2.3. Tissue preparation:

At the end of experiment (the tenth day), all rabbits were sacrificed under chloroform anesthesia to confirm the incidence of calcium crystal deposits. Their kidneys were isolated, washed, rapidly fixed with 10% formalin and subjected to histo-pathological examination to evaluate the microscopic changes of the renal tissue by using of light-microscopy, the sectioning of kidney samples was done at the laboratory of Baghdad at Al-Diwaniya city.

3.2.4. Biochemical analysis of serum

Blood urea nitrogen (BUN) and serum creatinine levels were measured calorimetrically (Thomas, 1998) by using spectrokinitic-photometer (CECIL, CE 2031-England) and the following kits:

*RANDOX (Randox Laboratories Ltd, United Kingdom): It had been used for determination of BUN.

*BIOLABO Reagents (Biolabo SA, France): Reagent for quantitative determination of creatinine in serum or plasma. .

3.2.5. Urinalysis:

Urine collection had been done at the 3 rd and 10 th days of experiment. Urine samples of all animal groups had send directly after collection to the laboratory of Al-Belad at Al-Diwaniya city to be subjected to macroscopic examination including physical and chemical tests with the aid of urine dipstick, and microscopic test of the urine sediment to detect the presence or absence of calcium oxalate crystals.

3.2.6. Statistical analysis:

In the present study, the obtained quantitative values were presenting as (mean±standard error). Student *t*-test was used for assessing the efficiency of employed therapy for the experiment rabbits by using SSPS version 12. The differences were accepted as significant if the calculated value for (*t*) was lessor than its tabulated value at $P<0.05$, whereas it was considered as non-significant if $P>0.05$ (Hill, 1991).

CHAPTER FOUR

RESULTS

4. Results:

BUN values were gradually and clearly increased in the control group after 3, and 10 days from induction of renal calcium crystals by oxalic acid administration **(43±0.2, 52±0.2** mg/dl respectively) (Table 4.1) when it compared with the normal value at 0 day (**35 ±0.07**mg/dl). S.creatinine levels were also elevated in the control group that measured after 3, and 10 days of oxalic acid administration (**2.1±0.5, 3.2±1.2** mg/dl respectively) in comparison to the normal value at 0 day (**1.2±8.9** mg/dl) (Table 4.2).

Levels of BUN of all animals in the treatment groups that measured at 3, and 10 days from oxalic acid administration were significantly ($p<0.05$) lowered in comparison to the control group. The values were; **21.2±0.08, 19±0.12** mg/dl respectively for the animals treated by lemon juice. In addition, BUN levels were clearly declined by treatment with *Boswellia Carterii* infusion all over the periods of blood sampling as follows; **25.5±0.13, 20±0.15** mg/dl respectively. S.creatinine levels of all animals in the treatment groups also could be measured after 3, and 10 days of oxalic acid administration, they were significantly ($p<0.05$) and gradually lowered in the group of lemon juice (**1.3±0.94, 1±1.16** mg/dl respectively), and in the group of *Boswellia Carterii* infusion (**1.1±1.39, 0.8±1.04** mg/dl respectively) when it compared with the control group.

Physical examination of urine samples that collected from control and both treatment groups at the 3 rd day of experiment showed yellow turbid appearance of urine sample. At the 10 th day of experiment, urine samples of control group still had the yellow turbid appearance besides an obvious decrement in urinary PH, while urine specimens obtained from rabbits treated with lemon juice and those treated with *Boswellia Carterii*

infusion revealed clear appearance indicating significant improvement when compared to the control group. In addition, urine sediments were also examined microscopically to prove the efficacy of tested plants in comparison to the control group. Epithelial cells, leukocytes or pus cells and calcium oxalates were recorded during urinalysis after 3 days of oxalic acid administration. Leukocytes and desquamation of epithelial cells in urine samples were significantly lowered by the *Boswellia Carterii* infusion and lemon juice at the tenth day in comparison to the control group. Excretion of Calcium oxalate in urine of control group had been continued until the end of experiment (10 th day) which clearly obvious under microscope. Lemon juice and *Boswellia Carterii* infusion had reduced the urinary excretion of calcium oxalates gradually until they disappeared at the tenth day of experiment (Table 4.3, 4.4)

The histopathological examination of kidney sections of all animals had been performed to confirm the induction of calcium crystallization within renal tissues and to compare the severity of renal changes of the control group with that of the treatment groups in order to determine the improvement, which may result from using of the tested agents.

Renal sections prepared from kidneys of the control rabbits had shown that the administration of oxalic acid resulted in necrosis of the glomerulus tissue, the capillaries of boundary zone are greatly distended and the stroma is edematous, with separation of tubules, the scarring is usually confined to the cortex, sparing the medullary pyramids a feature that helpful in distinguishing between infarcts and pyelonephritis. There are many black granules, which represent deposition of calcium in and around the dead tissue (Figure 4.1). These changes were compared with the normal renal tissue (Figure 4.2).

