International Journal of PharmTech Research
CODEN (USA): IJPRIF, ISSN: 0974-4304, ISSN(Online): 2455-9563
Vol.9, No.9, pp 519-533, 2016

# Potential Diuretic Activity of Olive Leaf Extracts

**Sahar Y. Al-Okbi[1]*, Zenab A. Hassan[2], Mohey M El-Mazar[3], Nagwa M. Ammar[4], LamiaT. Abou El-Kassem[4] and Hagar F. El-Bakry[1]**

[1]Food Sciences and Nutrition Department, National Research Centre, Cairo, Egypt
[2]Biochemistry Department, Faculty of Pharmacy, Helwan University, Egypt
[3]Faculty of Pharmacy, The British University of Egypt, Egypt
[4]Pharmacognosy Department, National Research Centre, Cairo, Egypt

**Abstract :** The aim of the present research was to study the diuretic effect of petroleum ether and aqueous methanol extract of olive leaves in rats in 3 different doses. The diuretic effect of co-administration of vitamin C with aqueous methanol olive leaf extract was assessed. The mechanism of the diuretic activity was studied through determination of saluretic, natriuretic and carbonic anhydrase inhibition indices, as well as glomerular and tubular functions in experimental model of lithium. Phytochemical analysis of aqueous methanol extract was assessed using chromatographic technique, UV and NMR spectroscopy. K/Na ratio was determined in olive leaf and its aqueous methanol extract. **Results**: The tested extracts showed diuretic activity in all the studied doses compared to control with different degrees and variation in saluretic effect. Co-administration of vitamin C with olive leaves aqueous methanol extract enhanced its diuretic effect. Urinary electrolytes produced by rats given olive leaf extracts were significantly lower than that of furosemide but significantly higher than control. Significant increase in creatinine clearance in rats given olive leaf extracts was noticed compared to control in lithium model. K/Na ratio was high in olive leaf and its aqueous methanol extract. Flavonoid compounds represented by luteolin, apigenin and apigenin 7-O-β-D-neohesperopyranoside were identified in olive methanol extract. ***Conclusion:*** Olive leaf extracts showed diuretic activity. Vitamin C had synergistic diuretic action with aqueous methanol extract. The diuretic activity of aqueous methanol extract may be attributed to the presence of vitamin C, flavonoids, and the high K/Na ratio that were identified in the plant.
**Keywords :** Olive leaf extracts, flavonoids, diuretic effect, vitamin C, mechanism of action, rats, safety.

## Introduction

Olive, *Olea europaea* L., is a species of a small evergreen tree in the family Oleaceae, native to the coastal areas of the Mediterranean region. Leaves are evergreen, gray-green or silvery beneath and about 3-7 cm long. Flowers are small, fragrant, borne in panicles shorter than the leaves[1]. Olive contains fixed oil, and phenolic compounds[2], oleuropein represent the main constituent. It also contains triterpens, sterols and oleanolic acid[3]. The leaves contain glycosides, tannins, saponins, choline and unsaturated lactone elenolide[1]. Olive has also been reported to contain an active constituent, β-(3,4-dihydroxy-phenyl) ethanol, which has been proved to possess calcium channel blocking activity[4]. The leaves were reported to have hypoglycemic, astringent and anti-hypertensive activity[1,5]. Olive leaf extract possess anti cancer effect[6,7]. Long term treatment with olive leaf extract enhances antioxidant enzyme activity and protects the neurons against oxidative stress[8]. Olive leaf extract was shown to have therapeutic role towards nephrotoxicity, atherogenic lipid profile and

cardiac and hepatic toxicity[9-11]. Olive leaf extract also attenuates obesity in mice[12]. In Egyptian Folk medicine Olive leaves are used as anti-rheumatic, anti-inflammatory, antinociceptive, antipyretic, vasodilatory, hypotensive, anti-diabetic agents and diuretic.

Diuresis is important in the treatment of many diseases ranging from acute cases as renal failure to chronic cases as hypertension, cardiac and hepatic diseases[13,14]. The objective of the present research is to study the diuretic activity of olive leaves extracts along with their mechanism of action. Assessing the potential synergistic diuretic effect of vitamin C with the olive leaf aqueous methanol extract was among the aim of the present research.

## Materials and Methods

### Phytochemical part

#### Materials

NMR spectra were obtained on Jeol ECA-500 spectrometer. DMSO-d6 was used as solvent. All chemical shifts (δ) are given in ppm with reference to tetramethylsilane (TMS) as an internal standard; the coupling constants (*J*) are given in Hz. UV spectra were recorded on Shimadzu UV-visible spectrophotometer model UV 240 (Tokyo, Japan). Polyamide 6S (Riedel de Haen AG, Hannover, Germany); Sephadex LH-20 (Pharmacia Fine Chemicals, Uppsala, Sweden); Solvent mixtures, BAW (n-butanol:acetic acid:water, 4:1:5 upper phase), and paper chromatography (PC), Whatman No. 1 MM (46 X57cm) (Kent, England) were used.

#### Plant material

Olive leaves (*Olea europaea* L. Family Oleaceae) were obtained from local market and authenticated in the herbarium of Faculty of Science, Cairo University, Egypt.

#### Methods

#### Preparation of olive leaves:

Olive leaves were dried in hot air oven (temperature not more than 40°C) and reduced to powder.

#### Extraction, separation and identification of flavonoidal compounds

#### Extraction and separation

The dried powdered olive leaves were defatted with petroleum ether (40-60 ºC) in a Soxhlet apparatus, and then extracted with methanol until exhaustion. The methanol extract was evaporated under vacuum until dryness to a residue, and the methanol residue was suspended in water and partitioned successively with chloroform and ethyl acetate. The ethyl acetate extract was subjected to column chromatography using polyamide 6S and eluted with water followed by stepwise addition of methanol. All fractions obtained were recombined according to their paper chromatographic analysis to give 3 major fractions. Each fraction was purified by column chromatography (CC) on Sephadex LH-20 using methanol to get 3 compounds. Flavonoids **1**– **3** were detected as dark purple spots under UV light, changing to yellow when exposed to ammonia vapor and by spraying with aluminum chloride solution. Complete acid hydrolysis of the *O*-glycoside **1** was carried out, followed by paper co-chromatography with authentic samples to identify the aglycones and sugar moieties. The structures of the isolated compounds were determined from UV and NMR spectral data[15].

#### Acid hydrolysis

Compound 1 (2 mg) in a mixture of 8% HCl (1 ml) and methanol (4 ml) was refluxed for 2 h. The reaction mixture was reduced under pressure to dryness, dissolved in $H_2O$ (3 ml) and neutralized with $Na_2CO_3$. The neutralized products were subjected to PC (eluent, benzene:*n*-butanol:pyridine:$H_2O$, 1:5:3:3). The chromatogram was sprayed with aniline hydrogen phthalate followed by heating at 100°C. The sugars were identified after comparison with authentic samples.

**Biological and nutritional part**

**Materials:**

**1) Animals:**

**a) Rats:**

Male Sprague-Dawley rats of 100-120 g body weight were used in the present study.

**b) Mice:**

Male and female albino mice of 21-25 g body weight were used in the acute toxicity test.

The animal experiments were carried according to the Ethics Committee of the National Research Centre, Cairo, Egypt, and followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

**2) Diet:**

Balanced diet (Casein 10%, corn oil 10%, sucrose 25.17, corn starch 50.33%, salt mixture 3.5%, and vitamin mixture 1%) was fed to rats for evaluation of the safety of the olive extracts on long term.

**3) Drugs:**

Furosemide: Lasix® Aventis Pharma, Egypt.

