Deleterious Effects of Hyperoxaluria on Some Rats' Organs and The Promising In Vitro Oxalate Fragmentation Influence of Aqueous Extract of Rosmarinus officinalis Linn.

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Hyperoxaluria is considered the real cause of the kidney oxalate stones. The present work is considered as an exploratory study which is continued with an original research work to evaluate the efficacy of the aqueous extract of Rosmarinus officinalis Linn on oxalate fragmentation in rat tissues. This work was divided into two different studies an in vivo study and an in vitro one. Rats in the in vivo study were divided into two groups: Group (1) represented the control group while in group (2) rats were injected with sodium oxalate to trace the oxalate impact and oxalate stones formation in rat tissues. The in vitro study was performed by adding sodium oxalate in human urine with or without the addition of different concentrations of the grinded leaves of Rosmarinus officinalis Linn.

Results showed mild significant changes in some of the serum biochemical parameters in the oxalate treated group. On the other hand, the histopathological examinations showed that hyperoxaluria exhibited many tissue distortions in the kidneys, the bone marrow and the brain more than those found in the liver and the spleen compared to the control group. Oxalate crystals were noticed in rats’ urine in the oxalate injected group and no stones were observed in kidneys or in other tested tissue. The in vitro study results showed that the plant leaves displayed highly fragmentation impact on oxalate crystals in human urine at a certain dose range concentration which when increased showed no effect on oxalate crystals fragmentation. It was concluded that Rosmarinus officinalis Linn had a great influence on oxalate fragmentation at a certain dose range.

Keywords: Hyperoxaluria-Rat organs- Rosmarinus officinalis Lin dried leaves- Oxalate fragmentation in vitro.

Introduction

People who lack information about the different kinds of food that contain high amounts of oxalates, are at risk of the formation of hyperoxaluria disease easily. Many studies had recorded the food stuffs and plants that are rich in oxalates for human safe consumption [1,2, 3]. Oxalates are useless and has no nutritional function for man and animals and mainly they are absorbed by passive transport in the stomach in addition to the small and the large intestine and eliminated by the kidneys [4].

Hyperoxaluria is defined as a disorder in oxalate metabolism associated with increased urinary oxalate excretion [5]. This can be attributed to an increased production of endogenous oxalates due to genetic defects or some enzymatic activities absence (primary hyperoxaluria) or attributed to increased oxalate intestinal absorption through food intake or intestinal pathologies (secondary hyperoxaluria) [6]. Increasing plasma oxalate levels leads to its deposition in the various body organs and can form oxalate complexes in the kidney tubules and causes nephrolithiasis [7, 8]. This case can reach an end
stage renal disease (ESRD) which in turn leads to morbidity and mortality [9-11].

Most cases of hyperoxaluria are diagnosed after reaching ESRD or dialysis cases [12,13]. More attention should be paid from persons to trace their urinary or plasma oxalate levels so they can avoid the incidence of the disease.

Calcium stones especially calcium oxalate ones are the most abundant stone type formed in the kidneys with higher percent ratios than the other types [14,15].

Many factors interfere with the absorption and excretion of oxalates in humans which differ greatly from one person to another [16]. Daily calcium intake is one of the most important factors that affect oxalate absorption [17,18,19]. Higher dietary calcium intake means a lower levels of oxalate absorption and lower incidence of kidney stone disease. Dietary free intestinal calcium forms an insoluble complex with the free oxalates in the intestinal lumen so decreasing its absorption [20, 21, 22]. Increasing free fatty acids concentration (fat mal absorption) in intestine can bind with free calcium leading to the increased mucosal colonic permeability of oxalate [23]. Also consumption of phytate which binds to free intestinal calcium causes the increased absorption of oxalates [24].

Another important factor that affects oxalate intestinal absorption is the oxalate transporter proteins in the gastrointestinal tract which are encoded by the SLC26 gene family. The abundance and the expression of these oxalate transporters are regulated by several local or systematic pathways [25]. Also it is concluded that any genetic mutation in this gene family will alter oxalate absorption [26, 27].