Kidney sections of rabbits treated with fresh lemon juice showed the presence of large circled glomeruli with mild haemorrhage. Also there is tubular deposition and mild hyaline degeneration in the renal convoluted tubules with congestion in the interstitial tissue of kidney and tubular basophilia (Figure 4.3, 4.4)

After treatment with *Boswellia carterii* infusion, kidney sections of rabbits revealed the presence of large, normal and circled glomeruli with normal renal convoluted tubules which lined with normal epithelial cells besides congestion in the interstitial tissue (Figure 4.5, 4.6).

Table (4.1): Blood urea nitrogen (mg/dl) of the studied groups measured before administration of agent and after 3, 10 days of renal calcium crystallization induced by oxalic acid.

Period (day) / Experiment groups	0 day	3 rd day	10 th day
Control	35 ±0.07 A	43±0.2 A	52±0.2 A
Lemon juice	35±0.07 A	21.2±0.08 B	19±0.12 B
***Boswellia carterii* infusion**	36±0.07 A	25.5±0.13 C	20±0.15 B

*Different capital letters mean significant effect at P<0.05 for vertical values.

Table (4.2): S.creatinine (mg/dl) of the studied groups measured before administration of agent and after 3, 10 days of renal calcium crystallization induced by oxalic acid.

Period (day) / Experiment groups	0 day	3 rd day	10 th day
Control	1.2±8.9 A	2.1±0.5 A	3.2±1.2 A
Lemon juice	1.2±8.9 A	1.3±0.94 B	1±1.16 B
***Boswellia carterii* infusion**	0.9±8.9 B	1.1±1.39 B	0.8±1.04 B

* Different capital letters mean significant effect at P<0.05 for vertical values.

Table (4.3): Physical examination of urine samples collected from the studied groups after 3, 10 days of renal calcium crystallization induced by oxalic acid.

Period (day) / Experiment groups	3 rd day			10 th day		
	color	appearance	PH	color	appearance	PH
Control	yellow	turbid	9.6±0.1A	yellow	turbid	9.9±0.1A
Lemon juice	yellow	turbid	9±0.1 A	yellow	clear	8.3±0.3 B
***Boswellia carterii* infusion**	yellow	turbid	9±0.2 A	yellow	clear	8.1±0.5 B

* Different capital letters mean significant effect at P<0.05 for vertical values.

Table (4.4): Microscopic examination of urine samples collected from the studied groups after 3, 10 days of renal calcium crystallization induced by oxalic acid.

Period (day) / Experiment groups	3 rd day				10 th day			
	Epithelial cells	leucocytes	erythrocytes	Calcium oxalate	Epithelial cells	leucocytes	erythrocytes	Calcium oxalate
Control	1±0.1 A	3±1.7 A	nil	Full field	5±0.1 A	4±0.4	nil	Full field
Lemon juice	1±0.2 A	2±0.9 B	nil	Full field	0±0.2 B	nil	nil	nil
***Boswellia carterii* infusion**	1±0.1 A	2±0.1 B	nil	Full field	0±0.1 B	nil	nil	nil