**5) Major chemicals:**

**Urea**: Purchased from EL-Nasr Pharmaceutical Chemicals Company (ADWIC), Egypt.

**Lithium chloride**: Obtained from S.d.Fine-Chem ltd. Mumbai, India.

**Methods**

**Preparation of olive leaf extracts**

The dried powdered olive leaves were subjected to continuous extraction in a Soxhlet apparatus with petroleum ether (40-60 ºC), followed by aqueous methanol until exhaustion. The solvents of both extracts were evaporated under vacuum until dryness of the extracts.

**Preparation of different doses of the extracts:**

Petroleum ether, aqueous methanol extracts and a mixture of vitamin C with aqueous methanol extract were emulsified separately in water using gum acacia. Vitamin C was dissolved in water. All the doses were prepared instantaneously before given to rats.

**Evaluation of the diuretic activity of olive leaf extracts**

The test is based on urine excretion in test animals in comparison to rats treated with a high dose of urea. The method was described by Lipschitz *et al.*[16] and modified by Kau *et al*[17]. After an adaptation period of 1 week, animals were divided into different groups (control groups, reference groups and eight test groups), each of 7 rats. Rats were placed individually in stainless steel metabolic cages. The rats were given laboratory stock diet and water ad-libitum. Seventeen hours prior to the experiment food and water were withdrawn. Each dose of the tested extract was given orally to the rats in 0.5mL water per 100 g. body weight. Also each animal of the reference groups received orally urea as 1 g per kg rat body weight in 0.5mL water per 100 g rat body weight. Each rat of the control groups was given 0.5mL water per 100 g. body weight. Additionally 5ml saline per 100 g body weight was given by gavage for all groups. Urine excretion was recorded after 5 and 24 hours.

Petroleum ether and aqueous methanol extract of olive leaves were tested in 3 different doses (500, 600, 750 mg / kg rat body weight. Vitamin C was tested alone (200mg/kg). The aqueous methanol extract of olive

leaves (500mg/ kg rat body weight) was tested in combination with vitamin C (200 mg/kg). The mean of urine volume excreted per 100 g rat body weight was calculated for each group. Results were expressed as Lipschitz value; i.e., the ratio T/U, in which T was the urine volume in response to the tested extract, and U, was that of urea treatment. Indices of 1 and more were regarded as positive diuretic effect. Calculating this index for the 24-hour excretion period as well as for 5 hours indicated the duration of the diuretic effect. Mean urine volume of the different test groups that showed non significant change or highly significant increase compared to that of urea group reflected diuretic effect. The whole experiment was not carried out in one time, so a control and a reference group were run in each time.

**Evaluation of the saluretic activity of olive leaf aqueous methanol extract [18].**

Rats were divided into 3 groups, each of eight rats. The rats were placed individually in stainless steel metabolic cages provided with a wire mesh bottom and a funnel to collect the urine. Twenty-four hours prior to the test; food but not water was withdrawn. On the start of this step each rat of the first group (control group); received 0.5mL distilled water orally per 100 g. rat body weight. The second group (reference group); received 25 mg furosemide per kg orally in 0.5 mL distilled water per 100 g. rat body weight. Each rat of the third group; received orally 500 mg olive leaves' aqueous methanol extract per kg rat body weight in 0.5mL distilled water per 100 g. rat body weight. Urine volume was collected after 5 and 24 hours for each rat. For the 5 and 24-hour urine of each rat the following electrolytes were determined:

1. $Na^+$ and $K^+$ content using flame atomic absorption spectrophotometer.
2. $CL^-$ content using colorimetric method[19].

The sum of $Na^+$ and $CL^-$ excretion is calculated as a parameter of saluretic activity. The ratio $Na^+/K^+$ is calculated for natriuretic activity. The ratio $CL^-/(Na^+ + K^+)$ was calculated to estimate carbonic anhydrase inhibition activity**.** Carbonic anhydrase inhibition can be excluded at ratios between 1 and 0.8. With decreasing ratios slight to strong carbonic anhydrase inhibition can be assumed.

**Studying the effect of olive leaf aqueous methanol extract on kidney function using experimental model of lithium**

In the present experiment, the effect of olive leaf aqueous methanol extract (test) on glomerular filtration and proximal tubular reabsorption was studied. So, two groups, one control and one test group, each of 8 rats were run; rats of the control group received distilled water. Rats of the test group received aqueous methanol extract of olive leaf. In this experiment the effect of 500 mg of the tested extract / kg rat body weight on the urine volume, and on $Na^+$, $K^+$ and uric acid urinary excretions, glomerular and proximal tubular function, as well as $Na^+$ tubular handling, were investigated in 5mL/100 g body weight water-loaded rats in the individual metabolic cages for 3 hours. The amount of water load provided free water clearance in order to suppress the vasopressin effect on water re-absorption at the distal tubule segments and its effects on $Na^+$ re-absorption[20]. Glomerular and proximal tubular functions were measured, respectively, by creatinine clearance ($CL_{cr}$) and lithium clearance ($CL_{Li}$). As previously established[20-22], fourteen hour prior to urine collection, the rats received lithium chloride at 0.6 mEq/ kg body weight by gavage, and were allowed water but not food overnight. The 5mL/100 g body weight water load was administered in two stages: 3 and 2mL/100 g body weight, respectively, at 90 and 30 minutes before urine collection in the individual metabolic cages. At the second load, the control rats received water; for the experimental rats, 500 mg of the tested extract /kg rat body weight were added individually to the water. The urine was collected for 3 hours in the individual metabolic cages. Blood samples were withdrawn at the end from eye vein orbital of anaesthetized animal. Urine volume and pH were measured. Serum and urine $Na^+$, $K^+$, and $Li^+$ were determined using flame atomic absorption spectrophotometer, while creatinine[23] and uric acid[24] were determined using colorimetric methods. Creatinine clearance ($Cl_{Cr}$), lithium clearance ($Cl_{Li}$), uric acid clearance, fractional excretion (FE), $Na^+$ filtered load ($FL_{Na}$) and tubular handling: fractional proximal tubular reabsorption ($FPTR_{Na}$), fractional distal tubular reabsorption I ($FDTR_{Na}$-I), and fractional distal tubular reabsorption II ($FDTR_{Na}$-II) were calculated as mentioned previously [25].

**Evaluation of the safety of aqueous methanol extract of olive leaf**

**Long term study**

Rats were divided into 2 groups, each of 10 rats. Rats of the first group were given daily oral dose of the aqueous methanol extract of olive leaves as 500 mg/ kg rat body weight. The second group served as control and given only the same amount of vehicle as in the first group. The experiment lasted for one month, during which rats' body weight and food intake were followed twice weekly. At the end of the experiment, 24-hour urine was collected and measured. Rats were fasted; blood samples were withdrawn from rats' eye vein orbital after ether anesthesia. The blood was divided into two parts: The first for determination of blood hemoglobin[26] and packed cell volume[27]. The second part of blood was centrifuged at 4000 rpm for 10 min and the serum was separated for determination of glucose[28], Na[29], K[30], Mg[31], Ca[32], Zn[33] and Fe[34]. Assessment of kidney functions was carried out through determination of serum and urine creatinine[23], serum urea[35] and calculation of creatinine clearance. Liver function was estimated through determination of the activities of transaminases (ALT and AST)[36]. Serum total cholesterol[37], high density lipoprotein cholesterol (HDL-ch)[38,37], low density lipoprotein cholesterol (LDL-ch)[39,37], and triglycerides[40] were assessed by colorimetric methods. Very low density lipoprotein cholesterol (VLDL-ch) was calculated as previously[41].

Different organs (liver, kidney, heart and spleen) were separated and weighed. Their percentages to rats' body weights were calculated. Also total food intake (TFI), body weight gain (BWG) and protein efficiency ratio (FER), body weight gain/total food intake, were calculated.