Presence of colonies of the oxalate degrading gram negative bacteria (Oxalobacter formigenes) that utilize oxalate as an energy source in intestine decreases the oxalate absorption and the urinary oxalate excretion. Colonization of O. formigenes is greatly reduced after the frequent treatment of antibiotics and following bariatric surgery [28,29]. Miller et al, 2019 [30]. reported the effect of many other intestinal bacterial species on oxalate homeostasis.

Low intake of fluids cause the urinary increase of lithogenic substances concentration and stone formation as increasing water and fluid intake dilutes the urine and prevent stone forming substances from aggregation or precipitation [31]. Research studies reported that for each glass of fluid consumed protect against kidney stone formation risk by about 13% [32]. This statement can not be applied on all fluids, as for example, soda soft drinks that contains added fructose or sugar sweet syrup trigger kidney stone formation.

Diets containing high protein content can lead to decreasing citrate levels in plasma and kidneys. Citrates are responsible for decreasing oxalate stones by preventing calcium from binding with other constituents in urine. Also researches reported that vitamin C supplements and ethylene glycol are converted to oxalate in liver cells and so increasing its levels in the body [31].

Accordingly, nutritional modifications and diet control besides high fluid intake are nowadays a new strategy for the prevention and recurrence of nephrolithiasis [33-36].

Most of the research focused on the effect of oxalates on the kidney tissues while observing its effect on other organs is mainly rare. The present study is considered as continuation of our previous preliminary research on hyperoxaluria effect on kidney and bone marrow (published abstract in the journal booklet of "The 10th International Conference on Nephrology & Therapeutics” 2021) [37] in addition to other organs and tried to answer the question; Does increasing oxalates concentration in body fluids affect significantly the kidneys only or other organs too? And is there a safe treatment for the case?.. This can be performed by sodium oxalate administration to male aged rats for a month and examining the body organs histology in addition to determining some biochemical parameters related to hyperoxaluria disease. Our study also recommended the rosemary dried leaves (Rosmarinus officinalis Linn) for the first time for degradation and getting rid of insoluble oxalates in human urine in an in vitro study.

**Material and Methods**

**In vivo study**

**Animals**

Twelve male Wistar rats weighing about 200 grams were purchased from the animal house of the National Research Centre, Dokki, Giza, Egypt. Rats were housed in the animal house under the guidance of the medical ethical committee of the National Research Centre under the ethical number 1442032021.

**Ethics approval**

The study was performed in accordance with the guidelines of the medical ethical committee of the National Research Centre under the ethical number 1442032021.

**Chemicals and Kits**

Sodium oxalate salt was purchased from Sigma. Colorimetric kits used for biochemical analysis of serum parameters were obtained from Biodiagnostic Company, Egypt.

**Experimental design**

Rats were divided into two groups.

The first group was the control group (6 rats) which did not received any treatments.

The second group was the oxalate treated group (6 rats) in which rats were administrated sodium oxalate
salt dissolved in sterilized water through the intraperitoneal route. The rats were injected for a period of a month with the dose 70mg/kg as reported by Ilhan et al., [38]. In our experiment the oxalate dose was prepared in 2.5 ml/kg. Before ending the experiment, the rats were put in metabolic cages overnight to obtain 24 hrs urine samples for the detection of oxalate and phosphate crystals in rat urine under light microscope. After a month, blood was collected from the retro-orbital venous plexus of the eye using sterilized capillary tubes. Blood was then centrifuged for 10min at 4000rpm for obtaining the serum. Serum was stored at -20°C till used for biochemical analysis.

-Biochemical analysis
Serum liver and kidney functions were determined colorimetrically and lactate dehydrogenase was estimated kinetically as reported by Foda et al., 2016 and 2022 [39,40].

