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# The potential role of alkaline diets in prevention of calcium oxalate kidney stone formation

Doha A. Mohamed, * Hoda B. Mabrok, Asmaa A. Ramadan and Hagar F. Elbakry

Formation of kidney stones is considered a major global problem. Diet plays an important role in the management of kidney stone formation. The main goal of the present research was to evaluate the protective role of fruit and vegetable mixtures as models of an alkaline diet on formation of kidney stones in rats and to conduct molecular docking study. The chemical compositions, phenolic compound profile, β-carotene content, vitamin C and antioxidant activity of both mixtures were assessed. Fruit (−42.419 ) and vegetable (−11.13) mixtures recorded a negative potential renal acid load in the presence of macro-/micro-nutrients, β-carotene and phenolic compounds; chlorogenic acid was the major content in both mixtures. Both mixtures exhibited high antioxidant activity. Molecular docking study proved that rutin displayed the highest binding affinities for glycolate oxidase (−11.8 kcal $mol^{-1}$) and lactate dehydrogenase (−10.1 kcal $mol^{-1}$). The kidney stone model in rats exhibited metabolic acidosis in the urinary profile through reduction of citrate; Ca, Mg and K excretion and elevation of oxalate, creatinine, creatinine clearance, uric acid, urea and protein. Additionally, there was a significant reduction in plasma Ca, Mg and K levels, while liver and kidney function parameters improved significantly. Fruit and vegetable mixtures as models of an alkaline diet proved improvement in all the parameters. Histopathological examination of kidney sections of the kidney stone model showed crystal deposition, inflammation, and severe necrosis. Kidney sections of alkaline diet models indicated mild and moderate changes. *Conclusion*: The results of this study proved that both alkaline diet models were effective in protecting against kidney stone formation *in vivo* and in molecular docking studies.

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## Introduction

Acid–base balance plays an important role in regulation of human physiology. Diet is a risk factor for low-grade metabolic acidosis (pH lower than 7.35). Diets with high protein, especially animal protein, and saturated fat and high sugar contents are one of the main reasons for releasing higher numbers of precursors of acids into the bloodstream. Foods rich in potassium, calcium and magnesium are precursors of bases. Diet-induced acidosis leads to metabolic impairment and elevation in the incidence of diet-related diseases such as type-2 diabetes, cardiovascular diseases and kidney stone formation.[1,2] Potential renal acid load (PRAL) is used as an indicator of the acid base load of food.[3] Positive PRAL values indicate acid-containing foods, whereas negative PRAL values indicate alkaline foods. The more negative the potential renal acid load, the more alkaline the food. Therefore, consumption of food-based precursors (alkaline diet/food) is a good strategy for reducing the incidence of diet-related diseases, such as kidney stone formation.

The formation of kidney stones is considered a major global problem. The incidence of kidney stones increases globally from approximately 2% to 20% and constitutes a major burden on the healthcare system.[4] Kidney stone disease (KSD) is a disease that is more common in men than in women.[5] KDS appears in a wide range of ages, including children, adolescents, and adults.[6] Kidney stones may lead to irreversible damage, such as ureteral blockage, urinary tract infection, cystitis, and, eventually, end-stage renal failure.[7] The most common types of stones are calcium oxalate (CaOx) stones, representing approximately 80–90%. CaOx forms, grows, aggregates and accumulates due to urine saturation, and it is deposited in the kidneys and causes inflammation and injury in the kidney tubular cells in association with oxidative stress and reactive oxygen species (ROS).[5,8] CaOx kidney stone is one of the primary causes of chronic renal diseases, leading to heavy expenditure burden and poor life quality.[9] KSD can lead to several health problems, such as metabolic bone disease, hypertension, and vascular calcifications.[10] Therefore, the prevention of kidney stone disease is an important goal for the reduction of its burden on the health system and for the

*Nutrition and Food Sciences Department, Food Industries and Nutrition Institute, National Research Centre, Dokki 12622, Cairo, Egypt.*
*E-mail: dohamohamed@yahoo.com*

reduction of its ability as a risk factor involved in the incidence of many chronic diseases, such as hypertension and cardiovascular diseases.

Nutrition is probably one of the most important modifiable risk factors associated with the high prevalence of kidney stone disease in the general population.[11] Some dietary patterns, such as the Mediterranean diet pattern, are associated with a lower risk of KSD,[12] while the Western diet pattern has a negative impact on KSD due to the accumulation of metabolic acidosis.[13] The main differences in both diet types are the reduction of fruit and vegetable intake and elevation in consumption of saturated fats in the Western diet compared to the Mediterranean diet. The increment in consumption of acidic foods such as rich sources of phosphorus and proteins in association with the reduction in base foods, such as rich sources of potassium, calcium and magnesium, leads to disturbance in the acid–base balance, which is an important cause of many chronic diseases.[1] Fruits and vegetables are rich sources of vitamins, minerals, dietary fibers and phytochemicals, such as total phenolic, phenolic acids, polyphenols, flavonoids and isoflavons.[14,15] All these nutrients and phytochemicals present in fruits and vegetables enhance their activities as antioxidant, anti-inflammatory and cardio-protective candidates. Additionally, the consumption of both fruits and vegetables prevents humans from developing many chronic diseases, such as cancer and dyslipidemia.[16]

Fruits and vegetables (alkaline diets) play an important role in the prevention of all types of kidney stones, as reported previously.[17–20] The mechanism of action of fruits and vegetables in protection from kidney stones includes the high citrate content that elevates urinary citrate and prevents the formation of kidney stones,[21] and the alkalinizing effect on urinary pH. Phytate, citrate, potassium, and magnesium are the main stone inhibitors in fruits and vegetables.[22] Phytate consumption led to the progress of insoluble complexes with calcium in the gut, which suppressed crystal formation in the urine and reduced the risk of stone formation.[17,23] Dietary fiber prevents the formation of stones through its non-digestible ingredients that link to minerals and fat within the gastrointestinal tract, resulting in the suppression of the urinary excretion of oxalate.[17]

Recently, dietary intervention and the modification of food consumption have become very important for the reduction of kidney stone formation.[24] Most treatments used for the reduction of kidney stone formulations depend on inhibiting glycolate oxidase (GO), which leads to a reduction in glyoxylate (Glyo) production. Glyoxylate is mainly produced by the oxidation of glycolate by the GO enzyme. Lactate dehydrogenase (LDH) is responsible for the increase in oxalate formation in the kidney through the formation of the insoluble crystals of calcium oxalate.[25] The present research aimed to study the protective effects of fruit and vegetable mixtures as a model of an alkaline diet in rats. Beta-carotene, vitamin C, phenolic compounds and their profiles in both mixtures were estimated using HPLC. Molecular docking of the most promising phenolic compounds was studied as a potential inhibitor of liver glycolate oxidase (GO) and as a reducer of liver lactate dehydrogenase (LDH). The proximate composition of both mixtures and their mineral content (Na, K, Ca, P, and Mg) were evaluated. The antioxidant activity of the fruit and vegetable mixtures was estimated using DPPH. Fruits and vegetables used in the present study were collected according to their lower content of oxalate and potential renal acid load (PRAL), which means that all fruits and vegetables used in the study were alkaline foods.

## Materials and methods

### Plant materials

All fruits and vegetables were purchased from local markets (Giza, Egypt), including celery, eggplant, summer squash, carrot, red cabbage, red radish, onion, red pepper, yellow pepper, green pepper, broccoli, tomato, sweet potato, banana, avocado fruit, raisins, dried apricot, dried fig and dried dates.

### Animals

Male Sprague Dawley rats (100–138 g) were used in the present study. The National Research Centre Animal House was the provider of the animals. During the study, the animals were given clean water and food *ad-libitum*. The experimental procedure was approved by the National Research Centre, Ethical Committee No. 13050203.

### Animal's diets

Four types of diets were designed and fed to animals during the study period (Table 1). The AIN-93 vitamin and salt mixture was prepared according to the procedure employed by Reeves *et al.*[26] Dried vegetables and dried fruit mixtures were crushed into powder form to be used in the diet preparation.