**Short term study (Acute toxicity test)**

Acute oral lethal toxicity test of olive leaf aqueous methanol extract was carried out as reported previously[42]. The 24-hour mortality counts among equal sized groups of mice (8 animals/group) receiving progressively increasing oral dose (1, 2, 4, 6, 8, 10, 12g/ kg mouse body weight) of the tested extract were recorded, if any.

**Food chemistry**

Vitamin C was determined in fresh olive leaf using 2, 6- dichlorophenolindophenol visual titration method as previously[43]. The moisture and ash content were determined in olive leaves according to the method described in AOAC[44]. The mean of triplicates was calculated. Na, K, Ca, Mg, Zn, Fe, Mn and Cu were analyzed in the diluted solution of the ashed sample using flame atomic absorption spectrophotometer apparatus (Model I L 157 atomic absorption/ air ethylene) according to AOAC[44].

**Statistical analysis of the results**

Mean ± SE of the data of each group of the experimental rats was calculated. Results were analyzed using SPSS version 10 by applying one-way ANOVA test using LSD at a probability of 0.01 and 0.05.

## Results

**Evaluation of the diuretic activity of olive leaf extracts**:

In the present study the significant increase or the non significant change in urine volume of the test groups compared to urea group showed that the tested extract has diuretic activity that is higher or similar to urea group which is reflected by T/U (Lipschitz value) that was noticed to be more than one or very near to one, respectively. The significant increase in urine volume of the test groups compared to control may reflect some diuretic activity which if not similar to urea group may reflect weak diuretic activity. Data in table 1 showed five and twenty-four hours urine volume (ml /100 g rat body weight) of different test groups given 500, 600 or 750 mg petroleum ether or aqueous methanol extract /kg rat body weight, control group, urea group and the corresponding Lipschitz values. Table 1 also contains the urine volumes of rats given vitamin C and those given vitamin C with 500 mg aqueous methanol extract /kg rat body weight. It can be noticed that urine volume at both 5 and 24 hours of rats given urea was significantly higher than that of the control group. Urine volume at 5 hours of rats given petroleum ether extract of olive leaves in the different studied doses showed significant

higher values than the control group. However urine volume at 24 hours was only significantly higher than control on administration of 750 mg of petroleum ether extract /kg rat body weight.

Ingestion of petroleum ether extract of olive leaves showed that both 5 and 24 hours urine volumes were not significantly changed at the dose of 500 compared to that at 600 mg/kg, however both urine volumes at the dose of 750 mg/kg were significantly higher than that at the lower doses.

All the tested aqueous methanol extracts in the different studied doses produced significant increase in urine volume compared to control at both 5 and 24 hours.

Administration of aqueous methanol extract of olive leaves produced non significant change in 24 hours urine volume at the different administered doses (500, 600 and 750 mg/kg) compared to urea group. Ingestion of aqueous methanol extract of olive leaves, at the dose of 600 mg/kg produced non significant change in 5 hours urine volume compared to urea group.

On administration of aqueous methanol extract of olive leaves, the 5 hours urine volume showed non significant change between the dose of 500 and 600 while significant increase was noticed when the dose of 500 and 600 were compared with that at 750 mg/kg. The 24 hours urine volume of the groups given aqueous methanol extract of olive leaves showed that it did not significantly differ at the different doses.

**Table 1: Five and twenty-four hours urine volume (ml /100 g rat body weight) of control group (C), urea group (U), test groups (T)) and T/U values of the different test groups: Petroleum ether extract of olive leaves, aqueous methanol extract**, **aqueous methanol extract in combination with vitamin C and vitamin C.**

| ***Group*** | **5 hours** | | **24 hours** | |
|---|---|---|---|---|
| | Urine volume | T/U | **Urine volume** | T/U |
| *Control* | $0.7 \pm 0.046^{a}$ | | $2.2 \pm 0.104^{a}$ | |
| Urea (1g/kg rat body weight) | $2.5 \pm 0.117^{b}$ | | $3.8 \pm 0.081^{b}$ | |
| Aqueous methanol extract of olive leaves (500 mg/kg rat body weight) | $1.9 \pm 0.115^{c}$ | 0.76 | $4 \pm 0.071^{b}$ | 1.05 |
| Aqueous methanol extract of olive leaves (500 mg/kg rat body weight) + Vitamin C (200 mg / kg rat body weight) | $4.1 \pm 0.229^{d}$ | 1.64 | $6.4 \pm 0.133^{c}$ | 1.68 |
| *Control* | $0.8 \pm 0.049^{a}$ | | $2.3 \pm 0.119^{a}$ | |
| Urea (1g/kg rat body weight) | $2.7 \pm 0.133^{b}$ | | $4 \pm 0.117^{bc}$ | |
| Vitamin C (200 mg/kg rat body weight) | $2.2 \pm 0.122^{d}$ | 0.81 | $3.6 \pm 0.098^{c}$ | 0.9 |
| Control | $0.6 \pm 0.05^{a}$ | | $2.1 \pm 0.153^{a}$ | |
| Urea (1g/kg rat body weight) | $2.3 \pm 0.163^{b}$ | | $3.6 \pm 0.098^{b}$ | |
| Aqueous methanol extract of olive leaves (600 mg/kg rat body weight) | $2 \pm 0.059^{bc}$ | 0.87 | $3.8 \pm 0.101^{bc}$ | 1.06 |
| Aqueous methanol extract of olive leaves (750 mg/kg rat body weight) | $1.5 \pm 0.101^{d}$ | 0.65 | $3.6 \pm 0.083^{b}$ | 1 |
| Control | $0.6 \pm 0.05^{a}$ | | $2.1 \pm 0.153^{a}$ | |
| Urea (1g/kg rat body weight) | $2.1 \pm 0.111^{b}$ | | $3.4 \pm 0.097^{b}$ | |
| Olive leaves petroleum ether extract(500 mg/kg rat body weight) | $1 \pm 0.13^{c}$ | 0.48 | $2.3 \pm 0.106^{a}$ | 0.68 |
| Olive leaves petroleum ether extract(600 mg/kg rat body weight) | $1.2 \pm 0.069^{c}$ | 0.57 | $2.4 \pm 0.191^{a}$ | 0.71 |
| Olive leaves petroleum ether extract(750 mg/kg rat body weight) | $1.8 \pm 0.123^{d}$ | 0.86 | $3.3 \pm 0.113^{b}$ | 0.97 |

Values are expressed as mean ± S.E. where n = 7 rats per group. For each of the 5 and 24 hours urine collection same letters within a column means non significant difference; different letters means significant difference among the experimental groups; where $p < 0.05$ for 5 hour urine collection and $p < 0.01$ for 24 hour urine collection.

It can be noticed also that combination of vitamin C with the aqueous methanol extract of olive leaves (500 mg/kg) produced a synergism for its diuretic effect after 5 hours where T/U ratio was 1.64. This effect persisted for the 24 hours where T/U ratio was 1.68.

**Studying the effect of olive leaf methanol extract on kidney function using experimental model of lithium.**

In the experimental model of lithium, data in table 2 showed that administration of aqueous methanol extract of olive leaf showed no significant change in urine volume, urine pH, lithium clearance, uric acid clearance and its fractional excretion compared to control. Urine creatinine and creatinine clearance of rats given aqueous methanol extract of olive leaves were significantly higher than that of the control. The urinary excretion of sodium, lithium, potassium and uric acid in rats given orally distilled water (control) or 500 mg of aqueous methanol extract of olive leaf /kg rat body weight showed non significant difference. Rats given aqueous methanol extract of olive leaf were similar to the control group concerning $Na^+$ filtered load, fractional proximal tubular reabsorption, fractional distal tubular reabsorption I, and fractional distal tubular reabsorption II.

**Saluretic activity of aqueous methanol extract of olive leaf**

Table 3 showed that 5 and 24 hour urinary sodium were significantly higher in furosemide treated rats than the control group. The 5 and 24 hours urinary sodium of olive leaf aqueous methanol extract treated group showed significant higher value than control group but significant lower levels than furosemide treated group.