-Histological examination
After the blood collection, the rats of both groups were dissected by decapitation or dislocation method. The organs (kidneys, livers, brains, spleens and femur bones) were separated from the rats and were cleaned in saline solution and then were rinsed in 10% formalin to be prepared for staining as determined by Banchroft et al. [41].

-Statistical analysis
Data were analyzed with SPSS program (Statistical Package for the Social Sciences) version 7.5 software package (USA). Results were expressed as mean ± S.D. A p value of 0.05 or less was considered statistically significant.

In Vitro study
-Plant preparation
Rosemary dried green leaves were purchased from the Egyptian market. The leaves were then ground in the lab to fine powder by using a porcelain mortar and stored in a dry place until used for evaluation of its influence on oxalate fragmentation

-Experimental protocol
After the collection of 24-hrs urine samples from healthy humans, PH of the urine was adjusted to 6 (PH=6). Samples were then used in precipitating calcium oxalate crystals in vitro according to the method of Barros et al., [42]. Briefly, 40µl of 0.1M of sodium oxalate were added to 1 ml urine from each sample in different tubes every 30min at 37°C till reaching 90 min and crystals of oxalate were formed. Positive control (non-treated) urine samples contained only sodium oxalate crystals while treated urine samples contained the tested plant with different concentrations (from 0.01gm/ml to 0.1 gm/ml human urine) added to the samples tube before the formation of the oxalate crystals. All samples were kept for 24hrs then were centrifuged and tested under microscope

Results
-In vivo studies
-Effect of oxalate administration on urine samples in male rats.
The urine examination of control group did not show any crystals (Fig.1A). On the other hand, the urine examination in rats administrated oxalate showed the first sign for detecting hyperoxaluria in rat body. Presence of different crystals shapes that was observed with different volumes in the urine (Fig.1B-D).

-Histopathological examinations
-Effect of oxalates administration on kidney tissue and bone marrow in male rats.
Examining the control sections of kidney tissues showed normal cell structures (Fig. 2A-C). The deleterious effect of oxalates was clearly shown on kidney tissues. Widening of Bowman's capsule and destruction of blood vessels were observed besides the presence of inflammatory infiltration and severe necrosis and degeneration of epithelial lining renal tubules (Fig. 2 D, E, F & G). Bone marrow different layers was also affected by oxalate administration. Hypercellularity of hematopoietic tissue and decreased proportion of fat cells were observed (Fig. 3D & E) compared to their corresponding control group (Fig.3 A, B & C).

-Effect of oxalate administration on brain, liver and spleen tissues in male rats.
The brain tissue in the oxalate treated group displayed many pathological cases. Degeneration of neurons and axons was observed besides the increasing of Glial cells compared to the control group. On the other hand, the liver and the spleen tissues were also affected by oxalate administration to male rats but to a lesser extent (Figs. 4,5,6).

-Preliminary biochemical studies.
The effect of sodium oxalate administration on some biochemical parameters in rat serum.
Table (1) showed the mild effect of sodium oxalate on serum biochemical parameters in male rats compared to the histopathological examinations. The serum urea, creatinine and ALT levels were non-significantly changed while AST and LDH showed a significant (P<0.05) difference compared to control group.

-In-vitro study (magnification X40)
Effect of adding different concentrations of Rosmarinus officinalis Linn dried grinded leaves on experimental oxalate crystals formation in human urine.
After 24hrs from crystallization, the positive non treated samples showed the different large insoluble...
shapes of oxalate crystals that aggregated in the urine (Fig.7A, B, C& D).

Microscopic figures (represented in fig.7E, F& G) showed that applying different concentration of rosemary dried ground leaves had a great effect on the fragmentation of the insoluble urine oxalate crystals. From the minimum dose 0.01gm/ml urine to 0.03 g/ml urine there was an observed significant impact of the plant in minimizing of the oxalate crystal size compared to the positive non treated samples. Increasing the concentration of the plant from 0.04 to 0.1 g/ml urine, showed a gradual increase in oxalate crystals size formation (Fig.7H, I, J& K).

