Role of *Eruca sativa* in prevention of induced nephrocalcinosis in rabbits

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Keywords: Nephrocalcinosis, oxalic acid, hyperoxaluria, *Eruca sativa* , calcium oxalate crystals.

ABSTRACT:

Background: Nephrocalcinosis is a clinical pathologic condition characterized by abnormal deposition of calcium phosphate or calcium oxalate in the renal parenchyma, which can result in impaired kidney function.

Aim: This study aimed to evaluate the effect of *Eruca sativa* aqueous extract in prevention of nephrocalcinosis induced by a large dose of oxalic acid in rabbits.

Materials and Methods: Twenty four healthy domestic rabbits were equally allocated into three groups and were distributed into: normal group (without drug), oxalic acid group and *Eruca sativa* group. Both the first and second groups were received distilled water, while the third group received aqueous extract of *Eruca sativa* for ten consecutive days. Single high dose of oxalic acid was given to induce nephrocalcinosis in rabbits of the second and third groups (except normal group) after 2 hours from receiving of distilled water and *Eruca sativa* extract at the first day. Blood samples were collected from all animals for biochemical analysis. Urine analysis and histopathological examination were performed for all rabbits to verify the presence of crystals in urine and renal tissues.

Results: *Eruca sativa* aqueous extract produced highly significant reduction in blood urea nitrogen, serum creatinine , with significant reduction in serum Na^+ , and highly significant elevation in serum Ca^{2+} and serum K^+ levels, also it is produced highly significant reduction in the calcium oxalate crystals density in urine and renal tissues when compared with of oxalic acid group.

Conclusion: Current study found that the aqueous extract of *Eruca sativa* effectively prevented the formation and deposition of calcium oxalate crystals in renal tissues, thus preventing the occurrence of nephrocalcinosis.

دور نبات الجرجير في منع التكلس الكلوي المحدّث في الأرانب

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الكلمات المفتاحية: التكلس الكلوي، حامض الاوكزاليك، فرط أوكسالات البول، مستخلص نبات الجرجير، بلورات أوكسالات الكالسيوم.

يعد التكلس الكلوي حالة مرضية تتميز بترسب بلورات فوسفات الكالسيوم أو أوكزآلات الكالسيوم في النسيج الكلوي، من الممكن أن يؤدي الى الفشل في وظيفة الكلية. تهدف هذه الدراسة إلى تقييم تأثير المستخلص المائي لنبات الجرجير في منع التكلس الكلوي المحدّث باستخدام جرعة عالية من حامض الاوكزاليك في الأرانب. اجريت الدراسة على أربعة وعشرين أرنبا وزعت الى ثلاثة مجاميع متساوية على النحو التالي, المجموعة السوية (من غير دواء), مجموعة حامض الاوكزاليك ومجموعة الجرجير. جرعت كلا من المجموعة الاولى والثانية بالماء المقطر ,و جرعت المجموعة الثالثة بالمستخلص المائي لنبات الجرير لمدة عشرة ايام متتالية. اعطيت جرعة واحدة عالية من حامض الاوكزاليك لإحداث التكلس الكلوي في ارانب المجموعة الثانية والثالثة (ماعدا المجموعة الطبيعية) بعد مرور ساعتين من اعطاء الماء المقطر ومستخلص الجرير في اليوم الاول. جمعت عينات الدم من جميع الارانب للكشف عن وظائف الكلية. كما اجري فحص البول والفحص النسيجي لجميع الارانب للتحقق من وجود البلورات في البول والأنسجة الكلوية .

اظهر المستخلص المائي لنبات الجرجير انخفاضا معتدا عاليا في مستوى نيتروجين اليوريا والكرياتينين مع انخفاضا معتدا في تركيز الصوديوم. كما بب ارتفاعا معتدا عاليا في تركيز الكالسيوم والبوتاسيم , اضافة الى ذلك بب انخفاضا معتدا عاليا في كثافة بلورات أوكزآلات الكالسيوم في البول والأنسجة الكلوية عند مقارنته مع مجموعة حامض الاوكزاليك . استنتج من هذه الدراسة, ان المستخلص المائي لنبات الجرجير منع بشكل فعال تكوين وترسب بلورات أوكزآلات الكالسيوم في الأنسجة الكلوية، وبالتالي منع حدوث التكلس الكلوي.