It can be noticed that the 5 and 24 hour urinary potassium were significantly higher in furosemide treated rats than the control group. Administration of olive leaves aqueous methanol extract produced significant increase in urinary potassium compared to furosemide and control group after 5 and 24 hours. The mean values of 5 and 24 hour urinary chloride were significantly higher in furosemide treated rats than the control group. The 24 hours urinary chloride of rats given olive leaves aqueous methanol extract was significantly lower than that of furosemide. Olive leaf aqueous methanol extract produced significant increase of urinary chloride after 24 hours compared to control. The urinary chloride of rats given olive leaves aqueous methanol extract after 5 hours showed non significant difference with the control.

It can be noticed that administration of furosemide produced significant increase in saluretic, natriuretic and carbonic anhydrase inhibition indices after 5 and 24 hours compared to control. The 5 hours saluretic, and the 5 and 24 hours natriuretic and carbonic anhydrase inhibition indices of the group given olive leaves aq. methanol extract showed significant decrease compared to that of furosemide treated group. The values of 5 and 24 hours saluretic indices of olive leaves aq. methanol extract group were significantly higher than control.

**Evaluation of the safety of methanol extract of olive leaves**

Data in table (4) showed that daily oral administration of 500 mg aqueous methanol extract of olive leaves /kg rat body weight for 30 days did not affect most of the biochemical parameters compared to control group. However serum calcium, AST activity and total cholesterol level significantly decreased in the group given the methanol extract compared to control. No significant change was noticed in organ weight of liver, kidney, heart and spleen as percentage of body weight on administration of methanol extract compared to the control group. In table (5) it was noticed that oral administration of the aqueous methanol extract of olive leaves in a dose of 500 mg/kg for 30 days did not produce any significant changes in final body weight, body weight gain, total food intake or the food efficiency ratio compared to that of control group.

Acute toxicity test revealed that the aqueous methanol extracts of olive leaves caused no mortality up to the highest tested dose (12 g / kg mice body weight).

**Table 2: Urine volume (V), pH, creatinine ($Cl_{Cr}$), lithium clearance ($Cl_{Li}$), uric acid clearance, fractional excretion (FE) and $Na^+$, $K^+$, $Li^+$, creatinine and uric acid urinary excretions (µmol/ min/ 100g body weight) of rats of the control and the test group. The table also contains $Na^+$ filtered load ($FL_{Na}$) and tubular handling: fractional proximal tubular reabsorption ($FPTR_{Na}$), fractional distal tubular reabsorption I ($FDTR_{Na}$-I), and fractional distal tubular reabsorption II ($FDTR_{Na}$-II) of rats of the control and the test group.**

| Groups / Parameters | Control | Olive leaves aqueous methanol extract |
|---|---|---|
| V (µl/ min/100 g body wt) | 12.44 [a]± 0.69 | 13.17 [a]± 0.84 |
| pH | 6.65 [a]± 0.09 | 6.84 [a]± 0.11 |
| $Cl_{Cr}$ (µl/ min/100 g body wt) | 213 [a]± 13.111 | 262.8 [b]± 14.989 |
| $Cl_{Li}$ (µl/ min/100 g body wt) | 20.94 [a]± 4.355 | 18.58 [a]± 4.54 |
| Uric acid clearance (µl/min/100g body wt) | 61.3 ± 10.926[a] | 52.05 ± 11.627 [a] |
| Uric acid FE (%) | 27.75 ± 3.406[a] | 19.74 ± 4.452 [a] |
| $Na^+$ | 0.0095 ± 0.003 [a] | 0.0092 ± 0.001 [a] |
| $K^+$ | 0.0054 [a]± 0.0009 | 0.0075[a]± 0.0011 |
| $Li^+$ | 0.0017 [a]± 0.0003 | 0.0017 [a]± 0.0004 |
| Creatinine | 0.007 [a]± 0.0004 | 0.011 [b]± 0.0007 |
| Uric acid | 0.0013[a]± 0.0002 | 0.0013[a]± 0.0003 |
| FLNa (µmol/min/ 100 g body weight) | 25.13 ± 3.957 [a] | 36.9 ± 3.22 [a] |
| FPTRNa (%) | 91.58 ± 1.167 [a] | 92.46 ± 1.993 [a] |
| FDTRNa-I (%) | 99.43 ± 0.121[a] | 99.48 ± 0.145 [a] |
| FDTRNa-II (%) | 8.37 ± 1.164 [a] | 7.51 ± 1.992 [a] |

Results are represented as mean ± S.E. where n= 8 rats per group, within a row same letters means non significant difference; different letters means significant difference among the experimental groups ($P < 0.05$).

**Table 3: The first five and the twenty-four hours urinary sodium, potassium, chloride, Na+Cl, Na/K and Cl/Na+K of the different experimental groups.**

| Groups / Parameters | | Control | Furosemide (25 mg/kg) | Olive leaves (aq. methanol extract) (500 mg/kg) |
|---|---|---|---|---|
| Urinary sodium (mmol) | 5 hours | 1.3 ± 0.064 [a] | 4.03 ± 0.069 [b] | 2.01 ± 0.039 [c] |
| | 24 hours | 3.27 ± 0.03 [a] | 6.51 ± 0.043 [b] | 3.99 ± 0.02 [d] |
| Urinary potassium (mmol) | 5 hours | 3.8 ± 0.173 [a] | 4.9 ± 0.207 [b] | 5.9 ± 0.096 [c] |
| | 24 hours | 5.3 ± 0.133 [a] | 7.01 ± 0.1 [b] | 9.1 ± 0.046 [c] |
| Urinary chloride (mmol) | 5 hours | 1.7 ± 0.054 [a] | 5.1 ± 0.135 [b] | 1.89 ± 1.498 [ad] |
| | 24 hours | 3.5 ± 0.131 [a] | 8.29 ± 0.141 [b] | 5.05 ± 0.084 [e] |
| Urinary Na+Cl | 5 hours | 3.01 [a] ± 0.105 | 9.13 [b] ± 0.098 | 3.9 [d] ± 0.061 |
| | 24 hours | 8.57 [a] ± 0.125 | 13.55 [b] ± 0.115 | 13.09 [c] ± 0.041 |
| Na/K | 5 hours | - | 0.83 [b] ±0.031 | 0.34 [a] ±0.007 |
| | 24 hours | - | 0.93 [b] ±0.014 | 0.439 [c] ±0.004 |
| Cl/Na+K | 5 hours | - | 0.58 [b] ±.029 | 0.24 [e] ±0.006 |
| | 24 hours | - | 0.61 [b] ±0.014 | 0.386 [a] ±0.006 |

Results are expressed as mean ± S.E., where n=8 rats per group; within a row, different letters means significant difference among the experimental groups ($P < 0.05$).