### Preparation of plant materials

Celery aerial parts, eggplant, summer squash, carrot, red cabbage, red radish, onion, red pepper, green pepper, broccoli, tomato, sweet potato, banana and avocado fruit were washed with tap water and then cut into small slices. All plants were

**Table 1** Composition of different experimental diets (g per 100 g)

| Ingredients | Balanced diet | Kidney stone diet | Fruit mixture diet | Vegetable mixture diet |
|---|---|---|---|---|
| Casein[a] | 12 | 12 | 11.242 | 9.97 |
| Corn oil | 10 | 10 | 8.27 | 9.69 |
| Sucrose | 23.5 | 23.5 | 23.5 | 23.5 |
| Starch | 47 | 42 | 28.02 | 28.5 |
| Salt mix. | 3.5 | 3.5 | 3.5 | 3.5 |
| Vitamin mix. | 1 | 1 | 1 | 1 |
| Cellulose | 3 | 3 | — | — |
| Potassium oxalate | — | 5 | 5 | 5 |
| Fruit mixture | — | — | — | 20 |
| Vegetable mixture | — | — | 20 | — |

[a] 12 g casein was determined to contain 10 g protein using AOAC.[27]

dried separately in an air-circulated oven at 40 °C to complete dryness. All dried plant materials were reduced separately into powder form and stored in plastic bags in a refrigerator at 4 °C.

### Preparation of vegetables and fruit mixtures

Vegetable mixture was prepared from the dried powders of different vegetables, as mentioned previously. Carrot, red cabbage, red radish, broccoli, sweet potato, onion, eggplant, and summer squash were mixed with 10% of each. Then, red pepper (5%), green pepper (5%), celery aerial parts (5%) and tomato (5%) were mixed and used as vegetable mixture. Fruit mixture was prepared by mixing powders of banana fruit, raisins, avocado fruit, and apricot by 20% from each; then, 10% from fig and 10% from date were added. These mixtures were used for proximate analysis and for the preparation of diets that were fed to the rats.

### Proximate analysis, vitamin C, phenolic compounds content and β-carotene of fruits and vegetables mixtures

Fruits and vegetable samples were analyzed to determine moisture, protein, fat, crude fiber, and ash and to calculate carbohydrates according to the methods of AOAC.[27] Total dietary fiber was determined in the studied mixtures using AOAC methods.[27] Potassium, sodium, calcium, magnesium and phosphor were determined using a PerkinElmer 2380 atomic absorption spectrophotometer according to the method of AOAC.[27] Vitamin C was determined according to the method of Turak *et al.*[28] using HPLC. Folin–Ciocalteu reagent was used for the estimation of total phenolic content, and the results were represented as gallic acid equivalents (GAE) in mg per 100 g sample.[29] β-Carotene was extracted from fruit and vegetable mixtures using petroleum ether and acetone in a ratio of 3 : 2, according to the method of Ranganna.[30] The results were expressed as follows: β-Carotene (mg per 100 g) = [¼Absorbance × 13.9 × 104 × 100]/Weight of sample (g) × 560. The results of all determined parameters were expressed as the mean ± SD for the three replicates.

### High-performance liquid chromatography (HPLC) analysis of phenolic compound profiles of fruit and vegetable mixtures

The phenolic compound profile was analysed using the HPLC system (Agilent-1260 series) with a Zorbax Eclipse Plus C18 column, 4.6 mm × 250 mm (5 μm). The eluting phenolic acid was monitored using a multi-wavelength diode array detector at 280 nm. The flow rate was 0.9 ml $min^{-1}$, injection volume was 5 μl and column temperature was maintained at 40 °C. The gradient programme was applied with eluent A (water) and eluent B (0.05% trifluroacetic acid in acetonitrile) as follows: 82% A for 1 min, 75% A for 10 min, 60% A for 7 min, and 82% A for 6 min, resulting in a total duration of 24 min. Phenolic compounds extracted from each mixture (100 mg) were dissolved in 1 ml methanol (HPLC grade) and filtrated through a 0.2 μm filter sterilized membrane before the injection. Samples were determined in triplicates. The retention times of the identified compounds were recorded and compared with the respective retention times of known standard reference materials. The concentration of each phenolic compound was quantified by measuring the peak area and comparing it to the relative standards. The standards used were gallic acid, chlorogenic acid, catechin, methyl gallate, syringic acid, caffeic acid, coumaric acid, pyrocatechol, rutin, vanillin, ferulic acid, naringenin, rosmarinic acid, quercetin, daidzein, cinnamic acid and hesperetin.

### DPPH radical scavenging activity

The DPPH radical scavenging ability of methanolic extracts obtained from the powder of fruit and vegetable mixtures was assessed.[31] The methanolic extracts of both mixtures were dissolved in 1.0 mL of 0.1 mM DPPH solution at room temperature. After 30 min of incubation, the absorption was measured at 515 nm. The results were expressed as percentage inhibition using the following equation:

$$\%\ \text{inhibition} = (A_0 - A_s/A_0) \times 100$$

$A_0$ is the absorbance of the control; $A_s$ is the absorbance of the sample.

Then, the % of inhibitions was plotted against respective concentrations. $IC_{50}$ values were calculated as the concentration of the extract required to produce 50% DPPH radical scavenging activity.

### Determination of the pH of fruit and vegetable mixtures

The pH of fruits and vegetable mixtures was measured according to AOAC[27] method. In brief, 10 g of vegetable and fruit mixtures were extracted using distilled water (1 : 3 w/v) in a water bath of 50 °C for 30 min; the samples were mixed from time to time with a glass rod. The electrode was introduced into the sample extract at a temperature of 20 ± 2 °C. The measurements were recorded for three replicates from the same sample extract.

### Determination of potential renal acid load (PRAL) of the studied fruit and vegetable mixtures

The potential renal acid load (PRAL) of fruit and vegetable mixtures was calculated according to the formula of Adair & Bowden:[3]

$$\begin{aligned}\text{Potential renal acid load} &= 0.4888 \times \text{dietary protein (g)} \\ &+ 0.0366 \times \text{dietary phosphorus (mg)} - 0.0205 \\ &\times \text{dietary potassium (mg)} - 0.0125 \times \text{calcium (mg)} \\ &- 0.0263 \times \text{magnesium (mg)}.\end{aligned}$$

### Molecular docking study of fruit and vegetable mixtures against glycolate oxidase (GO) and lactate dehydrogenase (LDH)

The crystal structures of the glycolytic enzymes glycolate oxidase (GO) (PDB ID: **5QIF**) and lactate dehydrogenase (LDH) (PDB ID: **4RLS**) were retrieved from the Protein Data Bank.[32] The proteins were prepared for docking using AutoDock Tools 1.5.7. Potential binding pockets were identified based on the

positions of co-crystallized ligands. Ligand structures were obtained from the PubChem database.[33] The ligands were energy minimized using the MMFF94 force field in Avogadro 1.2.0.[34] Molecular docking simulations were carried out using AutoDock Vina.[35] The prepared protein and ligand structures were used as inputs. The docking results were visualized and analysed using Biovia Discovery Studio Visualizer 2020.[36]

### Kidney stone model in rats

The rats were divided into four groups ($n$ = 7 per group) and kept at 25 ± 2 °C with a 12 h light and dark cycle. Group I served as a normal control, which received a balanced diet. Group II received a balanced diet containing 5% potassium oxalate and served as a kidney stone control for 28 days.[37] Groups III and IV received balanced diets containing 5% potassium oxalate and 20% vegetable or fruit mixtures, respectively. These two groups served as preventive groups. At the end of the experimental period, rats were placed in the metabolic cages, and 24 h urine was collected in the presence of sodium azide 0.02% for the prevention of bacterial growth. Blood samples were collected from fasting rats to separate plasma and different biochemical analyses. Kidneys were collected from all rats for histopathological studies.

### Urine examinations

After collecting urine over sodium azide 0.02%, urine was used for microscopic studies to detect crystals in urine. Urine volume and pH (an indicator of the net endogenous production of acids) were measured. An aliquot of each urine sample was saved at −20 °C for different assays. Urinary citrate, oxalate, calcium, phosphor, sodium, potassium, magnesium, total protein, uric acid, creatinine and urea were estimated using a commercial kit. Creatinine clearance was calculated using the following equation:

$$\text{Creatinine clearance (ml min}^{-1}) = [\text{urine creatinine (mg d}^{-1})] \times (\text{urine volume 24 h})/\text{plasma creatinine (mg dl}^{-1}) \times 1440.$$

### Blood metabolites and minerals

Plasma samples of all rats were used to determine creatinine, urea, blood urea nitrogen (BUN), uric acid, total protein, albumin (ALB), gamma glutamyl transferase (GGT), alkaline phosphatase (ALP), sodium, potassium, magnesium, phosphorus and calcium using a commercial kit. Ionized calcium was calculated according to the equation of Foster *et al.*:[38]

$$\text{Ionized calcium} = 0.9 + 0.55 \times \text{Total Calcium} - 0.3 \times \text{ALB}.$$

### Statistical analysis

The SPSS v26 statistical programme was used to analyse the data, and one-way ANOVA with Tukey multiple comparison tests was performed to evaluate whether the means were statistically different. The results of vegetable and fruit mixtures analysis were statistically analysed using Student's $t$-test. Differences were considered significant at $p \leq 0.05$.