INTRODUCTION:

Nephrocalcinosis (NC) is a clinical pathologic condition of kidney characterized by abnormal deposition of calcium mainly in the form of calcium phosphate or calcium oxalate in the renal parenchyma, which can result in impaired kidney function (1). It can arise from several conditions including, hypercalcemia , hypercalciuria, hyperoxaluria, hypocitraturia, hyperparathyroidism, vitamin D toxicity, sarcoidosis, renal tubular acidosis, medullary sponge kidney, renal cortical necrosis, renal tuberculosis, hyperphosphatemia, hyperphosphaturia, and use of certain medicines such as furosemide, acetazolamide, amphotericin B, and triamterene (2).

Fragments of calcium oxalate or calcium phosphate may break free from the kidney and provide nuclei for the formation of kidney stones (nephrolithiasis) . Therefore, nephrocalcinosis may eventually result in acute or chronic obstructive uropathy, leading to eventual kidney failure (3).

Oxalic acid provides a useful animal model to study the mechanisms and therapeutic approaches of the nephrocalcinosis. Therefore , ingestion of a high dose of oxalic acid developed hyperoxaluria, which can lead to formation and deposition of insoluble calcium oxalate(CaOx) crystals in the renal tissues, causing inflammatory reaction, cell damage and renal dysfunction, therefore it is necessary to search for new natural agents of plant origin that might improve the nephrocalcinosis (4,5) .

Eruca sativa (*E. Sativa*) commonly known as Rocket salad, is one of the nutritious perennial vegetables of Mediterranean origin , belonging to the Brassicaceae family, widely grown all over the world and immensely used as salads and cooking spice. It has diversified medicinal and therapeutic properties such as astringent ,diuretic , analgesic, digestive ,emollient ,tonic , laxative ,rubefacient, stomachic, aphrodisiac and stimulant properties , also

it has antimicrobial, anti-inflammatory, antioxidant ,antifungal, antitumour, antiulcer ,antiplatelet, antithrombotic antihyperlipidemic, antidiabetic ,antinephrolithiatic, nephroprotective and hepatoprotective activities (6,7,8,9).

MATERIALS AND METHODS:

Plant collection and aqueous extract preparation :

Fresh leaves of *E. sativa* were purchased from the local market of Al-Nasiriya city, Iraq. The plant leaves were washed, dried and milled into fine powder. 200 grams of powdered plant leaves were infused in two liters of distilled water and were heated at 80 ºC for 30 minutes with stirrings, then cooled the extract at room temperature and filtered through Whatman paper No. 1 filter paper. Then evaporate the filtrate under reducing pressure using rotary evaporator at 40°C to obtain crude extract .This extract was stored in a dark sterile screw bottle at 4°C until use (6).

Animals:

Twenty four healthy domestic rabbits of both sexes weighing between 1000 to 1800 grams were used in this study. They were housed in separated cages under good conditions and fed standard oxoid pellets with water *ad libitum*. The animals were randomly allocated into three groups(eight animals in each group) and were given a single daily dose of the followings at 9:00 a.m. for ten successive days.

Group 1 (normal group): received distilled water (3ml) orally.

Group 2 (oxalic acid group): received distilled water (3ml) orally .

Group 3(*Eruca sativa* group): received aqueous extract of *Eruca sativa* (500mg/kg) orally (10).

At 11:00 a.m. of the first day, all animals of group 2 and 3 (except group 1) were given orally single dose of oxalic acid (333 mg/kg) by gastric tube for induction of nephrocalcinosis (4).

Blood collection and analysis:

At the end of the treatment period, blood samples were aspirated from heart of each rabbit, then centrifuged at 3000 rpm for 15 minutes. The clear, non hemolysed supernatant sera were used to measure blood urea nitrogen (BUN), serum creatinine, Na^+ , Ca^{2+} and K^+ concentrations using commercially available kits by spectrophotometer method (11).

Urine collection and analysis:

At the end of experiment ,all animals were fasted overnight and kept in individual cages, urine samples were collected in the morning from each rabbit to determine the presence of calcium oxalate crystals in urine using light microscope. Crystals were counted and scored as follows: “0” no crystals; “1” 1 to 10 crystals per high power field (x400); “2” 11 to 20 crystals/ HPF; and “3” > 20 crystals/ HPF (12).