**Table 4: The different biochemical parameters after 30 days of daily oral administration of 500 mg/kg of the aqueous methanol extract of olive leaves compared with normal control group.**

| Parameter \ Group | | Control | aqueous methanol extract of olive leaves |
|---|---|---|---|
| **Blood haemoglobin (mmol/l)** | | 7.78 ± 0.165 [a] | 8.12 ± 0.231 [a] |
| **Haematocrite (% from whole blood)** | | 46.86 ± 1.3 [a] | 47.04 ± 1.14 [a] |
| **Serum glucose (mmol/l)** | | 5.18 ± 0.628 [a] | 5.95 ± 0.792 [a] |
| **Serum electrolytes** | Na (mmol/l) | 169.22 ± 14 [a] | 149.02 ± 2.1 [ab] |
| | K (mmol/l) | 6.78 ± 0.522 [a] | 8.71 ± 1.063 [a] |
| **Serum minerals** | Mg (mmol/l) | 0.867 ± 0.078 [a] | 1.05 ± 0.038 [a] |
| | Ca (mmol/l) | 1.96 ± 0.053 [a] | 1.66 ± 0.032 [b] |
| | Zn (µmol/l) | 17.63 ± 0.878 [a] | 16.6 ± 3.033 [a] |
| | Fe (µmol/l) | 37.23 ± 3.52 [a] | 45.41 ± 3.908 [a] |
| **Kidney function Tests** | Serum creatinine (mg/dl) | 1.13 ± 0.062 [a] | 0.96 ± 0.059 [a] |
| | Urinary creatinine (mg/dl) | 137.8 ± 7.049 [a] | 138.2 ± 6.421 [a] |
| | Creatinine clearance (ml/min) | 1.028 ± 0.161 [a] | 1.613 ± 0.25 [ab] |
| | Serum urea (mmol/l) | 6.1 ± 0.528 [a] | 6.22 ± 0.183 [a] |
| **Liver function Tests** | ALT (IU/ml) | 50.56 ± 2.161 [a] | 45.75 ± 1.88 [a] |
| | AST (IU/ml) | 90.78 ± 1.526 [a] | 71.85 ± 2.60 [b] |
| **Serum lipid profile** | TG (mmol/l) | 0.983 ± 0.102 [a] | 1.1 ± 0.108 [a] |
| | T-Cholesterol (mmol/l) | 2.2 ± 0.094 [a] | 1.94 ± 0.099 [b] |
| | HDL-cholesterol (mmol/l) | 1.245 ± 0.103 [a] | 1.307 ± 0.137 [a] |
| | LDL-cholesterol (mmol/l) | 0.417 ± 0.15 [a] | 0.357 ± 0.127 [a] |
| | VLDL-cholesterol (mmol/l) | 0.197 ± 0.02 [a] | 0.22 ± 0.022 [a] |
| Organ weight **(% Of FBW)** | Liver | 2.3 ± 0.052 [a] | 2.4 ± 0.156 [a] |
| | Kidney | 0.533 ± 0.021 [a] | 0.567 ± 0.056 [a] |
| | Heart | 0.3 ± 0.0001 [a] | 0.283 ± 0.031 [a] |
| | Spleen | 0.383 ± 0.031 [a] | 0.317 ± 0.017 [a] |

Values are expressed as mean ± S.E., n= 10 rats per group, within a row same letters means non significant difference; different letters means significant difference among the experimental groups ($P < 0.05$).

FBW: final body weight.

**Table 5: Nutritional parameters after 30 days of daily oral administration of 500 mg/kg the aqueous methanol extract of olive leaves compared with normal control group.**

| Group / *Parameter* | Control | aqueous methanol extract of olive leaves |
|---|---|---|
| IBW (g) | 189.3 ± 3.66 [a] | 190.8 ± 6.96 [a] |
| FBW (g) | 246.8 ± 7.814 [a] | 241.2 ± 10.28 [a] |
| BWG (g) | 57.5 ± 7.76 [a] | 50.4 ± 6.87 [a] |
| TFI (g) | 363.6 ± 17.8 [a] | 376.35 ± 18.66 [a] |
| FER | 0.1511 ± 0.02 [a] | 0.1323 ± 0.018 [a] |

Values are expressed as means ± S.E, where n = 10 rats per group; within a row same letters means non significant difference; different letters means significant difference among the experimental groups ($P < 0.05$).

IBW: initial body weight; FBW: final body weight; BWG: body weight gain; TFI: total food intake; FER: food efficiency ratio.

**Food chemistry:**

Vitamin C was shown to be 3 mg/100g fresh olive leaves. Ash content gives an indication of the total mineral content in the sample and it is used for determination of the individual minerals. It is 7g/100g dry olive leaf. It can be noticed that moisture content of olive leaves was 50.4%. In olive leaves, potassium content was higher than that of sodium, which was reflected in K/Na ratio which was 8. The contents of Na, K, Ca, magnesium, zinc, iron, manganese and copper were 117, 934, 5395, 330, 10.4, 35, 139, 2.8 as mg/100g dry olive leaf. Ash content of olive leave aqueous methanol extract was 6%.The K/Na ratio in olive leave aqueous methanol extract (8.1) was more or less equal to that of the mother plant (8). Na and K were 231 and 1864 mg/100g olive leave aqueous methanol extract. Calcium, zinc, iron and manganese content of the extract (585, 0.9, 28, 1.7 mg/100g) showed reduced values than that of the original plant. magnesium in the olive extract (324 mg/100g) was more or less similar to that in the plant. Copper content in the aqueous methanol extract of olive leaves (1.7 mg/100g) was lower compared with that in the original plant.

**Phytochemical Results**

**Identification of flavonoidal compounds from olive leaf:**

Three flavonoidal compounds were identified in olive leaf methanol extract. These are apigenin 7-*O*-β-neohesperopyranoside, luteolin and apeginin (Fig, 1).

**Apigenin 7-*O*-β-neohesperopyranoside:**

Yellow powder, PC $R_f$ 0.38 (BAW) and 0.23 (15% HOAc). Normal acid hydrolysis gave glucose, rhamnose and apigenin (CoPC). UV $\lambda_{max}$ (MeOH): 268, 331; (NaOMe): 276, 322 sh, 392; ($AlCl_3$): 276, 302, 346, 387 ($AlCl_3$/HCl): 276, 300, 340, 387; (NaOAc): 268, 300, 368; (NaOAc/$H_3BO_3$): 269, 297, 336. 1H NMR: δ = 7.9 (2H, d, *J* = 8.5 Hz, H-2'/6'), 6.9 (2H, d, *J* = 8.5 Hz, H-3'/5'), 6.84 (1H, s, H-3), 6.8 (1H, d, *J* = 1.5 Hz, H-8), 6.6 (1H, d, *J* = 1.5 Hz, H-6), 5.05 (1H, d, *J* = 7.5 Hz, H-1''), 4.06 (1H, br, H-1'''), 1.08 (1H, d, *J* = 6.1 Hz, $CH_3$-Rh)

**Luteolin:**

Yellow powder, PC $R_f$ 0.68 (BAW) and 0.06 (15% HOAc). UV $\lambda_{max}$ (MeOH): 253, 349; (NaOMe): 273, 409; ($AlCl_3$): 272, 330, 343, 421 ($AlCl_3$/HCl): 258, 277, 386; (NaOAc): 268, 296, 367; (NaOAc/$H_3BO_3$): 260, 306, 371. 1H NMR: δ = 7.3 (2H, dd, *J* = 8.0 & 1.5 Hz, H-2'/6'), 6.79 (1H, d, *J* = 8.0 Hz, H-5'), 6.57 (1H, s, H-3), 6.35 (1H, d, *J* = 1.6 Hz, H-8), 6.09 (1H, d, *J* = 1.6 Hz, H-6).

**Apigenin:**

Yellow powder, PC $R_f$ 0.65 (BAW) and 0.04 (15% HOAc). UV $\lambda_{max}$ (MeOH): 269, 298, 334; (NaOMe): 276, 322 sh, 394; ($AlCl_3$): 276, 302, 346, 382 ($AlCl_3$/HCl): 276, 300, 340, 382; (NaOAc): 280, 300, 368; (NaOAc/$H_3BO_3$): 269, 297, 337. 1H NMR: δ = 7.94 (2H, d, *J* = 8.1 Hz, H-2'/6'), 6.93 (2H, d, *J* = 8.1 Hz, H-3'/5'), 6.79 (1H, s, H-3), 6.48 (1H, d, *J* = 2.5 Hz, H-8), 6.18 (1H, d, *J* = 2.5 Hz, H-6).