## Results

### Proximate analysis, pH, phenolic compounds content, vitamin C, β-carotene and antioxidant activity of fruit and vegetable mixtures

The results of the chemical composition, pH and antioxidant activity of the studied fruit and vegetable mixtures are presented in Table 2. The vegetable mixture showed a significantly high content of protein (10.29%), ash (5.74%), dietary fibers (15.63%), vitamin C (93.21 mg per 100 g) and β-carotene (14.75 mg per 100 g), while the mixture of fruits showed a significant content of fat (8.66%) and phenolic compounds (2444 mg GAE per 100 g). The pH of the vegetable mixture (4.97) was non-significantly increased compared to the fruit mixture (4.54). The fruit mixture exhibited a significant elevation in antioxidant activity (54.71 μg ml$^{-1}$) than the vegetable mixture (24.47 μg ml$^{-1}$), as shown by the elevation of DPPH $IC_{50}$. All minerals were significantly higher in the vegetable mixture than in the fruit mixture except for magnesium and potassium, which were higher in the fruit mixture than in the vegetable mixture. The fruit mixture recorded a highly negative potential renal acid load (PRAL) of −42.419 compared with vegetable mixtures, which recorded −11.13 PRAL, which means that the fruit mixture was more alkaline than the vegetable mixture.

### HPLC phenolic profile of the fruit and vegetable mixtures

In this study, 13 and 15 phenolic compounds were identified in the fruit mixture and vegetable mixtures, respectively, by the HPLC (Fig. 1). As shown in Fig. 1, chlorogenic acid was the major compound present in fruit and vegetable mixtures by 801.8 μg g$^{-1}$ and 14 318.8 μg g$^{-1}$, respectively. Cinnamic acid and hesperetin were the highest compounds identified in the

Table 2 Chemical composition, pH and antioxidant activity of dried vegetable and fruit mixtures (mean ± SD)

| T | Fruit mixture | Vegetable mixture |
|---|---|---|
| Protein (g per 100 g) | $3.76^{b}$ ± 0.25 | $10.29^{a}$ ± 0.13 |
| Fat (g per 100 g) | $8.66^{a}$ ± 0.34 | $1.60^{b}$ ± 0.04 |
| Ash (g per 100 g) | $2.68^{b}$ ± 0.30 | $5.74^{a}$ ± 0.06 |
| Na (mg per 100 g) | $10.10^{b}$ ± 0.66 | $188.30^{a}$ ± 3.51 |
| K (mg per 100 g) | $2127.70^{a}$ ± 2.52 | $1077.70^{b}$ ± 7.51 |
| Ca (mg per 100 g) | $52.70^{b}$ ± 2.52 | $223.70^{a}$ ± 3.51 |
| P (mg per 100 g) | $93.10^{b}$ ± 3.00 | $282.30^{a}$ ± 3.06 |
| Mg (mg per 100 g) | $129.20^{a}$ ± 2.02 | $61.30^{b}$ ± 4.03 |
| Total carbohydrates (g per 100 g) | $84.89^{b}$ ± 0.89 | $82.40^{a}$ ± 0.22 |
| Total dietary fiber (g per 100 g) | $10.32^{b}$ ± 0.48 | $15.63^{a}$ ± 0.35 |
| Vitamin C (mg per 100 g) | $2.03^{a}$ ± 0.07 | $93.21^{b}$ ± 0.54 |
| Total phenolic (mg GAE per 100 g) | $2444.00^{a}$ ± 7.94 | $2274.70^{b}$ ± 6.81 |
| β-Carotene(mg per 100 g) | $11.13^{b}$ ± 0.42 | $14.75^{a}$ ± 0.50 |
| pH | $4.54^{a}$ ± 0.01 | $4.97^{a}$ ± 0.02 |
| DPPH $IC_{50}$ (μg ml$^{-1}$) | $54.71^{b}$ ± 0.86 | $24.47^{a}$ ± 0.50 |
| PRAL | −42.42 | −11.13 |

Similar letters mean a non-significant difference between the two mixtures ($p \leq 0.05$) in the same row. PRAL: potential renal acid load.

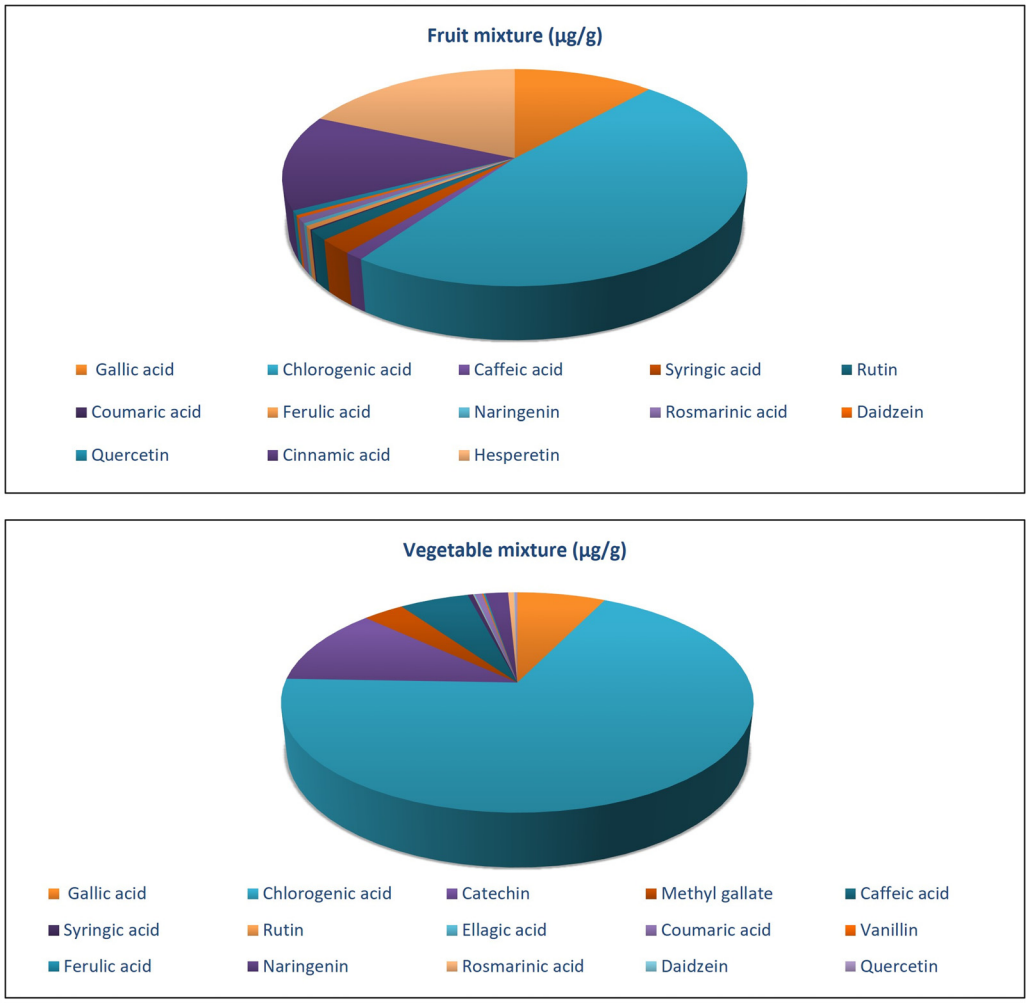


**Fig. 1** Phenolic compound profile of fruit and vegetable mixtures.

fruit's mixtures, while catechin, gallic acid, caffeic acid and methyl gallate were the highest compounds that appeared in the vegetable's mixture.

