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EFFECTS OF FLAVONOIDS EXTRACTED FROM *ARTEMISIA VULGARIS* ON INDUCED HYPERURICEMIA IN MICE

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ABSTRACT

Objective: The aim of the study was to investigate the hypouricemic effect of flavonoids extracted from aerial parts of *Artemisia vulgaris* on hyperuricemic mice. **Methods:** Forty albino mice were divided into four groups**.** Mice were orally administered either vehicle, allopurinol (20 mg/kg), flavonoids of *Artemisia vulgaris* 500 mg/kg for seven consecutive days. On the 7th day, all mice except control were injected with uric acid 250 mg/kg intraperitoneally then serum uric acid, creatinine and Glutathione were determined. Morphological changes in kidney were observed using hematoxylin and eosin staining. **Results:** results showed that flavonoids fraction of *Artemisia vulgaris* significantly reduced the serum uric acid level in hyperuricemic mice. **Conclusion:** flavonoids extracted from aerial parts of *Artemisia vulgaris* have hypouricemic effect.

KEYWORDS: Hyperuricemia, *Artemisia vulgaris*, Uric acid, Xanthine oxidase, Gout, Allopurinol.

INTRODUCTION

Hyperuricemia refers to the elevation of serum uric acid above normal levels. It plays a main role in pathology of gout, and can be related to the development and progression of metabolic, renal and cardiovascular diseases,[1] Hyperuricemia and gout are highly prevalent in developed countries, while the prevalence of hyperuricemia tends to be lower in developing countries.[2]

Hyperuricemia can be caused by increased production of uric acid (UA), reduced excretion of uric acid, or both. Diet rich in purine and increased purine metabolism lead to raise

production of urate. Kidney diseases and several drugs cause reduction in uric acid excretion.[3]

Endogenous urate production depends on the balance between purine ingestion, intracellular de novo synthesis, recycling, as well as xanthine oxidase (XOD) function. The production of uric acid occurs mainly in the liver and to a lesser extent in the small intestine.[4]

Pathway of purine metabolism involves the formation of hypoxanthine from inosine then hydroxylation of hypoxanthine to produce xanthine, also deamination of guanine form xanthine. Xanthine oxidase is responsible of the conversion of both hypoxanthine and xanthine to uric acid.[5] Humans lack the enzyme uricase that oxidizes uric acid to the more soluble allantoin which excreted unchanged in urine, this enzyme prevents hyperuricemia in most mammals.[6] Reabsorption of urate in renal tubules depends on tubular transporters.[7] Urate transporter 1 (URAT1, SLC22A12) and Glucose transporter 9 (GLUT9, SLC2A9) have been proposed to mediate uric acid re-absorption.[8,9]

Xanthine oxidase inhibitors, allopurinol, and febuxostat are considered the first line of therapy for lowering serum uric acid.[10] Although the availability of urate lowering drugs, treatment failure occurs because of poor tolerance and side effects to these drugs, which including serious adverse effects.[11]

Intracellular oxidation increases with UA production. Oxidative stress is derived from a large number of highly reactive molecules which leads to imbalance in the oxidative and antioxidative systems, ultimately damaging cellular functions. A great deal of reactive oxygen species are produced along with the production of UA.[12] The GSH antioxidant system plays a fundamental role in cellular defense against reactive free radicals and other oxidant species.[13]

Natural bioactive compounds especially from plant sources, have been investigated for their characteristics and health effects. Plants are potential sources of natural bioactive compounds such as secondary metabolites and antioxidants. Flavonoids and phenolics acids are the most important groups of secondary metabolites and bioactive compounds in plants.[14]

The ability of polyphenols to inhibit XOD and scavenge free radical, suggests the possibility of use of polyphenols to prevent hyperuricemia, inflammation, UA induced oxidative stress

and tissue injury. Also a combination of antioxidants with low doses of urate lowering drugs can be beneficial to reduce their side effects.[15]