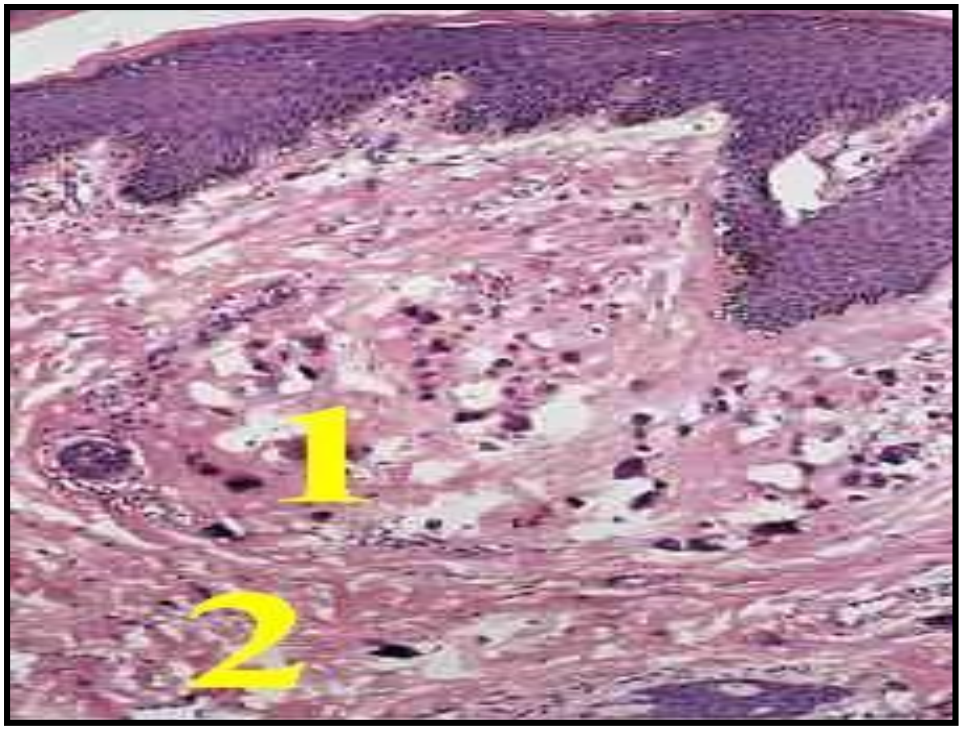

Figure (4.1): Glomerulus of the control group revealed necrosis (1) the capillaries of boundary zone are greatly distended and the stroma is edematous, with separation of tubules (2).the scarring is usually confined to the cortex, sparing the medullary pyramids a feature that helpful in distinguishing between infarcts and pyelonephritis. There are many black granules which representing deposition of calcium in and around the dead tissue (H&E stain, 400X).

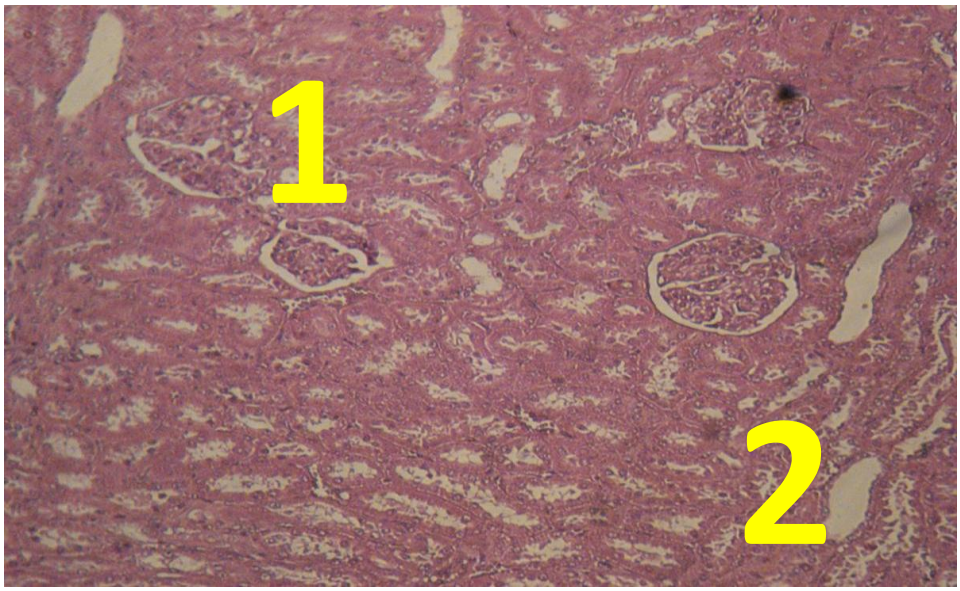

Figure (4.2): Normal kidney texture note normal glomeruli (1) and renal tubules (2) (H&E stain, 100X)