Histopathological study:

At the end of the final urine collection, all rabbits were subjected to kidney resection under chloroform anesthesia, then the histopathological examination of the sections was done to check for calcium oxalate crystal deposits in renal tissues as well as histopathological changes in renal architecture by using a light microscope after fixation the section in 10% formalin and staining with hematoxylin & eosin. The number of crystal deposits were counted in five fields for each sample and their density was scored as: “0” no crystal deposition; “1” 1- 5 crystals/HPF; “2” 6- 10 crystals/HPF; and “3” > 10 crystals/HPF(12).

Statistical analysis:

Data were expressed as mean ± standard error of mean (SEM.) and unpaired t-test (Student t-test) was used to compare the difference between each two groups using statistical package for social science (SPSS) version 17. Differences were considered significant at the level of P≤0.05 and highly significantly at the level of P≤0.001 (13).

RESULTS:

In the present study, administration of a single large dose of oxalic acid 333 mg\kg orally to rabbits caused nephrocalcinosis manifested by a highly significant elevation in both BUN (30 ± 0.09 vs 15 ± 0.04) mg/dl and serum creatinine (1.6 ± 0.03 vs 0.6 ± 0.01) mg/dl, with highly significant reduction in serum Na^+ level (145 ± 0.39 vs 150 ± 0.49) mmol/L, serum Ca^{2+} level (9 ± 0.10 vs 11 ± 0.16) mg/dl and serum K^+ level (4 ± 0.04 vs 4.7 ± 0.03) mmol/L when compared to that of normal group (group 1)(Table 1).

Aqueous extract of *Eruca sativa* (group 3) showed highly significant reduction in in both BUN (16 ± 0.10 vs 30 ± 0.09) mg/dl and serum creatinine (0.7 ± 0.01 vs 1.6 ± 0.03) mg/dl, with significant reduction in serum Na^+ level (142 ± 0.36 vs 145 ± 0.39) mmol/L and highly significant elevation in serum Ca^{2+} level (10.8 ± 0.05 vs 9 ± 0.10) mg/dl and serum K^+ level (5 ± 0.03 vs 4 ± 0.04) mmol/L when compared with oxalic acid group (group 2)[Table 1].Whereas it is produced a highly significant reduction in serum Na^+ level (142 ± 0.36 vs 150 ± 0.49) mmol/L, and restored the BUN(16 ± 0.10 vs 15 ± 0.04) mg/dl , serum creatinine (0.7 ± 0.01 vs 0.6 ± 0.01) mg/dl, serum Ca^{2+} level (10.8 ± 0.05 vs 11 ± 0.16) mg/dl and serum K^+ level (5 ± 0.03 vs 4.7 ± 0.03) mmol/L nearly to the normal values when compared to that of normal group (Table 1).

The microscopic examination of urine of normal group animals did not contain any crystals. Oxalic acid group revealed a highly significant increase in the numbers (density) of CaOx crystals when compared with normal group. While *Eruca sativa* group showed a highly significant reduction in the numbers of CaOx crystals (0.6 ± 0.01 vs 2.5 ± 0.02) when compared with oxalic acid group (Table 2).

The histological examination of kidneys of normal rabbits showed a normal histological structure of renal parenchyma (glomeruli and tubules) without calcium oxalate crystals deposits(Figure 1A).Kidneys of rabbits in oxalic acid group showed deposition of highly numbers of calcium oxalate crystals (arrow) with sever tubular necrosis(Figure 1B) .While the kidney sections of rabbits in *Eruca sativa* group showed a highly significant reduction in the numbers of CaOx crystals deposits (0.4 ± 0.02 vs 2 ± 0.01) with better improvement in renal tissue integrity when compared with oxalic acid group, and was more similar to that of the normal group (Table 2, Figure 1C).