### Molecular docking study of fruit and vegetable mixtures against glycolate oxidase (GO) and lactate dehydrogenase (LDH)

Liver GO and LDH play an important role in the formulation of kidney stones. The binding affinity data presented in Table 3 revealed that all ligands of the phenolic compounds identified in the fruit and vegetable mixtures exhibited promising interactions with the GO and LDH proteins. Notably, rutin displayed the highest binding affinities for both GO ($-11.8$ kcal mol$^{-1}$) and LDH ($-10.1$ kcal mol$^{-1}$), followed by ligands such as ellagic acid, hesperetin, naringenin, chlorogenic, and rosmarinic, which showed high to moderately high binding affinities. Other ligands, such as catechin, daidzein, quercetin, and ferulic acid, also demonstrated favourable interactions with either GO or LDH. In contrast, ligands such as vanillin, gallic acid, syringic acid, cinnamic acid, coumaric acid, and methyl gallate exhibited relatively lower binding

**Table 3** $\Delta G$ binding affinity (kcal mol$^{-1}$) for each phenolic compound ligand with proteins GO and LDH

| Ligand | GO | LDH |
|---|---|---|
| Caffeic acid | −8.0 | −6.0 |
| Catechin | −9.6 | −7.7 |
| Chlorogenic | −9.8 | −8.1 |
| Cinnamic acid | −7.6 | −5.5 |
| Coumaric acid | −7.4 | −5.7 |
| Daidzein | −9.0 | −7.4 |
| Ellagic acid | −10.4 | −8.0 |
| Ferulic acid | 18.0 | −6.1 |
| Gallic acid | −7.0 | −5.5 |
| Hesperetin | 110.6 | −7.8 |
| Methyl gallate | −7.3 | −5.6 |
| Naringenin | −10.0 | −7.7 |
| Quercetin | −9.4 | −8.0 |
| Rosmarinic | −9.9 | −7.8 |
| Rutin | −11.8 | −10.1 |
| Syringic acid | −7.1 | −5.8 |
| Vanillin | −6.5 | −5.4 |

affinities for both proteins. These findings suggest the potential of certain ligands, particularly rutin, as inhibitors or modulators of GO and LDH enzymes.

According to binding affinity, the 3D and 2D interaction diagrams of the best compound (rutin) were analysed for the proposed activity (Fig. 2A and B). Because chlorogenic acid was the highest phenolic compound present in both studied mixtures, we also studied their 3D and 2D interaction diagrams (Fig. 3A and B). Rutin showed the lowest docked binding energy with Go (−11.8 kcal $mol^{-1}$) and LDH (−10.1 kcal $mol^{-1}$) proteins, which is advantageous. Rutin forms two hydrogen bond interactions with the amino acid residues ARG145 and ARG226 in the Go protein while forming a diverse set of interactions with LDH, including numerous conventional hydrogen bonds with residues such as ARG98, ASN137, ARG168, ASP194, TYR238, ALA97, and GLN99 (Fig. 2A and B). The formation of hydrogen bonds and the diverse range of interactions by rutin with the GO and LDH proteins, respectively, can be considered a significant factor contributing to its higher binding affinity (−11.8 kcal $mol^{-1}$ and −10.1 kcal $mol^{-1}$, respectively). Furthermore, the distance values of the hydrogen bond interactions between rutin and GO (2.57568 Å and 2.10368 Å) are within the optimal range for conventional hydrogen bonds, indicating favourable geometries and orientations for these interactions. Rutin forms interactions with a broader range of amino acid residues, suggesting a more extensive network of interactions within the binding pocket of LDH. Chlorogenic acid interacted with the active site of GO at the ARG226, ARG227, ASN253, and SER230 residues with conventional hydrogen bonds and Pi-Alkyl. Chlorogenic acid interacted with the active site of LDH at the VAL30, HIS192, THR94, and ILE251 residues with conventional hydrogen bonds, Pi-Sigma, and Pi-Alkyl. Moreover, unfavourable bounds (donor-donor) at the ALA29 residue were formed. The present results indicated the potential role of fruit and vegetable mixtures under investigation as inhibitors for the formation of oxalate crystals

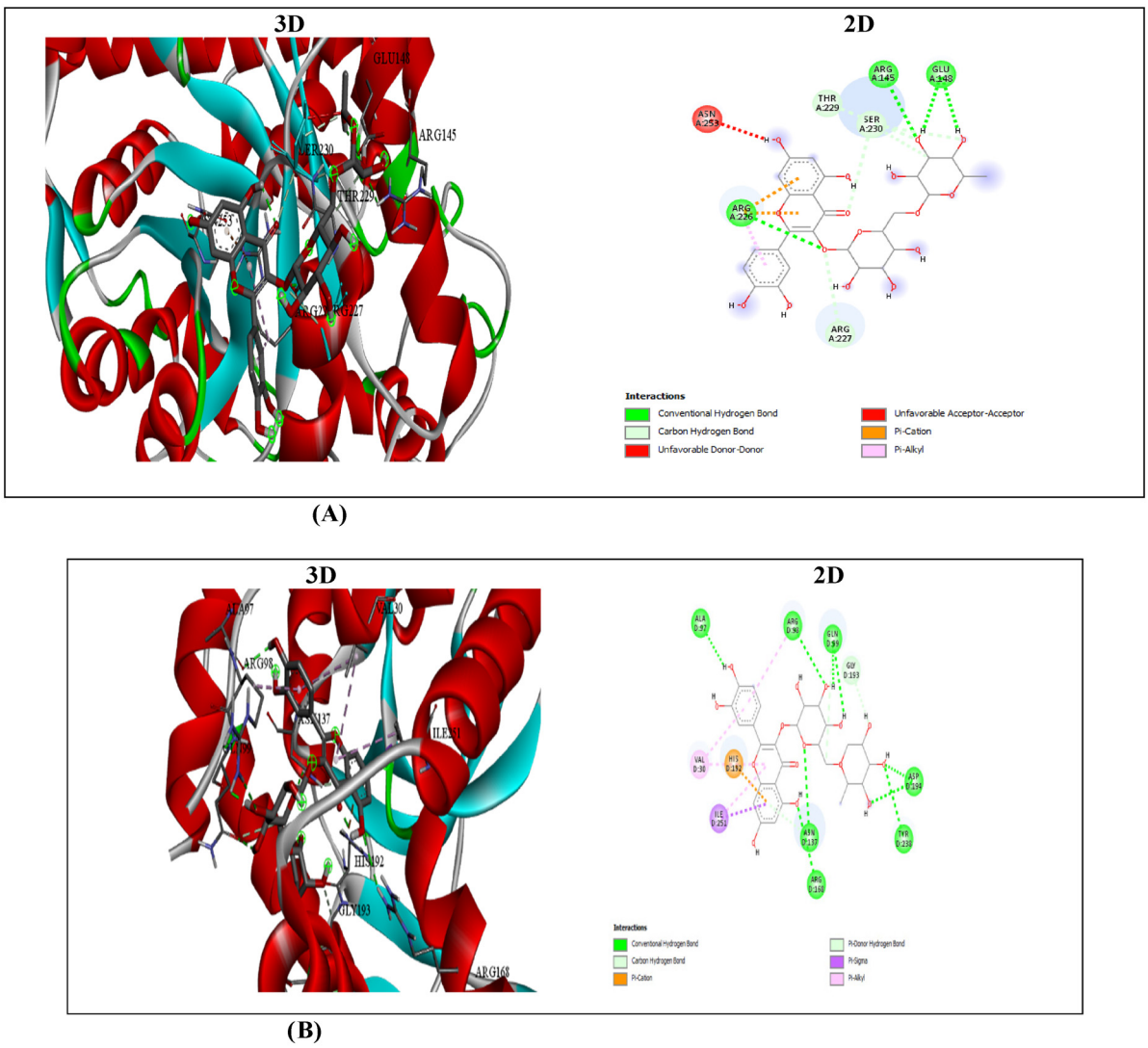


Fig. 2 (A) 3D and 2D of rutin interaction with glycolate oxidase. (B) 3D and 2D of rutin interaction with lactate dehydrogenase.