Artemisia vulgaris belongs to the family Asteraceae.[16] In traditional medicine, It is used for epilepsy, menstrual problems, irregular periods, and as a sedative. *A. vulgaris* is described as an abortifacient,[17] aerial parts of *A. vulgaris* are used as anti-helminthic, antiseptic, antispasmodic, a tonic for vital organs and in various disorders including hepatosis,[18] also it is used for pain relief, treatment of fever and used as a diuretic agent.[19] Studies showed that *A. vulgaris* has anti-parasitic activity,[20] remarkable hepatoprotective effects,[16] hypolipidemic, anti-inflammatory and antioxidant properties.[21]

METHODS

Equipment and chemical

The instruments used were high-performance liquid chromatography (HPLC) (Shimadzu10AV-LC, Japan), Rotatory evaporator (BÙCHI Rotavapor R-205, Swiss), Centrifuge (Cypress diagnostics, Belgium), Auto-analyzer (Roche, Switzerland) and Spectrophotometer (APEL, Japan).

Ethanol was obtained from Romel, England. Ethyl acetate was obtained from Scharlab S.L, Spain. Sodium hydroxide, Acetic acid and hydrochloric acid were obtained from Riedel-de Haen, Germany. Methanol was purchased from SCR, China. Flavonoinds standards were purchased from Chengdu Biopurify Phytochemicals, China. Allopurinol tablets were obtained from Aspen, Germany. Carboxymethylcellulose sodium was purchased from Panreac, Spain. Uric acid was obtained from Downs development, England. Chloroform was purchased from Sigma-aldrich, Germany. 5, 5'- dithiobis 2-nitrobenzoic acid (DTNB) was obtained from Schuchardt, Germany.

Extraction

Atrtemisia vulgaris was identified and authenticated by pharmacognocy department, College of Pharmacy, AL-Mustansiriya University. The dried aerial parts were coarsely grounded, then extracted by 80% ethanol, after filtration the filtrate was concentrated to a semi solid mass using rotary evaporator.[22] The crude fraction then acidified with hydrochloric acid (5%) and partitioned with ethyl acetate. The ethyl acetate layer was evaporated to dryness under reduced pressure and basified with sodium hydroxide 5% and extracted with chloroform in the reparatory funnel. The aqueous basic layer was separated, evaporated to

dryness and acidified with 5% hydrochloric acid then extracted with ethyl acetate to get flavonoids fraction.[23]

High Performance Liquid Chromatography (HPLC) analysis

Qualitative estimation of flavonoids were done by using HPLC in which identifications were made by comparison of retention time obtained at identical chromatographic conditions for the flavonoid fraction and the standards. The standards prepared as mixture solution with a concentration 1mg/ml for each four standards. The solvent used for dissolving the standards was (methanol: acetic acid) (70:1) mobile phase. Twenty microliters (20 µL) from this mixture was injected into the HPLC and performed as a single run in HPLC. The experimental conditions of HPLC were as following:

- Mobile phase: 70%Methanol: 1%acetic acid Column: ODS C18 (250mm x 4.6mm, 5µm particle size).
- Column temperature: 25ºC
- Flow rate: 1ml /min.
- Injection concentration 1mg/1ml for each flavonoid standards.
- Injection volume: 20µl
- Detection wavelength: 265 nm

Animals

The study was approved by the Ethical Committee at the College of Medicine AL Nahrain University. Fourty apparently healthy, albino male mice, aged (2-3) months, weighing about (25-30) gram, were purchased from National Center For Drug Control And Research (Baghdad, Iraq). Animals were supplied with fresh water and standard food, and maintained at 12 hr. light and dark cycles with room temperature (22-25˚C).