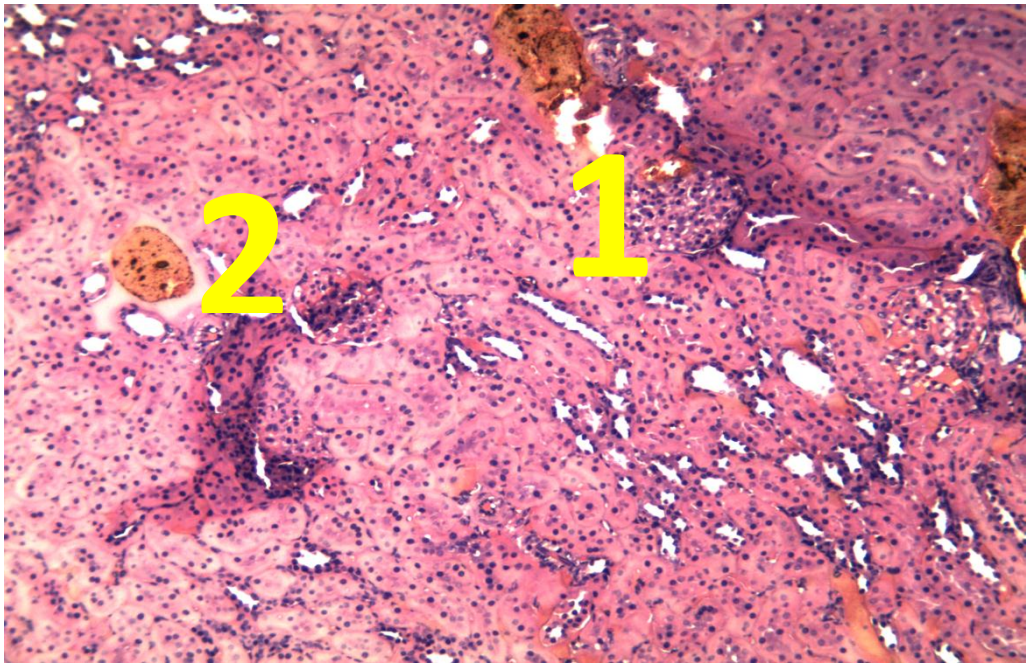

Figure (4.3): Kidney sections of lemon juice group showed the presence of large circled glomeruli with mild haemorrhage, also there is tubular deposition and hyaline degeneration in the renal convoluted tubules (1) with congestion in the interstitial tissue of kidney (2) (H&E stain, 100X).

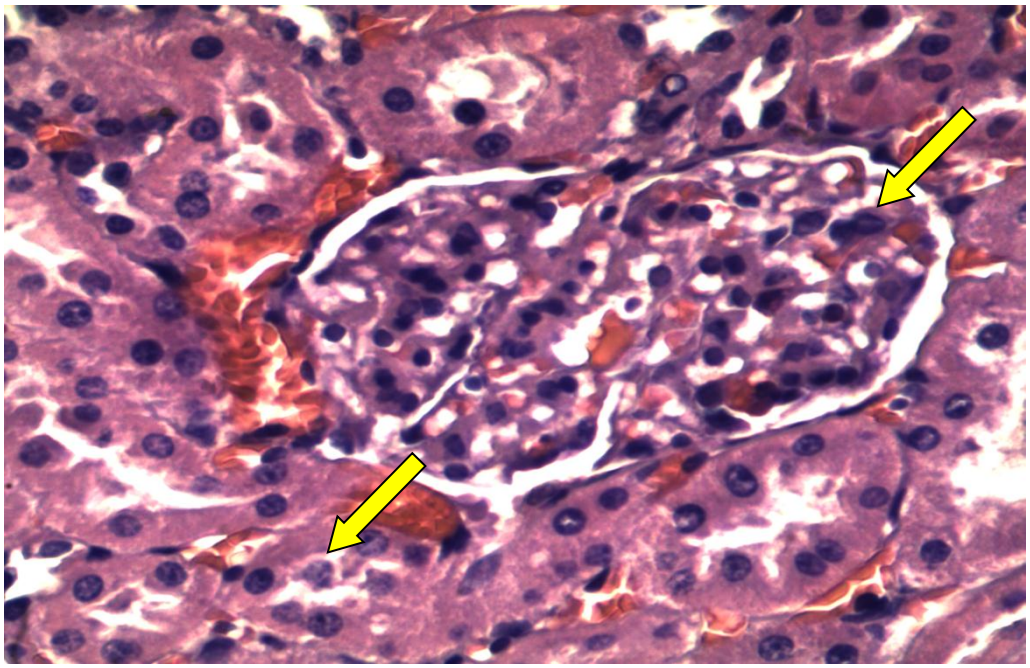

Figure (4.4): Kidney sections of lemon juice group revealed the presence of large circled glomeruli with tubular basophilia and there is normal convoluted tubules which lined by normal epithelial cells, but there is few tubules undergoing from mild hayaline degeneration. Also there is congestion and mild haemorrhage in the interstitial tissues (yellow arrows) (H&E stain, 400X).