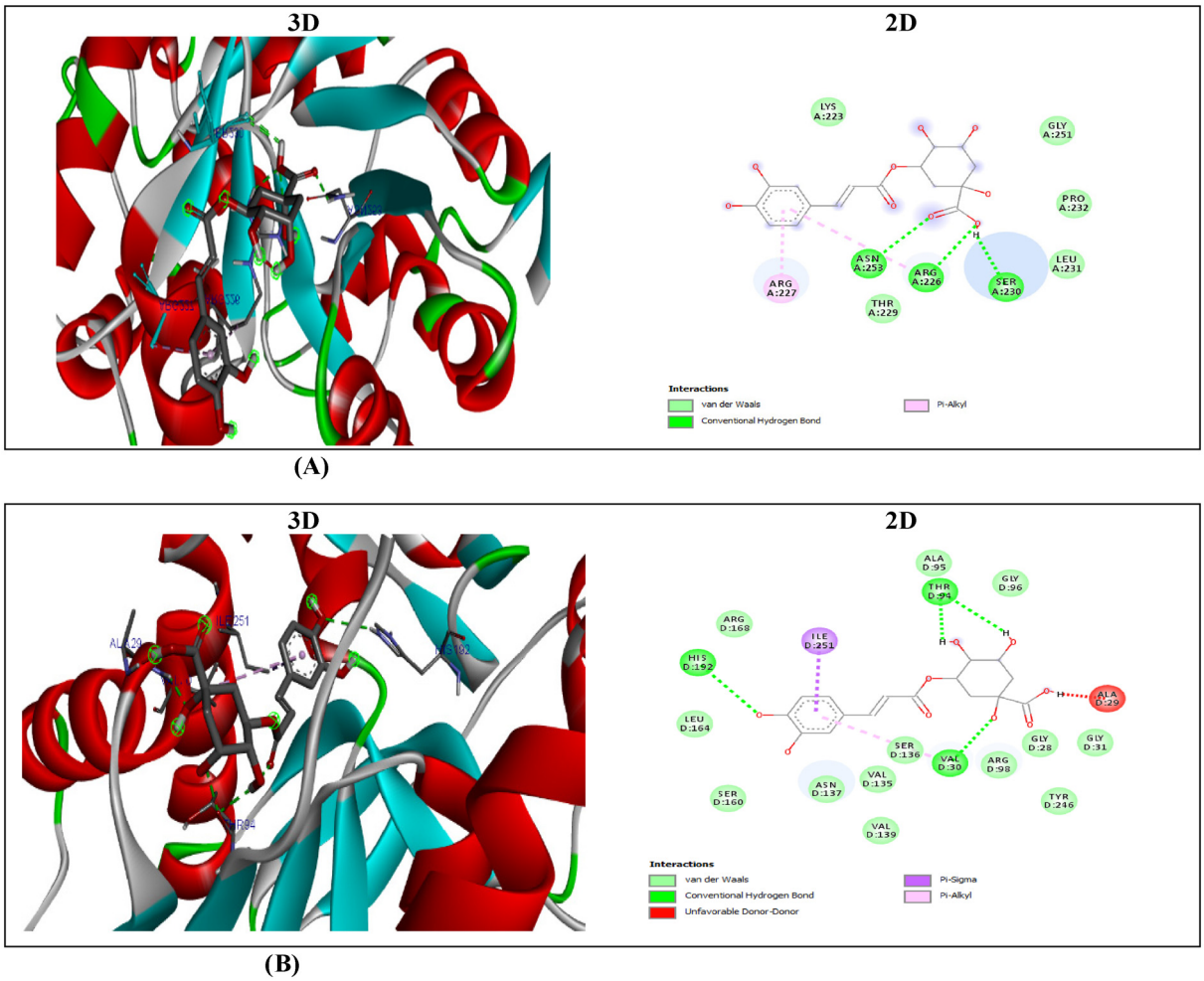


**Fig. 3** (A) 3D and 2D of chlorogenic acid interaction with glycolate oxidase. (B) 3D and 2D of chlorogenic acid interaction with lactate dehydrogenase.

through the inhibition of liver GO and the reduction of liver LDH.

### Biochemical parameters of urine and blood samples of kidney stone model and normal rats

**Urinary profile.** The urinary profiles of the different experimental groups are shown in Table 4. Urinary pH demonstrated a non-significant reduction in kidney stones in rats compared to the normal control, and pH increased non-significantly in the alkaline diet model compared with the kidney stone control. Urinary excretion of creatinine increased significantly in the experimental kidney stone control compared to normal control. The same findings were shown for creatinine clearance. Rats fed on a high oxalate diet containing 20% fruits or vegetable mixtures as an alkaline diet model showed a significant reduction in urinary creatinine. Urine urea, uric acid and protein excretion were elevated significantly in kidney stone control compared with normal control. Both rat groups fed on fruit or vegetable mixtures (alkaline diet models) recorded significant reductions in the urine levels of urea, uric acid and protein compared with kidney stone control.

Urinary oxalate and citrate are shown in Fig. 4(A and B). Urinary citrate (Fig. 4A) recorded a significant reduction in kidney stone control compared with the normal control, which increased significantly in fruit and vegetable mixtures feeding rats. Urinary oxalate (Fig. 3B) as an indicator of acidosis increased significantly in kidney stone control in comparison to normal control, and this elevation was reduced significantly in rat groups feeding on a diet containing an alkaline diet (20% fruit or vegetable mixtures). The studied alkaline diet models (fruits or vegetable mixtures) in the present study showed significant improvement in acidosis induced by a high oxalate diet.

The kidney and liver functions of the different experimental groups are presented in Table 5. Kidney function parameters (uric acid, urea, BUN and creatinine) demonstrated a meaningful rise in kidney stone control compared with normal control. These elevations in kidney function parameters were reduced significantly in rat groups fed on a high oxalate diet containing

**Table 4** Urinary profiles of different experimental groups

| Urine | Normal control | Kidney stone control | Fruits | Vegetables |
|---|---|---|---|---|
| Volume (ml/24 h) | $27.62^{a} \pm 0.56$ | $33.54^{b} \pm 0.97$ | $28.57^{a} \pm 0.83$ | $29.17^{a} \pm 0.41$ |
| pH | $8.04^{a} \pm 0.03$ | $7.87^{a} \pm 0.07$ | $7.93^{a} \pm 0.12$ | $7.93^{a} \pm 0.06$ |
| Creatinine (mg $dl^{-1}$) | $36.97^{a} \pm 0.29$ | $44.17^{b} \pm 0.76$ | $37.55^{a} \pm 0.50$ | $37.71^{a} \pm 0.41$ |
| Creatinine clearance (ml $min^{-1}$) | $0.98^{a} \pm 0.03$ | $1.17^{b} \pm 0.04$ | $1.23^{b} \pm 0.05$ | $1.20^{b} \pm 0.01$ |
| Urea (mg $dl^{-1}$) | $649.76^{a} \pm 4.13$ | $758.00^{b} \pm 8.61$ | $653.36^{a} \pm 3.72$ | $658.80^{a} \pm 5.11$ |
| Uric acid (mg $dl^{-1}$) | $6.95^{a} \pm 0.53$ | $14.24^{b} \pm 0.95$ | $9.24^{ac} \pm 0.60$ | $10.56^{c} \pm 0.47$ |
| Protein (mg $dl^{-1}$) | $40.86^{a} \pm 1.19$ | $62.77^{b} \pm 1.83$ | $43.48^{a} \pm 1.16$ | $49.72^{c} \pm 1.37$ |

Values are mean ± SE. Within the same row, means with different letters are significantly different at $p \leq 0.05$.

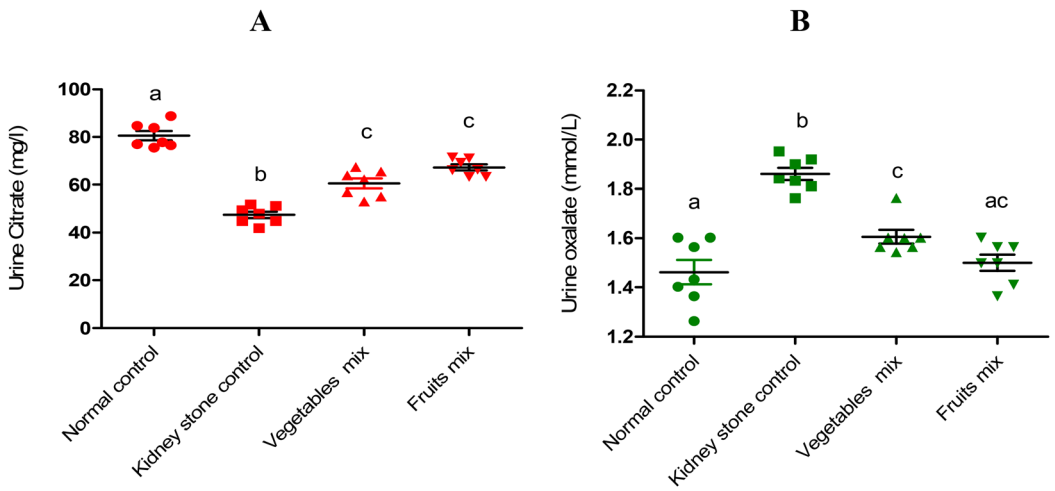


**Fig. 4** Urinary citrate (A) and oxalate (B) of different experimental groups. Different letters are significantly different at $p \leq 0.05$.