Table 1:Effect of aqueous extract of *Eruca sativa* on serum parameters .

group	BUN (mg/dl) M ± SEM	Creatinine (mg/dl) M ± SEM	Sodium (mmol/L) M ± SEM	Calcium (mg/dl) M ± SEM	Potassium (mmol/L) M ± SEM
Normal group	15 ± 0.04	0.6± 0.01	150 ± 0.49	11 ± 0.16	4.7 ± 0.03
Oxalic acid group	30 ± 0.09 a**	1.6 ± 0.03 a**	145 ± 0.39 a**	9 ± 0.10 a**	4 ± 0.04 a**
***E.sativa*+Ox.acid group**	16 ± 0.10 b**	0.7± 0.01 b**	142 ± 0.36 a**, b*	10.8±0.05 b**	5 ± 0.03 b**

*= Significant (P≤0.05), **= Highly significant (P≤0.001), a = as compared to group 1, b= as compared to group 2(n = 8/group).

Table 2:Effect of aqueous extract of *Eruca sativa* on calcium oxalate density in urine and renal tissue.

group	Crystal density	
	In urine M ± SEM	In renal tissue M ± SEM
Normal group	00 ± 0.00	00 ± 0.00
Oxalic acid group	2.5 ± 0.02 a**	2 ± 0.01 a**
***E.sativa*+Ox.acid group**	0.6 ± 0.01 b**	0.4 ± 0.02 b**

*= Significant (P≤0.05), **= Highly significant (P≤0.001), a = as compared to group 1, b= as compared to group 2 (n = 8/group).

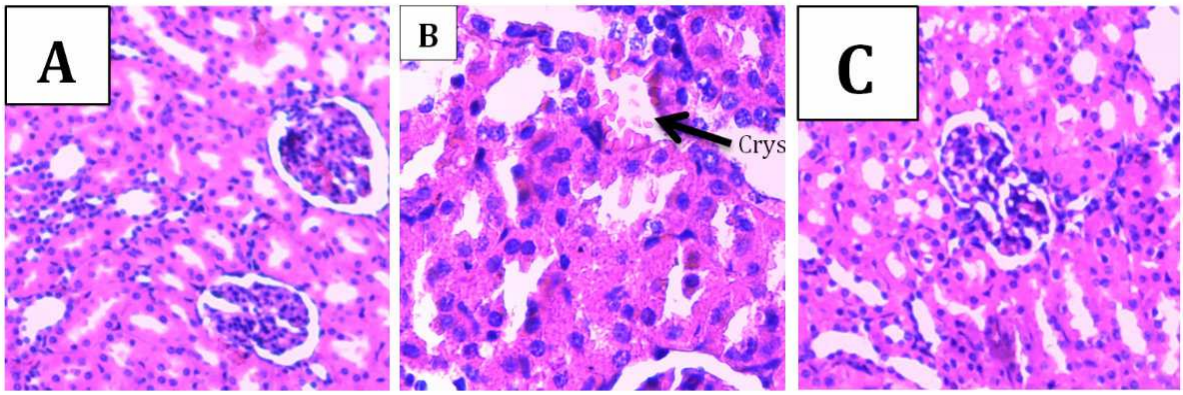

Figure 1: Sections of rabbits kidney of different groups. A= normal group, B= oxalic acid group, C= *Eruca sativa* group.

DISCUSSION:

Nephrocalcinosis is a state of deposition of calcium in the form of phosphate or oxalate in the tissue of the kidney that can impair kidney function.

In the present study, Nephrocalcinosis was induced by using a single large dose (333 mg/kg) of oxalic acid resulting in acute renal failure which is characterized by renal tubular necrosis and an accumulation of CaOx crystals in the urine and kidney tissues as well as significant elevation in BUN and serum creatinine. In addition oxalic acid produced highly significant reduction in serum Na^+ , Ca^{2+} and K^+ levels when compared with normal group, these results agreed with previous studies (4, 14). This finding can be explained by the fact that ingestion a high dose of oxalic acid produces hperoxaluria which can lead to CaOx supersaturation, nucleation and crystal formation association with production free radicals, followed by cell injury which facilitate crystal adherence and deposition in renal tissues, resulting in tubular obstruction , renal dysfunction and finally renal failure (15) .