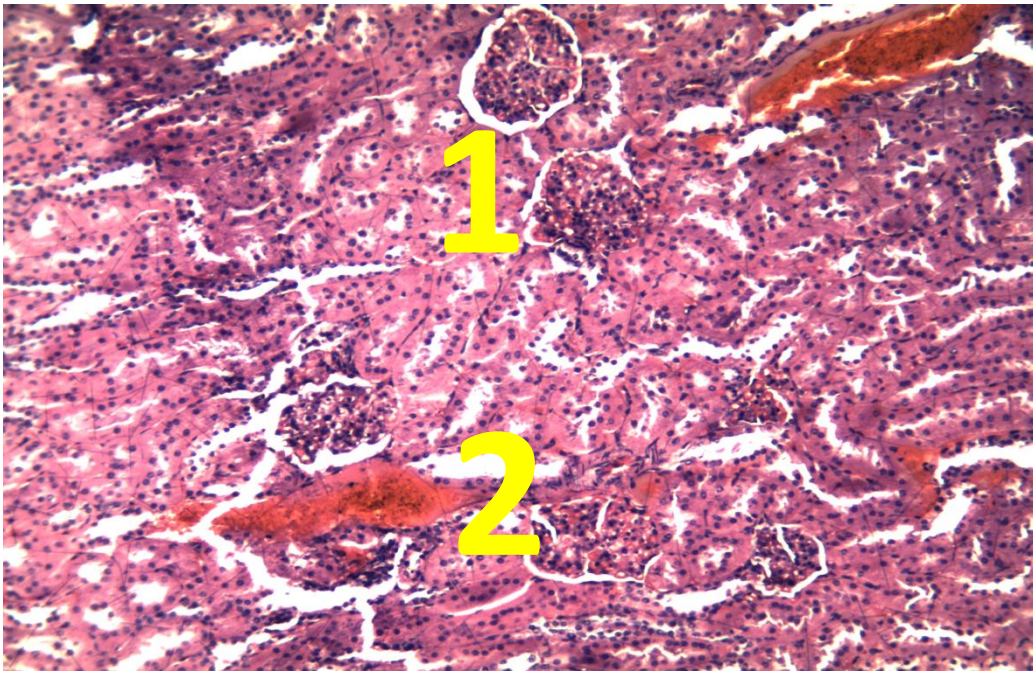

Figure (4.5): Kidney sections of ***Boswellia carterii*** **infusion** group revealed the presence of large, normal and circled glomeruli with normal renal convoluted tubules which lined with normal epithelial cells (1) and there is congestion in the interstitial tissue (2) (H&E stain, 100X).

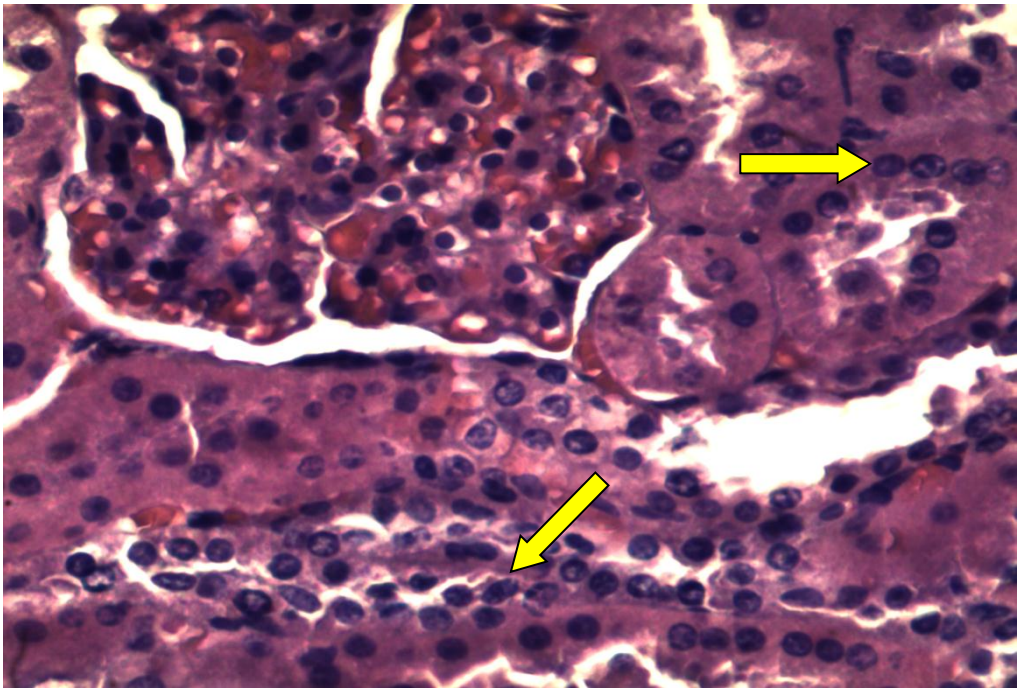

Figure (4.6): Kidney sections of ***Boswellia carterii*** **infusion** group revealed the presence of tubular basophilia (yellow arrows) (H&E stain, 400X)