**Table 5** Kidney and liver functions of different experimental groups

| Plasma parameters | Normal control | Kidney stone control | Fruits | Vegetables |
|---|---|---|---|---|
| Uric acid (mg $dl^{-1}$) | $1.26^{a} \pm 0.04$ | $2.29^{b} \pm 0.12$ | $1.39^{ac} \pm 0.05$ | $1.58^{c} \pm 0.05$ |
| Urea (mg $dl^{-1}$) | $32.23^{a} \pm 0.64$ | $42.11^{b} \pm 0.71$ | $35.00^{ac} \pm 0.71$ | $36.97^{c} \pm 1.03$ |
| BUN (mg $dl^{-1}$) | $15.05^{a} \pm 0.30$ | $19.67^{b} \pm 0.33$ | $16.35^{ac} \pm 0.33$ | $17.27^{c} \pm 0.48$ |
| Creatinine (mg $dl^{-1}$) | $0.72^{a} \pm 0.02$ | $0.88^{b} \pm 0.02$ | $0.61^{c} \pm 0.02$ | $0.64^{c} \pm 0.01$ |
| Protein (g $dl^{-1}$) | $7.46^{a} \pm 0.12$ | $5.48^{b} \pm 0.15$ | $6.32^{c} \pm 0.09$ | $5.87^{bc} \pm 0.23$ |
| Albumin (g $dl^{-1}$) | $3.29^{a} \pm 0.11$ | $1.81^{b} \pm 0.10$ | $2.48^{c} \pm 0.06$ | $2.24^{c} \pm 0.10$ |
| GGT (U $l^{-1}$) | $2.75^{a} \pm 0.22$ | $4.40^{b} \pm 0.26$ | $2.80^{ac} \pm 0.10$ | $3.03^{a} \pm 0.26$ |
| ALP(IU $L^{-1}$) | $166.24^{a} \pm 0.32$ | $242.40^{b} \pm 0.15$ | $176.57^{ac} \pm 0.29$ | $184.86^{c} \pm 0.34$ |

Values are mean ± SE. Within the same row, means with different letters are significantly different at $p \leq 0.05$. GGT: gamma glutamyl transferase; ALP: alkaline phosphatase.

20% fruit or vegetable mixtures. GGT and ALP recorded a significant increase in kidney stone control compared to the normal control, while rat groups feeding on fruit and vegetable mixtures showed a significant reduction in the plasma levels of GGT and ALP. Plasma protein and albumin levels were reduced significantly in kidney stone control in comparison with normal control. Rats feeding on fruit and vegetable mixtures showed significant elevation in plasma levels of protein and albumin to different degrees. The main finding is that alkaline diet models are effective in improving liver and kidney function alterations.

The mineral urinary excretion and plasma content of the different experimental groups are shown in Table 6. Urinary calcium, magnesium, sodium and potassium were reduced significantly in kidney stone control compared to normal control. Feeding rats on diets containing 20% fruit or vegetable mixtures recorded a significant increase in the previously mentioned urinary minerals. Urinary phosphorus increases significantly in kidney stone control in comparison with normal control. Urinary phosphorous was reduced significantly in rat groups fed on diets containing 20% fruit or vegetable mixtures compared with kidney stone control.

 

Table 6 Mineral urinary excretion and plasma content of different experimental groups

| Minerals | Normal control | Kidney stone control | Fruits | Vegetables |
|---|---|---|---|---|
| **Urinary** | | | | |
| K (mEq $L^{-1}$) | $120.81^{a} \pm 1.42$ | $100.59^{b} \pm 2.13$ | $119.04^{a} \pm 0.97$ | $115.54^{a} \pm 1.09$ |
| Na (mEq $L^{-1}$) | $144.80^{a} \pm 1.22$ | $129.70^{b} \pm 1.85$ | $142.26^{ac} \pm 1.38$ | $137.69^{c} \pm 1.40$ |
| P (mg $dl^{-1}$) | $46.77^{a} \pm 1.32$ | $55.98^{b} \pm 1.70$ | $47.63^{a} \pm 1.10$ | $49.17^{a} \pm 0.89$ |
| Mg (mg $dl^{-1}$) | $26.79^{a} \pm 0.72$ | $20.18^{b} \pm 0.90$ | $24.79^{ac} \pm 0.53$ | $22.55^{bc} \pm 0.64$ |
| Ca (mg/24 h) | $614.21^{a} \pm 8.16$ | $444.87^{b} \pm 10.65$ | $605.87^{a} \pm 10.08$ | $583.79^{a} \pm 10.09$ |
| **Plasma** | | | | |
| K (mEq $L^{-1}$) | $4.50^{a} \pm 0.11$ | $2.94^{b} \pm 0.11$ | $4.19^{ac} \pm 0.13$ | $3.69^{c} \pm 0.22$ |
| Na (mEq $L^{-1}$) | $143.26^{a} \pm 1.41$ | $154.34^{b} \pm 1.48$ | $141.75^{a} \pm 0.83$ | $136.23^{c} \pm 0.85$ |
| P (mg $dl^{-1}$) | $9.05^{a} \pm 0.24$ | $15.02^{b} \pm 0.41$ | $9.93^{a} \pm 0.38$ | $11.23^{c} \pm 0.26$ |
| Mg (mg $dl^{-1}$) | $3.51^{a} \pm 0.12$ | $2.12^{b} \pm 0.11$ | $3.00^{c} \pm 0.12$ | $2.76^{c} \pm 0.10$ |
| Total Ca (mg $dl^{-1}$) | $10.17^{a} \pm 0.20$ | $6.60^{b} \pm 0.33$ | $9.23^{d} \pm 0.15$ | $8.27^{c} \pm 0.16$ |
| Ionzd Ca (mg $dl^{-1}$) | $5.51^{a} \pm 0.13$ | $3.99^{b} \pm 0.20$ | $5.23^{ac} \pm 0.09$ | $4.77^{c} \pm 0.09$ |

Values are mean ± SE. Within the same row, means with different letters are significantly different at $p \leq 0.05$.

Data presented in Table 6 showed that plasma potassium, magnesium, calcium and ionized calcium reduced significantly in kidney stone control compared to normal control, while plasma sodium and phosphorus increased significantly compared with normal control. Rats fed on diets containing 20% fruit and vegetable mixtures showed a significant increase in potassium, magnesium, calcium and ionized calcium plasma levels in accordance with a significant increase in plasma sodium and phosphorus compared with kidney stone control.

The nutritional parameters of the different rat groups are presented in Table 7. Kidney stone control recorded a significant reduction in final body weight, body weight gain, total food intake and food efficiency ratio in accordance with the increase in kidney compared with normal control. Rats fed on diets containing 20% fruit and vegetable mixtures showed significant improvement in all the studied nutritional parameters with different degrees.

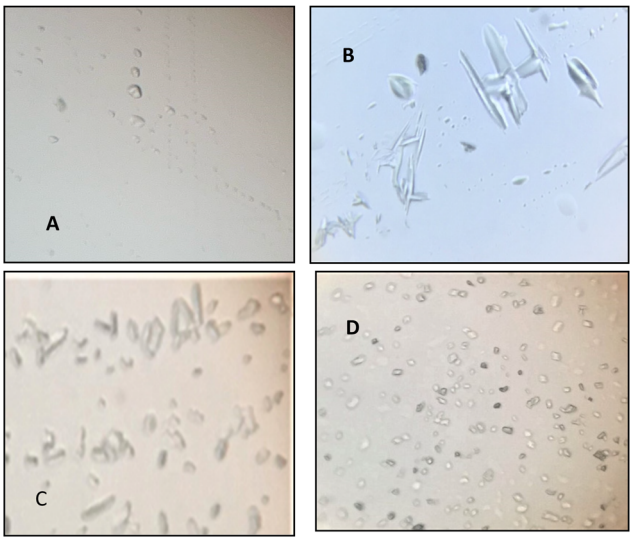


Fig. 5 Microscopy examinations of calcium oxalate crystals in the urine of different experimental groups. (A) Normal control group I, (B) kidney stone control group, (C) vegetable mixture group, and (D) fruit mixture group.