Apigenin 7-*O*-β-neohesperopyranoside

R= H : Apigenin
R=OH : Luteolin

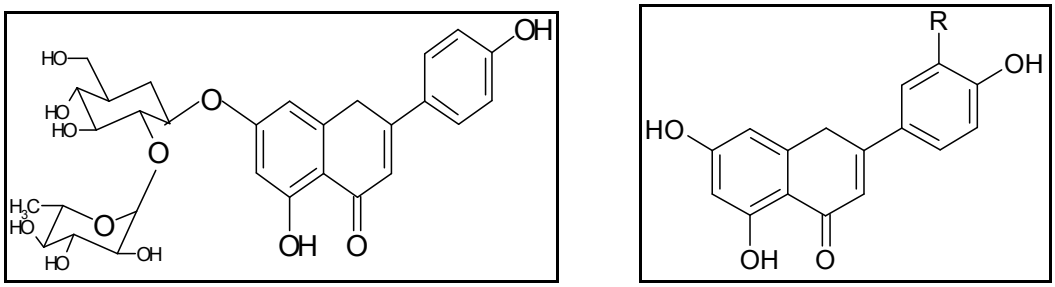


**Figure 1: Structure of Apigenin, luteolin and apigenin 7-*O*-β-neohesperopyranoside**

## Discussion

Diuretic activity of medicinal plants was related to the presence of phytosterols, flavonoids, saponins, volatile constituents, coumarins and vitamin C[45-47] as well as to plant K/Na ratio[48].

The diuretic effect of petroleum ether and aqueous methanol extract of olive leaves was studied in the present work. The mechanism of action, safety, chemical analysis of minerals and vitamin C and phytochemical analysis of the flavonoidal compounds were assessed in the aqueous methanol extract which showed the most efficient diuretic effect.

Lipschitz test showed that the tested extracts possess diuretic activity in all the tested doses with variable degrees. The aqueous methanol extract was more efficient than the petroleum ether extract which could be due to the flavonoidal content represented by luteolin, apigenin, apigenin 7-O-β-D-neohesperopyranoside detected in the present study. In addition, other constituents have been cited to have diuretic effect; in this respect, the diuretic effect of urosolic acid and oleanolic acid isolated from olive leaves was reported after 5 and 24 hours of their intraperitoneal injection which was comparable to that of urea[49]. It was proved that an active constituent, $\beta$ - (3.4 – dihydroxy – phenyl) ethanol, which was found in olive leaves has calcium channel blocking activity and thus has smooth muscle relaxing effect[4]. Such effect can decrease vascular resistance and thus increase the renal blood flow and the glomerular filtration rate. Also, olive leaf extracts could relax smooth muscles by direct action and thus increases blood circulation and urine secretion. Extracts rich in flavonoids were used as therapeutic agents in cases of small vessel permeability[50]. Flavonoids were reported previously to treat oedema[51] which could be due to their inhibiting activity of cyclic AMP phosphodiesterases[52]. Sugar alcohols as mannitol and sorbitol reported to present in olive leaves[49] are not mnetabolized in the body following systemic absorption from the alimentary tract and undergo glomerular filtration as such. As a result of hyperosmolality of the glomerular filtrate, water reabsorption in the proximal tubule is reduced and diuresis occurs, acting as osmotic diuretic[53]. However it was shown that an ethanol extract from the olive leaves administered orally produced no change in urine volume collected at 15, 30, 45, 60, 120 and 240 min compared to placebo[54]. This confliction of literature may be explained by the difference of the type of extract, the constituents of olive leaves variety, the time of urine collection and the dose level.

The diuretic effect of the petroleum ether extracts might be attributed to the presence of sterols and triterpens. Sterols were reported to be spironolactone analogues and could block $Na^+$ reabsorption in distal tubules, resulting in increase natriuresis without change in potassium excretion[3,53]. It was documented that olive leaves contain triterpens[3], which has been reported to increase the urine output[55].

On administration of vitamin C at 200 mg /kg rat body weight in the present study, it produced diuretic effect similar to urea after 24 hours. Long ago, it has been proven that vitamin C in green leafy vegetables has diuretic activity[45], so it was important to study vitamin C content in the studied plants.

Co-administration of vitamin C with olive leaves aqueous methanol extract, in the present study, produced a significant increase in diuretic activity compared to that of the extract alone, pointed to the synergistic effect of vitamin C which might be due to additional diuretic effect of vitamin C itself and a vitamin C enhancement of the bioavailability of this extract [56], by increasing their intestinal absorption, inhibiting their metabolism or enhancing their renal reabsorption[56].

Mineral elements in the medicinal herb especially K/Na ratio was reported to possess diuretic effect[48, 54, 57, 58]. From the present study it can be noticed that K/Na ratio was high in olive leaf aqueous methanol extract (8.7) which may participate in the diuretic activity of such extract.

In the saluretic experiment in the current study furosemide significantly increased excretion of sodium, potassium and chloride which go in line with our previous study[25] which could be ascribed to inhibition of $Na^+/K^+/2Cl^-$ Symporter in the ascending loop of Henle and reduction of carbonic anhydrase. Olive leaf extract produced significant increase of urinary sodium, potassium and chloride in 24 hours compared to control in the saluretic experiment. The excreted sodium and chloride was significantly lower than that of furosemide, but the potassium excreted on administration of the extract was significantly higher than furosemide. The high concentration of potassium excreted on administration of the extract was expected not to induce hypokalemia (as seen when administered for a month due to the high concentration of potassium in such extract as seen from

its chemical analysis in the present study which agreed with the work of Benjumea *et al.*[58]. In renal tubules; carbonic anhydrase catalyzes the production of hydrogen ions. This action could be inhibited by some diuretics, thereby an increased excretion of urinary sodium bicarbonate and K was noticed in the urine[59]. The ratio CL / (Na+K) could be a measure of carbonic anhydrase inhibition[11] if it is below 0.8. The lower the ratio the more is the carbonic anhydrase inhibition. Administration of furosemide in the present study produced a ratio of 0.58 and 0.61 in 5 and 24 hour urine, respectively which account for carbonic anhydrase inhibition which agreed with the work of Laurence and Bennett[60]. Olive leaf extract in the present study showed ratios of 0.24 and 0.386 in 5 and 24 hour urine, respectively which reflect a stronger carbonic anhydrase inhibition than furosemide. The high level of potassium in the extract as seen from the result may participate in the reduction of the ratio.

In the lithium model carried out in the present study; urinary excretion of creatinine and creatinine clearance were significantly higher than the control on administration of olive extract. So, it is postulated that olive leaf extract could increase the glomerular capillary hydrostatic pressure thereby increasing the effective filtration pressure and consequently the glomerular filtration measured by creatinine clearance. Also the extract might produce dilatation of mesangial cell thereby induce area increment that could affect filtration coefficient and glomerular filtration. In the present study urine pH of the group given olive extract was similar to control which indicated that the effect of any of the extracts on $H^+/K^+$ antiporter might be excluded. This is supported by the inability of the extract to decrease potassium reabsorption reflected by the non significant change in urine potassium in this step. The non significant change in lithium clearance on administration of olive leaf extract may suggest that the proximal tubular function was not involved in the mechanism of action of the olive extract as diuretic. This is because lithium clearance is considered proximal tubular marker[20, 22].