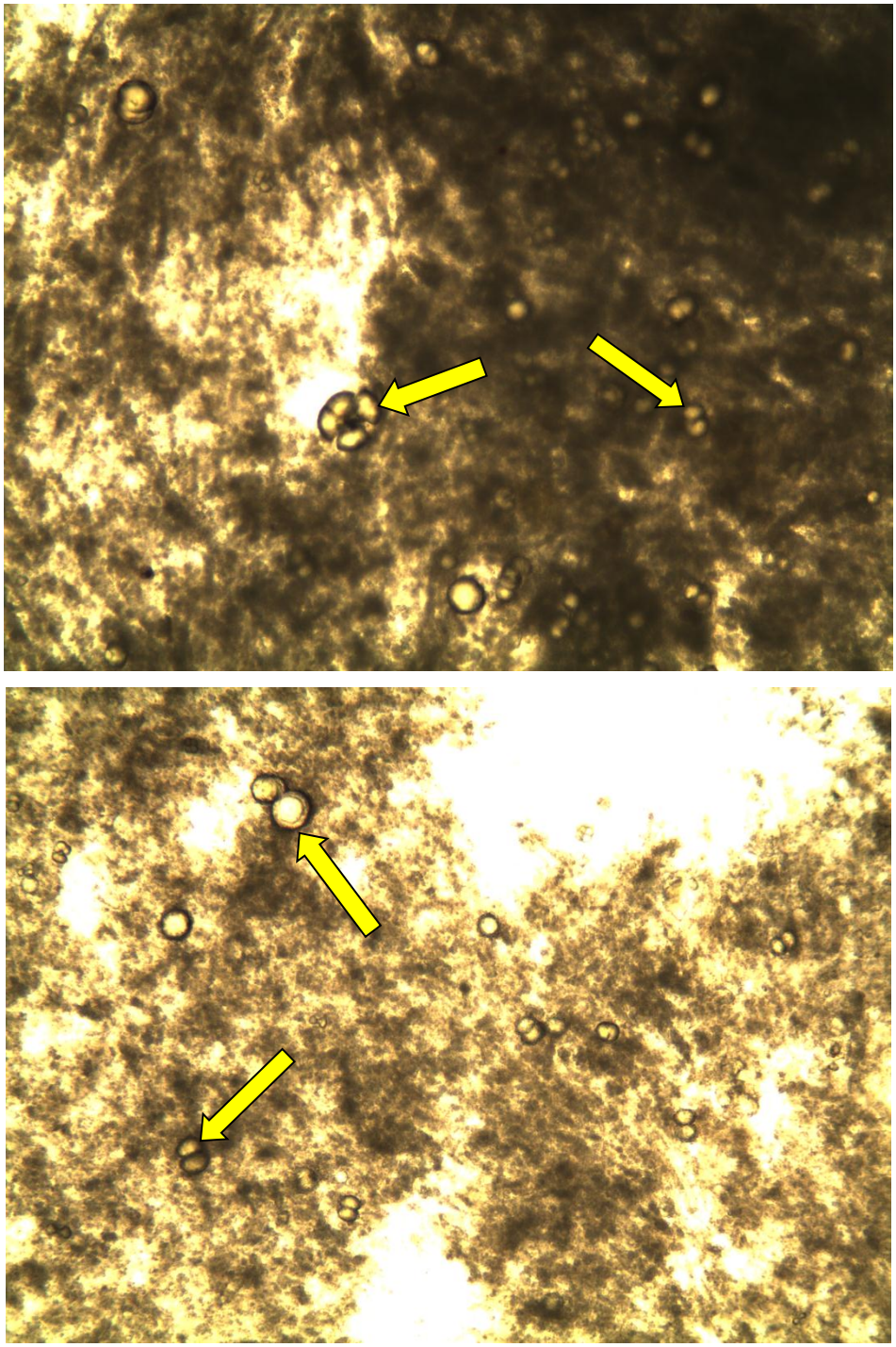

Figure (4.7): microscopic examination of urine sediment collected from control group revealing the accumulation of calcium oxalate crystals (monohydrate-type) (yellow arrows) (400 X)

CHAPTER FIVE

DISCUSSION

5. Discussion:

The present study found that the control group of rabbits had produced an elevation in the levels of BUN, serum creatinine levels, urinary excretion of epithelial cells, leukocytes, and calcium oxalate crystals after 3 and 10 days of oxalic acid administration in comparison to the normal values. The results were more evident at the last or tenth day of the experiment. Results of high BUN and serum creatinine levels revealed by control group as a result of calcium crystal deposition were similar to those recorded by by Ahmed, 1989.

5.1. Urinary crystallization of calcium salts:

Rabbits have an unusual calcium metabolism where the serum total calcium levels directly reflect the dietary intake. Dietary calcium is passively absorbed in the intestine through a mechanism that does not directly involve Vitamin D3 and that is independent of the rabbit's metabolic needs. The serum calcium is mainly excreted via the kidneys. Urine is therefore the major route of calcium excretion (45 per cent to 60 per cent of calcium is excreted by this route compared to two per cent in other mammals). Rabbits excrete alkaline urine. The excreted calcium precipitates in the alkaline urine, forming calcium crystals and giving rabbit urine its normal thick, creamy consistency (Vella and Donnelly, 2012; Mancinelli, 2013).