### Impact of fruit and vegetable mixtures in the microscopy examinations of calcium oxalate crystals in urine samples

Fig. 5 shows the calcium oxalate crystals in the different experimental groups. All experimental rats were examined in the presence of calcium oxalate crystals in their urine. Urine samples of rats in normal control group I (Fig. 5A) were devoid of any calcium oxalate crystals. Rats in group II (kidney stone control group) showed large calcium oxalate crystals (high score +3) in the urine samples (Fig. 5B). Rats in group III (Fig. 5C), which fed on a balanced diet containing potassium oxalate and 20% vegetable mixture showed a medium size of calcium oxalate crystals (score +2), while rats in group IV (Fig. 5D), which fed on a balanced diet containing potassium oxalate and 20% fruit mixture, showed the smallest size of

Table 7 Nutritional parameters of the different rat groups

| | Normal control | Kidney stone control | Fruits | Vegetables |
|---|---|---|---|---|
| Initial body weight (g) | $119.50^{a} \pm 2.65$ | $119.50^{a} \pm 2.20$ | $119.83^{a} \pm 5.29$ | $119.50^{a} \pm 4.71$ |
| Final body weight (g) | $192.50^{a} \pm 6.54$ | $136.67^{b} \pm 3.04$ | $152.67^{b} \pm 4.88$ | $149.00^{b} \pm 5.66$ |
| Body weight gain (g) | $73.00^{a} \pm 5.81$ | $17.17^{b} \pm 1.66$ | $32.83^{c} \pm 1.92$ | $29.50^{c} \pm 1.12$ |
| Total food intake (g) | $285.00^{a} \pm 2.24$ | $279.17^{a} \pm 4.55$ | $323.33^{c} \pm 4.41$ | $314.17^{c} \pm 4.73$ |
| Food efficiency ratio | $0.26^{a} \pm 0.02$ | $0.06^{b} \pm 0.01$ | $0.10^{cd} \pm 0.01$ | $0.09^{bd} \pm 0.00$ |
| Kidney (%) | $0.75^{a} \pm 0.06$ | $1.08^{b} \pm 0.04$ | $0.74^{a} \pm 0.04$ | $0.93^{c} \pm 0.03$ |

Values are mean ± SE. Within the same row, means with different letters are significantly different at $p \leq 0.05$.

calcium oxalate crystals (score +1). Calcium oxalate crystals (stones) were confirmed in the urine samples in the present study by their shape under a microscope. Calcium oxalate crystals exist in monohydrate and dehydrate forms, which can be differentiated by the shape of their respective crystals. The shape of the calcium oxalate crystal is octahedral. Calcium oxalate monohydrate crystals vary in shape, such as prisms, dumbbells, spindles, ovals or picket fences.

### Histopathological study results of different groups

Photomicrographs of kidney sections from various treatment groups are presented in Fig. 6. Histopathological examination of sections from rat kidneys of the normal control group (Fig. 6A) showed non-significant pathological changes (H & E stain, ×200). Kidney sections of the kidney stone control group of rats' (Fig. 6B) showed perivascular haemorrhage, the presence of renal casts inside renal tubules and severe necrosis, degenerations of the epithelial lining renal tubules (atrophied renal tubules) and inflammatory cell infiltrations (H & E stain, ×400). Additionally, kidney stone control rats (Fig. 6C) showed deposition of large areas of crystals (homogenous calcifications) in-between/and inside necrotic and degenerated renal tubules (nephrocalcinosis) (H & E stain, ×400). Kidney sections of the rat group fed on a high oxalate diet containing 20% vegetable mixture (Fig. 6D and E) indicated moderate crystal deposition, moderate necrosis, inflammatory cell infiltrations and degenerations of the epithelial lining renal tubules (H & E stain, ×200). Kidney sections of the rat group fed on a high oxalate diet containing 20% fruit mixture (Fig. 6F) revealed few crystal deposition, mild necrosis, low inflammation and degenerations of the epithelial lining renal tubules, and the presence of renal casts inside renal tubules (H & E stain, ×200). From these results, both mixtures are effective in protecting against calcium oxalate-induced kidney stones in rats. Fruit mixture was superior to vegetable mixtures.

## Discussion

Kidney stone disease (KSD) is common in almost all areas of the world, with a constantly rising incidence globally.[4] KSD is a widespread urinary tract illness.[39] Kidney stones are associated with high morbidity and high health care cost.[40] The most common types of stones are calcium oxalate (CaOx) stones, representing approximately 80–90%. Urinary oxalate is derived from endogenous oxalate synthesis and dietary oxalate intake. Endogenous oxalate metabolism occurs predominantly in the liver and is affected by the dietary intake of precursors, such as ascorbic acid.[41] Anionic oxalate is absorbed through the paracellular route in the gut. In a normal situation, the eliminated oxalate is not reabsorbed beyond the proximal tubule. Oxalate concentration correlates with water reabsorbed along the nephron, so it can crystalize with calcium.[42] In the current study, feeding rats on a high oxalate diet (5% potassium oxalate) induced metabolic acidosis, as shown by severe inflammation (kidney injury) and deposition of calcium oxalate crystals in the kidney tissues. Additionally, metabolic acidosis led to the progression of acidosis in urine, as revealed by increased urine excretion, increase in oxalate and reduction of citrate. The increment in urinary excretion observed in the kidney stone model is considered one of the risk factors for

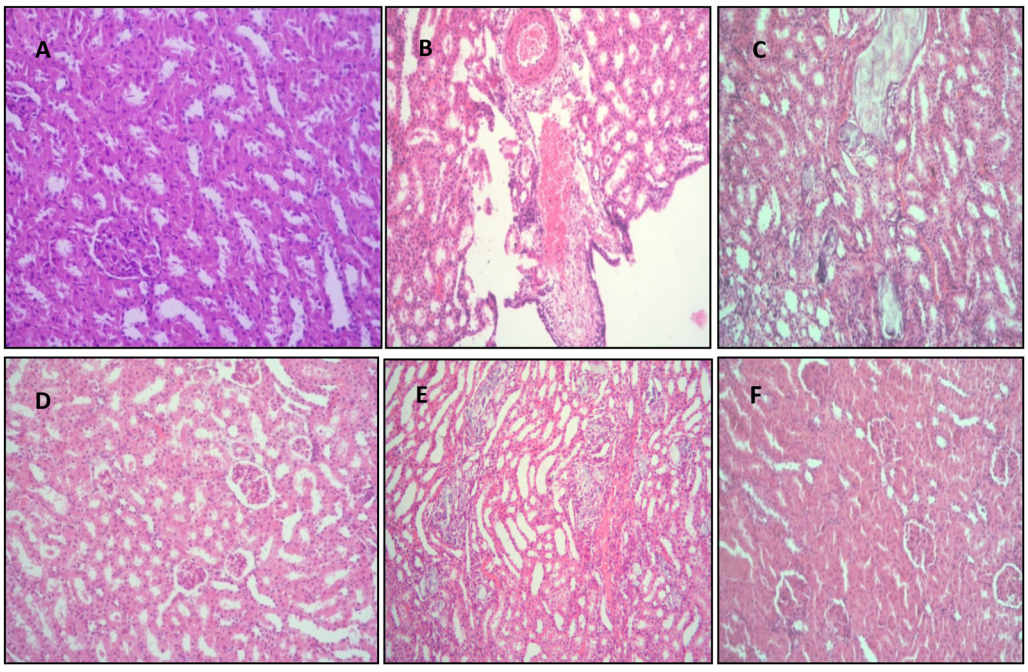


Fig. 6 Photomicrographs of kidney sections in different experimental groups. (A) Normal control group, (B) and (C) kidney stone control group, (D) and (E) vegetable mixture group, and (F) fruit mixture group.