Concerning the safety of the aqueous methanol extract of olive leaves after daily oral administration for a month, it can be noticed that their safety on liver and kidney functions was verified during this study. No significant change has been noticed in serum potassium, magnesium, zinc and iron. Previously it has been cited that synthetic diuretics produced reduction in some elements and elevation in others[61, 62]. Also it can be noted that although urinary excretion of potassium was high on administration of such extract in the saluretic evaluation experiment however there was no hypokalemia noticed in the present prolonged administration which may be explained by the high potassium content of such extract. Also the presence of magnesium, zinc and iron in the extract as shown from its analysis may prevent the reduction of their serum level. Also there was non-significant change in the different nutritional parameters, serum glucose level and the majority of lipid parameters reflected the secure usage of such extracts. Reduction in total cholesterol after administration of the aqueous methanol extract of olive leaf clarified the possibility of beneficial effect during hyperlipidemia, which is important when the extracts were used as diuretics in hypertensive patients. The reduction in the serum total cholesterol might be due to the presence of flavonoids in the extract. This active constituents was reported previously to possess hypocholestrolemic effect[63]. It can be noticed here that although there was reduction in serum total cholesterol however it was still within the normal range of rats, clarifying that the extract has no negative effect on normal. However one drawback was the significant reduction of serum calcium in the test group. It has been reported by Zhu *et al.*[64] that diuretics could cause desensitization of the vascular smooth muscle cells to the rise of the intracellular calcium. So, olive leaves extract may work with a mechanism differ from that of thiazides.

Toxicological study has shown that olive leave extract was very safe up to 12 g / kg mice body weight. This dose corresponds to 93 g / 70 kg man body weight for human when extrapolated to corresponding estimates in human adopting interspecies dosage conversion scheme[65].

## Conclusion:

Olive leaf aqueous methanol extract showed superior diuretic activity than the petroleum ether extract in the three studied doses. Vitamin C had synergistic diuretic action with aqueous methanol extract. Aqueous methanol extract of olive leaves showed complete safety except for reduction of serum Ca. The diuretic activity of aqueous methanol extract may be attributed to the presence of vitamin C, flavonoids, and the high K/Na ratio that were identified in the plant. Within the extreme of the present study; the mechanism of action of the diuretic activity of olive leaf aqueous methanol extract might be attributed to carbonic anhydrase inhibition and enhancing glomerular filteration. Proximal tubular function was suggested to be not involved in the mechanism of action.

## Acknowledgement:

This work was totally financed by National Research Centre, Cairo, Egypt. The work was carried out in National Research Centre, Cairo, Egypt.

### Conflicts of interest

There are no conflicts of interest to declare

### References:

1. Hussein F.T.K., Medicinal plants in Libya, 1st edition, Arab encyclopedia house, Lebanon, 1985.
2. Visioli F. and Galli C., Alpha-linolenic acid and cardiovascular disease, Am. J. Clin. Nutr., 2002, 75(6), 1121.
3. Cortesi N., Mosconi C. and Fedeli E., High performance liquid chromatography in the analysis of *O. europaea* leaf extracts, Chemical Abstracts, 1985, 102, 859.
4. Rauwald H.W., Brehm O. and Odenthal KP., Screening of nine vasoactive medicinal plants for their possible calcium antagonistic activity. Strategy of selection and isolation for the active principles of *Olea europaea* and *Peucedanum ostruthium.,* Phytother. Res., 1994, 8, 135.
5. Romero M., Toral M., Gómez-Guzmán M., Jiménez R., Galindo P., Sánchez M., Olivares M., Gálvez J. and Duarte J., Antihypertensive effects of oleuropein-enriched olive leaf extract in spontaneously hypertensive rats, Food Funct., 2016, 7(1), 584-93.
6. Marchetti C., Clericuzio M., Borghesi B., Cornara L., Ribulla S., Gosetti F., Marengo E. and Burlando B., Oleuropein-enriched olive leaf extract affects calcium dynamics and impairs viability of malignant mesothelioma cells, Evid Based Complement Alternat. Med., 2015, DOI: 10.1155/2015/908493.
7. Goldsmith C.D., Vuong Q.V., Sadeqzadeh E., Stathopoulos C.E., Roach P.D. and Scarlett CJ. Phytochemical properties and anti-proliferative activity of Olea europaea L. leaf extracts against pancreatic cancer cells, Molecules, 2015, 20 (7), 12992-3004.
8. Mehraein F., Sarbishegi M. and Golipoor Z., Different effects of olive leaf extract on antioxidant enzyme activities in midbrain and dopaminergic neurons of Substantia Nigra in young and old rats, Histol. Histopathol., 2016, 31(4), 425-31.
9. Mostafa-Hedeab G., Sati L.M., Elnaggar H.M., Elgatlawey Z.O., Eltwab A.A., Elsaghayer W.A. and Ali H. Ameliorating effect of olive leaf extract on cyclosporine-induced nephrotoxicity in rats, Iran. J. Kidney Dis., 2015, 9(5), 361-8.
10. Olmez E., Vural K., Gok S., Ozturk Z., Kayalar H., Ayhan S. and Var A., Olive leaf extract improves the atherogenic lipid profile in rats fed a high cholesterol diet, Phytother. Res., 2015, 29(10), 1652-7.
11. Kumral A., Giriş M., Soluk-Tekkeşin M., Olgaç V., Doğru-Abbasoğlu S., Türkoğlu Ü. and Uysal M., Effect of olive leaf extract treatment on doxorubicin-induced cardiac, hepatic and renal toxicity in rats, Pathophysiology, 2015, 22(2), 117-23.
12. Shen Y., Song S.J., Keum N. and Park T. Olive leaf extract attenuates obesity in high-fat diet-fed mice by modulating the expression of molecules involved in adipogenesis and thermogenesis, Evid. Based Complement. Alternat. Med., 2014, DOI: 10.1155/2014/971890.
13. Fogel M.R., Sawhney V.K., Neal E.A., Miller R.G., Knauer C.M. and Gregory P.B., Diuresis in the ascitic patient: a randomized controlled trial of three regimens, J. Clin. Gastroenterol., 1981, 3 (Suppl. 1), 73- 80.
14. Waller D.G., Renwick A.G., Hillier K., eds. Medical pharmacology and therapeutics. Hardlines, Oxford. 2001, 191- 199.
15. Mabry T.J., Markam K.R. and Thomas M.B., eds. The systematic identification of flavonoids, Berlin, Springer-Verlag, 1970.
16. Lipschitz W.L., Hadidian Z. and Kerpcsar A., Bioassay of diuretics., J. Pharmacol. Exp. Ther., 1943, 79, 97– 110.
17. Kau S.T., Keddie J.R. and Andrews D. A method for screening of diuretic agents in the rat, J. Pharmacol. Meth., 1984, 11, 67– 75.
18. Muschaweck R. and Sturm K. Diuretika, In : Elhart G, Ruschig H, eds., Arzneimittel Entwicklung – Wirkung- Darstellung, Germany: Verlag Chemie.Weinheim Bergstrasse, 1972, Vol 2, 317–328.
19. Schales O. and Schales SS., A simple and accurate method for the determination of chloride in biological fluids, J. Biol. Chem., 1941, 140, 879-884.