Mechanisms of calcium crystallization in kidney or nephrocalcinosis must be produced by urine being saturated with calcium and oxalate ions. Intra-tubular precipitation of calcium oxalate crystals seems likely in cases of marked hyperoxaluria, and mild degrees of hyperoxaluria may allow heterologous nucleation of calcium oxalate around calcium phosphate crystals (Evan *et al*., 2003) intra-luminal calcium oxalate crystals appear to cause an epithelial reaction, conductive to inflammation, urothelial

development and movement of crystals into the interstitium (Evan *et al*., 2004; Sayer *et al*., 2004).

When hyperoxaluria is induced in lab animals by oxalate salts or ethylene glycol, there is an increase in urinary excretion of oxalates within 2 days, hyperoxaluria is established within 3 days, calcium oxalate crystalluria develops within 2 weeks, and calcium oxalate nephrolithiasis develops within 4-6 weeks. Urinary PH and excretion of citrates are significantly decreased causing hypocitraturia (Khan, 2004).

5.2. Effect of lemon juice:

The second group was treated by using freshly squeezed lemon juice as a single daily dose of 6 ml/ kg B.W. orally started 2 hours before administration of oxalic acid and continued until the tenth day of this study. Among all of citrus juices that studied or consumed in an ordinary diet, lemon juice has proved to contain the highest concentration of citrus. A few studies tried to determine the effects of lemon juice supplementation on cases of calcium oxalate deposition within kidney, but the findings were faulty by the observational design of the study or by the small size of sample that limited the intensity of statistical analyses and the reliability of the consequences.

Lemon juice produces an increase in urine volume and urinary citrate excretion, besides lowering of urinary calcium. It has a high antioxidant power due to the presence of citrate, vitamin C, E and flavonoids such as eriocitrin, hesperetin (Miyake *et al*., 1998; Minato *et al*., 2003) and limonoids (Yu *et al*., 2005). Vitamin E can prevent deposition of calcium oxalate crystal in renal tissues by preventing peroxidative damage of the renal tubular membrane surface induced by hyperoxaluria (Huang *et al*., 2000; Thamilselvan and Menon, 2005) which in turn can prevent

attachment of calcium oxalate crystal and subsequent development of urolithiasis (Santhosh and Selvam, 2003).

Previous studies confirmed the beneficial effects of lemon juice as an inexpensive and well-tolerated source of citrate, hence it is rising the chance to be used as a supplemental therapy in patients with hypocitraturia (Penniston *et al*., 2007; Aras *et al*., 2008) Long-term lemonade therapy was able to reduce the stone formation rate from 1.00 to 0.13 stones per patient per year (Kang *et al*., 2007). Studies done by Seltzer *et al*., 1996; 2007; Penniston *et al*., 2007; and Kang *et al*., 2007 concluded that lemonade therapy as a potential treatment for hypocitraturic urolithiasis in a total of 226 patients with hypocitraturia had resulted in increment of urinary citrate levels. Lemon juice therapy is not able to deliver a urinary alkali load due to the low PH of lemon juice. On the other hand, The citraturic action of other citrate compounds especially potassium citrate may be related to the effect of alkali load brought by oxidation of citrate to bicarbonate associated with the direct citrate excretion in urine. (**Suarez and Youssef,** 2015; Wang *et al*., 2016).

Aras *et al.,* 2008 had demonstrated that fresh lemon juice at a dose of exactly 60 m.Eq/day (approximately 85 ml/day) could be an alternative to potassium citrate in the treatment of urinary calcium lithiasis in patients with hypocitraturia.

Touhami *et al*., 2007 had investigated the curative effect of a solution containing 100%, 75% or 50% lemon juice (6 µl solution/gm body weight) against calcium oxalate urolithiasis induced by ethylene glycol in male Wister rats over a period of 10 days. They recorded that renal histology of rats treated with either 100% or 75% lemon juice had no deposits of calcium oxalate crystals, which indicates subsequent prevention of urolithiasis development in rats.

5.3. Effect of *Boswellia carterii* infusion:

Our findings showed that the oral administration of *Boswellia carterii* infusion at a dose of 1gm/10 ml D.W./ rabbit could prevent the deposition of calcium crystals in renal parenchyma and significantly reduced the tissue damage via lowering of BUN, serum creatinine levels and urinary excretion of calcium oxalate. This curative efficacy can be related to the ability of plant extract to prevent the oxidative effect of oxalic acid on parts of kidney. As general, little studies are available about the activity of *Boswellia carterii* infusion in treatment of urolithiasis, but it was well known in the folkloric use to treat renal failure which may attributed to the antioxidant effect of *Boswellia* species as reported by several researchers (Raihan *et al*., 2012; Narayan *et al*., 2012).