Discussion
The present work revealed the deleterious effect of sodium oxalate administration which reached most of the organs in the rats' bodies. The kidneys, the spleen, the brain and the bone marrow were greatly affected and displayed distortion in their cells shape. The presence of oxalate crystals in the urine satisfied the detection test of hyperoxaluria. The histopathological changes in the kidneys tissues were shown clearly in spite of the normal ranges of serum creatinine and urea. The presence of renal tubule degeneration and necrosis, inflammation widening and of Bowman’s capsule may interpret the significant increase in serum AST levels and non-significant changes ALT as shown in table (1). We can say also that these increasing levels were not attributed to the liver malfunction as there was mild noticed degeneration and necrosis in hepatocytes. The liver displayed slightly inflammatory aggregations in the portal area besides the widely dilated sinusoids. Oxalates was found to be non-toxic to hepatocytes. The liver is the place where the oxalate is metabolized and the hepatocytes are programmed to pour the synthesized or metabolized oxalate normally in the blood so the liver was not affected greatly by the oxalate harmful effect [43, 44].

Accordingly, urea and creatinine serum levels in the present study did not represent the correct biochemical markers for the detection of kidney functions during hyperoxaluria in the short run. These results are opposing to Crestani et al., [45] as they found an increase in plasma urea and creatinine levels during the first 3 weeks in Wistar rats administrated sodium oxalate rich diets. They also reported no changes in blood glucose levels and PH.

On the other hand, the determination of histopathological changes in kidney tissues were observed in experimental animals when injected intraperitoneally with oxalates was attributed to the generation of malondialdehyde (MDA) and reactive oxygen species (ROS) [45-50]. The spleen plays a critical role in protection of multi-organs injury including the acute kidney injury (AKI) through controlling the expression of cytokines and inflammatory markers associated with the organ's injuries [51]. Inflammation leads to the progression of chronic renal failure. Large spleen sizes were observed in older patient cases undergoing dialysis [52] compared to controls. Many discussions on the mechanisms of the spleen in host defence and controlling acute kidney injury were reported [53]. Physiologic and molecular mechanisms of distant organs interactions in AKI include lymphocyte activation and infiltration and generation of inflammatory cytokienes/chemokines and endothelial injury. Oxidative stress increased levels and ROS are important mechanisms of AKI-induced distant organ dysfunction [54].

In our study the spleen tissue of aged rats underwent lymphocytic degeneration which reflected the spleen dysfunction and the disability in defending multi-organisms of the body from the inflammatory storms that existed during hyperoxaluria injury.

In the present study, the brain tissue showed many pathological distortions such as neuronal necrosis, degeneration of nerve fiber, perineural edema and accumulation of glial cells. This histopathological picture pointed to the beginning of the death and loss of the neurons as nerve fibers that connect them exhibited a degeneration state, remembering that nerve cells lack the ability of regeneration causing dementia at last [55, 56].

Our results are in accordance with Berini et al., 2015 [57] they reported the effect of calcium oxalate on brain structure and function discussing the mechanisms causing nerve injury in hyperoxaluria. Many researches focused on the brain status during the existence of acute kidney disease in the long run [58, 59] and the effect of the accumulated blood wastes on the brain due to kidney malfunction. But in the present study the blood wastes were not yet increased significantly So we can attribute these accelerated distortions in the brain tissue in our study to performing the experiment on male aged rats as the brain of aged males may be more susceptible to oxalate effect than young ones.

The hypercellularity of the hematopoietic tissue in the bone marrow of rats treated with oxalate in our study was attributed to the compensation of the bone marrow to the lost precursors of RBCs and WBCs due to the harmful effect of oxalate administration [60].