20. Thomsen K., Lithium clearance as a measure of sodium and water delivery from proximal tubule., Kidney Int. , 1990, 27 (suppl. 28), S10- S16.
21. Kirchner K.A., Effect of diuretic and antidiuretic agents on lithium clearance as a marker for proximal delivery., Kidney Int., 1990, 27 (suppl. 28), S22- S25.
22. Koomans H.A. and Mees E.J.D., Lithium in renal physiology: post – conference discussion and consensus., Kidney Int., 1990, 27 (suppl. 28), S78-S79.
23. Bartels H., Böhmer M. and Heierli C., Serum creatinine determination without protein precipitation., Clin. Chim. Acta., 1972, 37, 193-197.
24. Fossati P., Prencipe L. and Berti G., Use of 3,5-dichloro-2-hydroxybenzenesulfonic acid/4-aminophenazone chromogenic system in direct enzymatic assay of uric acid in serum and urine., Clin. Chem., 1980, 26, 227- 231.
25. Al-Okbi S.Y., Hassan Z.A., ElMaza M.M., Ammar N.M., Abou El-Kassem L.T. and El-Bakry H.F., Diuretic Activity of Polar and Non Polar Parsley Seed Fractions. Egypt. Pharm. J., (NRC), 2011, 10(2), 213-234.
26. Van Kampen E.J. and Zijlstra W.G., Determination of haemoglobin and its derivatives., Adv. Clin. Chem., 1965, 8, 141- 187.
27. Strumia M.M., Sample A.B. and Hart E.D., An improved micro-haematocrite method., Am. J. Clinical Pathology, 1954, 221, 1016.
28. Trinder P., A method for estimation of plasma glucose., Ann. Clin. Biochem., 1969, 6, 24.
29. Trinder P., A method for estimation of serum sodium., Analyst, 1951, 76, 596.
30. Sunderman F.W. Jr., Sunderman F.W., Studies in serum electrolytes. XXII. A rapid, reliable method for serum potassium using tetraphenylboron., Am. J. Clin. Pathol., 1958, 29(2), 95- 103.
31. Tietz N.W., Caraway W.T., Freier E.F., Kachmar J.F., Rawnsley H.M., eds. Fundamentals of clinical chemistry USA: WB Saunders Company, 1982.
32. Gindler E., Melvin J.D., King M.D., Rapid colorimetric determination of calcium in biological fluids with methyl thymol blue., Am. J. Clin. Path. 1972, 58, 376.
33. Makino T., Saito M., Horiguchi D. and Kina K., A highly sensitive colorimetric determination of serum zinc using water- soluble pyridylazo-dye., Clinica Chimica Acta, 1982, 120, 127-135.
34. Dreux C., Analysis of human serum: assay of iron II using bathophenanthroline. Ann. Biol. Clin., 1977, 35 (3), 275- 277.
35. Fawcett J.K. and Scott J.E., A rapid and precise method for the determination of urea., J. Clin. Pathol., 1960, 13, 156- 159.
36. Reitman S. and Frankel S. Colorimetric methods for aspartate and alanine aminotransferase., Am. J. Clin. Pathology, 1957, 28, 55.
37. Allain C.C., Poon L.S., Chan C.S., Richard W. and Paul C.F., Enzymatic determination of total cholesterol., Clin. Chem., 1974, 20, 470-474.
38. Burstein M., Scholnick H.R. and Morfin R., Rapid method for the isolation of lipoproteins from human serum by precipitation with polyanions., J. Lipid. Res., 1970, 11(6), 583- 595.
39. Wieland H. and Seidel D., A simple specific method for precipitation of low density lipoproteins., J, Lipid Res., 1983, 24(7), 904- 909.
40. Fossati P, and Prencipe L. Serum triglycerides determined colorimetrically with an enzyme that produces hydrogen peroxide., Clin. Chem. 1982, 28(10), 2077-80.
41. Friedwald W.T., Levy R.T. and Fredrickson D.S. Estimation of concentration of low density lipoprotein by use of the preventive ultracentrifuge. Clin. Chem., 1972, 18, 499.
42. Goodman A.G., Goodman L.S. and Gilman A, eds. Principles of toxicology., 6th edition, Macmillan, New York, 1980, 1602-1615.
43. Freed M., eds. Methods of Vitamin Assay, 3rd edition., John Wiley & Sons, New York. 1966, 294-299.
44. AOAC, Association of analytical chemists. Official Methods of Analysis. 15*th* edition., Washington, DC, 2000.
45. Abbasy M.A., Hill A. and Harris D., The Diuretic Action of Vitamin C., Lancet, 1936, II, 1413.
46. Ahlenstiel T., Burkhardt G., Köhler H. and Kuhlmann MK., Bioflavonoids attenuate renal proximal tubular cell injury during cold preservation in Euro-Collins and University of Wisconsin solutions. Kidney Int., 2003, 63, 554- 563.
47. Wang T., Hicks K.B. and Moreau R., Antioxidant activity of phytosterols, oryzanol and other phytosterol conjugates., J. Amr. Oil Chem. Soc., 2002, 79, 1201- 1206.

48. Szentimihalyi K., Kery A., Lakatos B., Sandor Z. and Vinkler P., Potassium -sodium ratio for the characterization of medicinal plant extracts with diuretic activity., Phytother. Res., 1998, 12, 163- 166.
49. Somova L.I., Shode F.O., Ramnanan P. and Nadar A., Antihypertensive, antiatherosclerotic and antioxidant activity of triterpenoids isolated from *Olea europaea*, subspecies *Africana* leaves., Journal of Ethnopharmacology, 2003, 84, 299- 305.
50. Christie S., Walker A.F. and Lewith G.T., Flavonoids - a new direction for the treatment of fluid retention., Phytother. Res., 2001, 15, 467 – 475.
51. Weiss R.F., Herbal medicine, 6th edition, UK: Beaconsfield publishers, Beaconsfield, 1988.
52. Galati E.M., Trovato A., Kirjavainen S., Forestieri A.M., Rossitto A. and Monforte M.T., Biological effects of hesperidin, a citrus flavonoid: Antihypertensive and diuretic activity in rat., Farmaco, 1996, 51, 219- 221 .
53. De-Stevens G. eds. Chemistry and Pharmacology of Medicinal Plants. New York , London: Academic press, 1963, 146-147.
54. Ribeiro R.A., De Barros F., De Melo M.M., Muniz C., Chieia S., Wanderley M.D., Gomes C. and Trolin G. Acute diuretic effects in conscious rats produced by some medicinal plants used in the state of Sao Paulo, Brasil. J. Ethnopharmacol., 1988, 24, 19– 29.
55. Anand R., Patnaik G.K., Kulshreshta D.K. and Dhawan B.N., Antiurolithiatic and diuretic activity of lupeol. Proc 24$^{th}$ and 25$^{th}$ Soc Conf., A10., 1991, from: Evaluation of the Effect of Triterpenes on Urinary Risk Factors of Stone Formation in Pyridoxine Deficient Hyperoxaluric Rats
56. Lee M.G. and Chiou W.L., Mechanism of ascorbic acid enhancement of the bioavailability and diuretic effect of furosemide., The American Society for Pharmacology and Experimental Therapeutics, 1998, 26, 401- 407.
57. Chodera A., Dabrowska K., Sloderbach A., Skrypczak L. and Budzianowski K., Effect of the flavonoid fraction of the solidago genus plants on diuresis and electrolyte concentration., Acta Pol. Pharm., 1991, 48, 35- 37.
58. Benjumea D., Abdala S., Hernandez-Luis F., Perez-Paz P. and Martin-Herrera D. Diuretic activity of *Artemisia thuscula*, an endemic canary species. J. Ethnopharmacol., 2005, 100, 205– 209.
59. Harper H.A., Review of physiological chemistry., 15*th* edition, Californea: Lange medical publication, 1977, 411.
60. Laurence D.R. and Bennett PN., Clinical Pharmacology., UK: Churchill, Livingstone, Medical Division of Longman Group UK Ltd, 1987, 545.
61. Mountokalakis TD, ed. Metal ions in biology and medicine, Vol 2. Paris: John Libbey Eurotext, 1992, 209-214.
62. Suki W.N., Effects of diuretics on calcium metabolism., Adv. Exp. Med. Biol., 1982, 151, 493-500.
63. Abu Mweis S.S., Vanstone C.A., Ebine N., Kassis A., Ausman L.M., Jones P.J. and Lichtenstein A.H., Intake of a single morning dose of standard and novel plant sterol preparations for 4 weeks does not dramatically affect plasma lipid in humans., J. Nutr., 2006, 136, 1012- 1016.
64. Zhu Z., Zhu S., Liu D., Cao T., Wang L. and Tepel M., Thiazide-like diuretics attenuate agonist-induced vasoconstriction by calcium desensitization. Hypertension, 2005, 45 (2), 233- 239.
65. Laurrence D.R. and Bacharach A.L., eds. Evaluation of drug activities: Pharmacometrics, Vol. (1), London and New York: Academic Press, 1974, 135 - 140.

*****