All the above findings of the present study suggest that renal crystals can be induced successfully by oxalic acid. Similar case can be produced by dietary intake of plant containing oxalate such as some grains, nuts, cocoa, and coffee resulting in deposition of calcium oxalate crystals within the glomeruli. This effect can be prevented or corrected by using agents that can enhance urinary excretion of oxalate and protect the kidney tissue against the degenerative and necrotic effect of oxalic acid in both animals and human.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusions

1. Results recorded by control rabbits showed that oxalic acid administration as a single dose is of high equality agent in induction of serious hypercaliciuria, hyperoxaluria, and hypocitraturia *in vivo.*
2. The present study found that the administration of lemon juice effectively prevented the development of renal calcium crystals in rabbits. These findings support the use of lemon juice as an alternative medicine to prevent urolithiasis.
3. Water extracts of *B. carterii* by infusion is efficacious in lowering urinary calcium oxalate excretion and calcium crystals deposits in kidneys in a way resembling the actions of common diuretic drugs.

5.2. Recommendations

1. Further research is necessary to clarify the mechanism underlying the prophylactic effect of lemon juice.
2. Studies on standardization, characterization, efficacy, long-term side effects, toxicity and plant drug interaction are recommended.
3. Isolation of β-boswellic acid, one of the main active components of *B. carterii* to evaluate its activity against urolithiasis as pure drug.
4. Finally, it is recommended to investigate the efficiency of lemon juice and olibanum extract on other types of crystal deposit-stones such as ammonium phosphate, magnesium phosphate and urate stones.

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الخلاصة

الخلاصة:

لقد هدفت الدراسة الحالية الى تقييم كفاءة منقوع راتنج اللبان الذكر وعصير الليمون الطازج مقارنة مع مجموعة ارانب حقنت بحامض الاوكزاليك. ولتحقيق هدف الدراسة لجأنا الى اثنا عشر ارنبا" مختبريا" ومن كلا الجنسين مقسمة الى ثلاث مجاميع (أربع أرانب في كل مجموعة).

استخدم حامض الاوكزاليك بجرعة 333 ملغم/كغم من وزن الجسم عن طريق الفم لغرض استحداث مرض التبلور الكلسي في كلى ارانب المجاميع الثلاثة على السواء وذلك قبل ساعتين من إعطاء الماء المقطر بجرعة 3 مل/ كغم من وزن الجسم فمويا" الى مجموعة السيطرة الموجبة (المجوعة الأولى)، قبل ساعتين من تجريع عصير الليمون الطازج بجرعة 6 مل/ كغم من وزن الجسم فمويا" الى ارانب المجموعة الثانية (مجموعة العلاج الأولى)، قبل ساعتين من تجريع منقوع راتنج اللبان الذكر بجرعة 1غم/ 10 مل ماء/ أرنب فمويا" الى ارانب المجموعة الثالثة (مجموعة العلاج الثانية).

كل المجاميع أعلاه تم تجريعها بالمواد المختبرة كجرعة مفردة كل يوم عن طريق الانبوب المعدي وعلى طول فترة التجربة التي استمرت 10 أيام.

وقد تم التحري عن التأثيرات العلاجية للمواد المختبرة من خلال تقييم وظائف الكلية بواسطة قياس مستويات نتروجين يوريا الدم وكرياتينين المصل (ملغم/ديسيليتر) في عينات الدم المسحوبة من كافة حيوانات التجربة في اليوم الثالث والعاشر منها وكذلك فحص عينات الادرار التي أمكن جمعها من الحيوانات في اليوم الثالث والعاشر أيضا" بهدف استخدامها كدلائل على تحسن الكلى بواسطة الفحص المجهري لراسب الادرار. هذا الى جانب الفحص النسيجي–المرضي للمقاطع

النسيجية المحضرة من كلى حيوانات التجربة التي تم التضحية بها في اليوم الأخير من الدراسة (اليوم العاشر).

كاستنتاج نهائي، فان كلا النباتين المختبرين أسفرا عن تأثير علاجي معنوي من خلال خفض مستويات نايتروجين الدم وكرياتينين المصل والطرح البولي لبلورات أوكزالات الكالسيوم. كما أظهر الفحص النسيجي المرضي لمقاطع النسيج الكلوي تحسن واضح في غينات الكلى نتج عن تقليل ترسب الكالسيوم والتغيرات المرضية الأخرى التي حدثت للكبيبات الكلوية بسبب حامض الاوكزاليك.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة القادسية
كلية الطب البيطري

دراسة الفاعلية العلاجية لعصير الليمون ومنقوع اللبان الذكر على التبلور الكلوي لأملاح الكالسيوم في أرانب التجربة

بحث مقدم الى مجلس كلية الطب البيطري/ جامعة القادسية كجزء من متطلبات نيل درجة البكالوريوس في علوم الطب البيطري

من قبل

إسراء سالم

إشراف

أ.م. جنان عبد الأمير صبيح الحسيني

نيسان **شعبان**

2018 م **1439 هـ**