The bone marrow is strongly affected by the over production or over doses of oxalate in the body. This case may reach the deposition of calcium oxalate crystals in the bone marrow tissues (Systemic oxalosis) leading to its failure [61-63].

The present study recommended a natural, available, cheap and safe therapeutic agent to minimize the
harmful effect of oxalates on body organs. Our choice was focused on the green dried leaves of *Rosmarinus officinalis* Linn. The plant was recommended for the first time as anti-uroliathic candidate *in vitro*. *Rosmarinus officinalis* Linn or rosemary is a plant that exists in the Mediterranean region and possesses a wide spectrum as antimicrobial, anti-inflammatory, analgesic, and antioxidant agent [64-67].

Many research studies reported its effect as an anti-toxicant and as prophylactic therapy for many case diseases related to the kidney and the liver in addition to improving nervous disorders[68-72]. Our results showed that the plant leaves has a high impact on oxalate fragmentation from the dose beginning from 0.01 g/ml till the dose of 0.03 g/ml. The results showed also increasing the plant concentration has no influence on the oxalate crystals size. These results may point to the useful use of small concentrations of the dried grinded rosemary leaves in fragmentation of oxalate crystals and the higher concentrations of the plant has no influence on oxalate fragmentation.

These results encourage us for performing more studies on evaluating the rosemary plant effect on kidney stone formation in rats. This study is now under investigation.

**Conclusion**

*Rosmarinus officinalis* Linn can be used as a powerful anti-urolithic candidate when monitoring the dose.

**Acknowledgement**

The authors thank gratefully the National Research Centre for providing the facilities to produce and publish this work.

**Conflict of interest.**

The authors declare that there is no conflict of interest.

**Funding statement**

This work was not supported by any fund.

**Author’s Contribution**

DSF put the experimental design, contribute to the practical work and wrote the manuscript, NEI contribute to the practical work and revised the manuscript.
- *In vivo* study.

- Microscopic examination for rat urine.

Fig. 1(A) Showing no crystals were observed in control rat urine (pH=9).

Fig. 1(B,C&D) illustrated the presence of different crystals shapes (black arrow) and (white arrow) in rat urine (pH=9) accompanied to sodium oxalate injection displaying the same urine pH as in control group (magnification X40).

Kidney tissue

- Control group

Fig. 2(A,B,C). Represented microscopic sections of control kidney tissue showing normal structure of renal tubules (arrows) and glomeruli (asterisk). (H&E X200).

- Oxalate treated group:

Fig. 2(D,E,F and G) represented microscopic sections of kidney tissue in oxalate treated rat. D section revealed inflammatory cells infiltration (triangle), widening of Bawman’ s capsules (asterisk), congested blood vessels and necrosis and degeneration of epithelial lining renal tubules (arrow). E illustrated severe necrosis and degeneration of epithelial lining renal tubules (arrows) and infiltration with inflammatory cells (triangle). F showed increase and widening of Bawman’ s capsules. G displayed vaculation and destruction of endothelial lining of blood vessels (white arrow). (H&E X200).
Fig. 3 (A&B). Sections displaying normal structure, normal hematopoietic tissue (arrow) and adipose cells (triangle). Fig.3(C): section showing mild decrease in hematopoietic tissue. (H&EX200).

-Oxalate treated group

Fig. 3 (D & E) represented microscopic sections in bone marrow of oxalate treated rat revealing hypercellularity (arrow) of hematopoietic tissue and decreased proportion of fat cells (asterisk). (H&EX200).
Brain tissue
Control and oxalate treated group

Fig. 4(A). Represented microscopic section of brain control group showing normal histological structures with normal neurons (arrow). Fig.4 (B-H) represented brain sections in oxalate treated rats. B Section in cerebrum showing diffuse gliosis (arrows). C Brain cerebrum showing necrotic neurons, perineural edema and ghost like neurons (asterisk). D Brain cerebrum showing neuronal necrosis and an area of encephalomalacia (white arrow). E Brain showing focal gliosios that mean accumulation of glial cells (asterisk). F Brain showing fragmentation and perineural edema (arrow). G Brain showing fragmentation and degeneration of nerve fiber and neuronal necrosis (arrow). H Brain showing encephalomalacia (white arrow). H&EX200
Spleen tissue
- Control and oxalate treated group.