 

kidney stone formation. Increased urinary excretion diluted the inhibitory factors, such as citrate and magnesium, for stone formation.[43] Calcium oxalate stones formed by crystal deposition in tubular epithelial cells are associated with an increase in oxidative stress due to an increase in reactive oxygen species (ROS). The increments in ROS lead to inflammation in kidney tissues. Inflammation is an important factor in the pathogenesis of kidney injury. Additionally, oxidative stress elevation due to higher levels of oxalate can lead to injury in the tubular and stone formation[44] as observed in the present study.

Nutrition is probably one of the most important modifiable risk factors associated with the high prevalence of kidney stone disease in the general population.[11] Diet plays an important role in the management of kidney stone formation. Thus, diet alteration is a useful tool for adjusting kidney stone formation risk factors, especially calcium oxalate.[34] Consumption of both fruits and vegetables prevents humans from many chronic diseases, such as cancer, chronic kidney diseases and dyslipidemia.[16] Nutrition plays an important role in the pH of blood and urine. The potential renal acid load (PRAL) score is used as an indicator of the acid base load of food.[3] Positive PRAL values indicate acid-containing foods, whereas negative the PRAL values indicate alkaline foods. PRAL score of foods is used as an indicator of dietary acid–base load, which is then assessed for CKD patients and used as a guide when adopting a low-acid diet.[3] In the present research, two mixtures of fruits and vegetables were studied as models of an alkaline diet owing to their potential protective effect from kidney stone formation in rats fed on a high oxalate diet (5% potassium oxalate). The studied fruit mixture (−42.419) and vegetable mixture (−11.13) recorded negative PRAL scores, which means that these mixtures are alkaline foods. PRAL provides an estimate of the production of endogenous acid that exceeds the level of alkali produced for given amounts of foods ingested daily.[45] The effect of an alkaline diet against kidney stone formation in the present study was noticed through alkaline urine, reduction of inflammation and deposition of oxalate crystals in kidney tissues and increased excretion of urinary oxalate in association with elevation of citrate concentration in urine. Low consumption of alkaline foods leads to a reduction of urine pH and excretion of magnesium, potassium and citrate, which increases the incidence of kidney stone formations.[17] The fruit mixture recorded a higher content of magnesium than the vegetable mixture as observed in proximate composition. Magnesium exhibited a protective role against kidney stone formation through its ability to bind free oxalate ions in urine, leading to an increase in the solubility of oxalate and subsequently inhibiting calcium-oxalate formation.[24,46] Increased oral magnesium intake may lead to decreased CaOx stone formation by binding intestinal oxalate, leading to decreased absorption and/or binding urinary Ox to decrease urinary supersaturation. Dietary magnesium decreases urine supersaturation.[46] The studied mixtures recorded a low sodium content. High consumption of sodium increases urinary calcium excretion and deposition of CaOx crystals in the kidney.[4] Therefore, the mineral content of the studied mixtures plays an important role in the management/prevention of kidney stone formation through the reduction of the supersaturation of urine with calcium oxalate. Fruit and vegetable mixtures as alkaline diet models led to the reduction of inflammation and the deposition of oxalate crystals in the kidney tissues, which may be attributed to the presence of phenolic compounds. The studied mixtures recorded a high content of phenolic compounds and demonstrated antioxidant activity against DPPH radicals. Phenolic compounds exhibited anti-inflammatory and antioxidant activities.[47]

A molecular docking study revealed that all the phenolic compounds present in the studied fruit and vegetable mixtures exhibited promising interactions with the glycolate oxidase (GO) and lactate dehydrogenase (LDH) proteins. Rutin displayed the highest binding affinities for both GO (−11.8 kcal $mol^{-1}$) and LDH (−10.1 kcal $mol^{-1}$) through the formation of hydrogen bonds and the diverse range of interactions with the GO and LDH proteins, respectively. In the present study, chlorogenic acid showed binding affinities less than rutin for both GO (−9.8 kcal $mol^{-1}$) and LDH (−8.1 kcal $mol^{-1}$). Chlorogenic acid was present in the fruit and vegetable mixtures by 80.18 and 1431.88 mg per 100 g, respectively, while rutin was present in a very small amount (2.43 and 0.879 mg per 100 g) in the fruit and vegetable mixtures, respectively. It was reported in a previous study that rutin and some dietary phytophenols, such as chlorogenic acid, were efficient in the prevention of kidney stone[20] due to their activities as diuretic, antispasmodic, and antioxidant activity, as well as an inhibitory effect on crystallization, nucleation, and aggregation of crystals. In another study, rutin (20 mg $kg^{-1}$ RBW) for 25 days inhibited calcium oxalate urolithiasis in rats administered ethylene glycol through its anti-inflammatory and antioxidant activities.[48] Recently Salem *et al.*[49] reported that rutin exhibited anti-inflammatory and anti-urolithiatic properties, promoting the dissolution of calcium oxalate (CaOx) crystals. Rutin dissolute CaOx stones in a dose-dependent manner at tested concentrations of 0.25, 0.5, and 1 mg $ml^{-1}$ specifically, at the highest tested dose of 1 mg $mL^{-1}$, rutin achieved 86% dissolution. The observed inhibition percentage ($IC_{50}$) for rutin was 0.37 mg $ml^{-1}$.

Most treatments used for the reduction of kidney stone formulations depend on inhibiting the GO, which leads to a reduction of glyoxylate (Glyo) production. Glyoxylate is mainly produced from the oxidation of glycolate by the GO enzyme; then, glyoxylate is converted to oxalate by applying LDH.[50] LDH is responsible for the increase in oxalate formation in the kidney through the formation of the insoluble crystals of calcium oxalate.[25] Hence, the present results indicated the potential role of fruit and vegetable mixtures under investigation as inhibitors for the formation of oxalate crystals through the inhibition of liver GO and the reduction of liver LDH. Flavonoids, a group of polyphenols abundant in fruit and vegetables function as LDH inhibitors. Polyphenols, such as rutin, quercetin and epigallocatechin gallate, can bind to the active site of LDH, reducing its activity levels. LDH inhibi-

tors have also been investigated as potential therapeutics for cardiovascular diseases and other chronic diseases, such as kidney disease and cancer.[51] Various natural compounds can inhibit LDH activity through different mechanisms. The most common approach involves direct binding to the enzyme's active site, leading to the inhibition of pyruvate conversion to lactate. Polyphenols can bind to the active site of the LDH, thereby reducing its activity levels. Natural products can inhibit LDH through the regulation of LDH gene expression. Different compounds, such as resveratrol and quercetin, can suppress LDH expression by reducing LDH gene transcription or promoting LDH protein degradation.[51,52] In contrast to small molecule inhibitors, RNA-based inhibitors target LDH expression by interfering with mRNA. RNA interference (RNAi) and antisense oligonucleotides (ASOs) are commonly used to inhibit LDH expression, which is achieved at the mRNA level. RNAi-based LDH inhibitors target the mRNA sequences responsible for encoding LDH, resulting in its downregulation and decreased LDH activity.[53]

## Conclusion

The results of this study highlight the efficiency of fruit and vegetable mixtures as alkaline diet models in the prevention of kidney stone formation in rats and in molecular docking studies. The potential preventive role of the alkaline diet models may be attributed to their negative potential renal acid load (alkaline diet) and polyphenol and mineral contents, especially potassium and magnesium.

### Study limitation

One limitation of the present study is that we do not include molecular biology studies, such as qPCR, to analyse the expression of genes related to kidney stone formation. Therefore, in the future studies of our project, we will study kidney stone formation and its impact on the expression of proteins and genes in an animal experiment and how intervention with different dietary supplements may prevent stone formation.

## Ethical statement

Animal procedures followed the guidelines of the National Research Centre for the Use of Animals in Experimental Studies and the Guide for Care and Use of Laboratory Animals by the U.S. National Institutes of Health (Publication No. 85-23, revised 1996). This study was permitted by the Medical Research Ethics Committee, National Research Centre, with approval number 13050203.

## Author contributions

D. A. M. is the principal investigator of the project, prepared all plant materials, evaluated the phytochemicals, chemical composition and antioxidant activity and designs the study and wrote the final manuscript with the interpretation of the results. A. A. R. contributed in the experimental work and microscopy of urine samples. H. B. M. molecular docking, statistical data analysis and contributed in the preparation of the manuscript. H. F. E. Analysed the urine and blood analysis of the samples and entry the data on the excel sheet. All authors contributed in the animal experiment. All authors read and approved the final manuscript.

## Data availability

All data generated or analysed during this study are included in this published article.

## Conflicts of interest

The authors declare no conflicts of interest.

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