In the present study, *Eruca sativa* significantly improved the functions and histopathological changes of the kidney and prevented the development of nephrocalcinosis, which was reflected in a highly significant reduction in the levels of BUN , serum creatinine, and number of CaOx crystals in urine and renal tissues when compared with oxalic acid group, as well as *E. sativa* restored Ca^{2+} and K^{+} levels nearly to the normal value with significant reduction in serum Na^{+} level . These effects could be attributed to the diuretic effect of *Eruca sativa* which is related to its active ingredients such as flavoniods, saponins or organic acids (10) .The diuretic effect lead to increase urine volume and urinary output, thus preventing the supersaturation of urine by calcium and oxalate and thereby CaOx formation, resulting in reduced deposition of CaOx crystals in urine and renal tissues, thereby preventing the elevated serum biochemical levels due to elimination of these in urine (16).

In the present study, the diuretic effect of *Eruca sativa* cause significant reduction in serum Na^{+} ,while did not lead to K^{+} depletion because it is rich in potassium ,making it a safe diuretic (17).

In this study ,*Eruca sativa* significantly decrease urinary and parenchyma CaOx crystals because it has large amount of chlorophyll that may inhibit the growth of calcium oxalate dehydrate which considered to be a primary phase in calcium oxalate crystals formation (18,19).

Other study found that acidic urine pH promotes CaOx crystallization, crystal-cell adhesion and crystals retention. Therefore, alkalinization of the urine may help to prevent CaOx deposition and nephrocalcinosis (20). Ansari (10) showed that *Eruca sativa* increase urinary pH making urine unsuitable for CaOx crystallization. In addition, increased urinary pH reduces the renal citrate reabsorption, thus increasing urinary citrate which is a potent inhibitor of CaOx crystals formation through its binding with free calcium ion ,then excreted as calcium-citrate complex in urine, thereby making calcium unavailable to bind with oxalate and prevents crystallization. In addition, citrate prevents aggregation and growth of preformed crystals through its ability to bind to the crystal's surface and also prevent their adhesion renal epithelial cells (21,22).

Previous studies found that the level of magnesium is slightly decreased in hyperoxaluric rat urine due to its interaction with oxalate. Magnesium therefore can reduce the supersaturation of calcium oxalate and subsequently reduce the growth and nucleation rates of calcium oxalate crystals (23). Similarly, *Eruca sativa* contains large amount of magnesium, so it may reduce free oxalate in intestine and urine, decreasing its availability for Ca^{2+} ion in renal tubules and prevent CaOx crystals formation (17).

Dietary content of certain cations has clinically important effect in prevention of CaOx crystal formation, so intake of dietary calcium inhibits the absorption of oxalate by increasing the formation of calcium-oxalate complexes in intestinal luminal, thereby reducing the pool of free oxalate available for absorption, hence reducing urinary oxalate excretion and CaOx crystals formation (24) . On the other hand, high dietary intake of potassium appears to reduce the risk of crystals formation because potassium promotes the urinary excretion of citrate, an inhibitor of urinary crystal formation (25). Therefore, in this study *Eruca sativa* may be working through these mechanisms because they contain a certain amount of calcium and potassium which can be observed by reducing the number of CaOx crystals in the urine and renal tissues and restoring serum Ca^{2+} and K^{+} levels nearly to the normal value (17).

Several recent studies have been shown that the naturally antioxidant compounds play an important roles in prevention calcium oxalate crystal deposition in the kidney by preventing hyperoxaluria-induced peroxidative damage to the renal tubular membrane surface, which in turn can prevent calcium oxalate crystal attachment and subsequent development of nephrocalcinosis and stones (26). In addition, saponins and flavonoids prevent renal calcium and oxalate deposition through disintegrating mucoproteins, which have a high affinity for calcium oxalate crystal surfaces and thus promote the growth and deposition of crystals (27). *Eruca sativa* possess a potent antioxidant and anti-inflammatory activities due to their natural compounds including, vitamins, carotenoids, flavonoids, saponins and polyphenols, which may be prevent super saturation of calcium oxalate and thus decreased their deposition in renal tubules ,resulting in inhibition of epithelial cell injury and inflammation induced by CaOx crystals (28).This confirms through another study showed that *E.sativa* seed extract possess a potent antioxidant and renal protective activity against mercuric chloride induced renal damage (29).

In conclusion, the present study showed that the administration of aqueous extract of *Eruca sativa* effectively prevented the development of nephrocalcinosis by inhibiting the formation and deposition of calcium oxalate crystals in renal tissues and protection of renal epithelial cells, it is likely to be used in management of nephrocalcinosis after confirmation of their effect in clinical trials.

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