Fig. 6(A).
Section in spleen of control rat showing the normal structure of red and white pulp with normal lymphoid follicles.

Fig. 6 (B&C). Spleen section in oxalate treated rats showing lymphocytic depletion (arrow) (H&EX200).

Liver tissue
- Control and oxalate treated groups

Fig. 5(A).
Section in liver tissue of control rat showing normal hepatocytes (arrow) and portal area (asterisk).

Fig. 5 (B&C): Liver hepatocytes section in oxalate treated group. (B) showing individual cell necrosis (triangle) and the hepatic sinusoids were widely dilated (black arrows) and congested with blood (white arrow), inflammatory cell infiltration in the portal area (asterisk). (C) Section showing individual cell necrosis dilated sinusoids. (H&EX200).
TABLE 1. Effect of Sodium oxalate administration on some biochemical parameters in rat serum.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Units</th>
<th>Control group</th>
<th>Sodium oxalate treated group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>(mg/dl)</td>
<td>95.54±10.8</td>
<td>107.38±23.55</td>
</tr>
<tr>
<td>Urea</td>
<td>(mg/dl)</td>
<td>60.30±1.82</td>
<td>65.14±6.35</td>
</tr>
<tr>
<td>Creatinine</td>
<td>(mg/dl)</td>
<td>0.20±0.02</td>
<td>0.22±0.045</td>
</tr>
<tr>
<td>ALT</td>
<td>(U/ml)</td>
<td>9.55±0.19</td>
<td>10.38±1.08</td>
</tr>
<tr>
<td>AST</td>
<td>(U/ml)</td>
<td>42.56±0.28</td>
<td>45.06±1.55*</td>
</tr>
<tr>
<td>LDH</td>
<td>(U/l)</td>
<td>208.84±7.28</td>
<td>152.186±12.22*</td>
</tr>
</tbody>
</table>

Values (mean±SD) bearing asterisk in a row differ significantly compared to control group \( (P \leq 0.05) \). ALT: alanine aminotransferase, AST: aspartate aminotransferase, LDH: lactate dehydrogenase.

- In vitro study.

- Positive control without adding *Rosmarinus officinalis Linn* to 24-hrs urine human samples containing added sodium oxalate.

Fig. 7 (A, B, C & D). Microscopic figures represented the oxalate crystals precipitated in 1ml of 24-hrs human urine samples (X40).

- Effect of *Rosmarinus officinalis Linn* dried grinded leaves on the fragmentation of the added oxalate in 24-hrs human urine samples.

Fig. 7 (E, F, G, H, I, J & K). Microscopic figure represented the effect of different doses of dried leaves of rosemary plant on added oxalate fragmentation in 1ml human urine. [E] showing the effect of the dose 0.01gm/ml, [F] 0.02gm/ml, [G] 0.03gm/ml, [H] 0.04gm/ml, [I] 0.06gm/ml, [J] 0.08gm/ml and [K] 0.1gm/ml.
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The effects of the rosemary extracts on some parameters in the gut flora of rats. 1.

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The antibacterial activity of rosemary extracts was determined by the agar diffusion method. The results showed that the extracts had a significant inhibitory effect on the growth of bacteria. Furthermore, the extracts were found to be effective in preventing the growth of fungi. The results of the antioxidant activity test showed that the extracts had a significant antioxidant effect. These findings suggest that rosemary extracts can be used as a natural preservative in the food industry. Additionally, the results of the cytotoxicity test showed that the extracts had a significant cytotoxic effect on cancer cells.