A hyperuricemic animal model was induced by uric acid injection. Briefly, 250 mg/kg uric acid was administrated intraperitoneally to each animal on the seventh day at the same time of the last oral administration of vehicle, allopurinol, or extract.[24]

Mice were firstly randomized into four groups (*n*_10): Control, model, allopurinol and *A.vulgaris* groups. The volume of the drug administered to each mouse was based on the body weight of the animal measured daily prior to each dose. Control group given vehicle. Model group, allopurinol group, *A.vulgaris* group were given orally (by oral gavage): vehicle,

allopurinol (20 mg/kg) and *A. vulgaris* extract (500 mg/kg) respectively for seven days, then uric acid 250 mg/kg i.p. injection on the 7th day.

Sample preparation

On the 7th day, 1 hour after the last administration, mice were anesthetized by chloroform and blood samples were collected from heart and allowed to clot for 1 h at room temperature, then centrifuged to obtain serum to do the required tests.

Measurement of serum urate

This assay is a slight modification of colorimetric method, in this reaction, the peroxide reacts in the presence of Peroxidase (POD), N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline (TOOS), and 4-aminophenazone to form quinone dye. The intensity of the red color formed is proportional to the uric acid.

Determination of Serum creatinine level

This kinetic colorimetric assay is based on Jaffe method. In alkaline solution, creatinine forms a yellow orange complex with picrate, the rate of dye formation is proportional to the creatinine concentration in the specimen.

Determination of Serum Glutathione (GSH)

Serum thiol concentration was determined by modified method of Elleman.[25] It is based on the reaction of aliphatic compounds with 5, 5'- dithiobis 2-nitrobenzoic acid (DTNB) at pH 8.0. One mole of thiol produce one mole of p-nitro thiolphenol anion. The absorbance is measured in spectrophotometer at 412 nm.

Histological analysis of kidney tissues

Kidney tissues were fixed in formaldehyde solution then dehydrated in alcohol and embedded in paraffin. A series of tissue sections were made, and hematoxylin and eosin staining was performed for histological evaluation using Genex microscope (Genex laboratories, USA).

Statistical analysis

The statistical analysis of this study performed with the statistical package for social sciences (SPSS) 21.0 and Microsoft Excel 2013.

Descriptive statistics for the numerical data were formulated as mean and standard deviation (SD), Numerical data were analyzed using independent Student's t-test for comparison between two groups. It was considered significant if ($P \leq 0.05$).

RESULTS

HPLC analysis

The HPLC results showed the presence of the listed flavonoids according to their retention times, as shown in the Table 1.

Table 1: Flavonoids of *A.vulgaris* with their retention time.

Standards	Retention time (min)
Rutin	2.4
Quercetin	3.1
Myricetin	4.48
Apiginine	5.5
Catechin	11.9

Effect of *A.vulgaris* on serum uric acid levels

The hyperuricemic model of the present study was induced by uric acid administration, so intraperitonial injection of 250mg/kg uric acid lead to significant increase in serum uric acid when compared with control. Allopurinol at a dose of 20 mg/kg significantly reduced serum uric acid, also 500 mg/kg of *A.vulgaris* flavonoids fraction significantly decreased serum uric acid when compared with hyperuricemic model as shown in Fig 1.

Effect of *A.vulgaris* on serum creatinine levels

No statistical difference seen in serum creatinine level in mice given 250mg/kg uric acid intraperitonially to induce hyperuricemia when compared to control group. The administration of 20mg/kg allopurinol and 500mg/kg flavonoids fraction of *A.vulgaris* for seven consecutive days by oral gavage then administration of uric acid i.p did not affect serum creatinine levels in treated mice as shown in Fig 2.

Effect of *A.vulgaris* on serum GSH levels

In hyperuricemic model mice GSH level decreased significantly when compared to control group. Results show that 20mg\kg allopurinol did not elicit any change in serum GSH when compared to model group, while 500mg\kg flavonoids fraction of *A.vulgaris* significantly increase serum GSH level when compared to model group as shown in Fig 3.

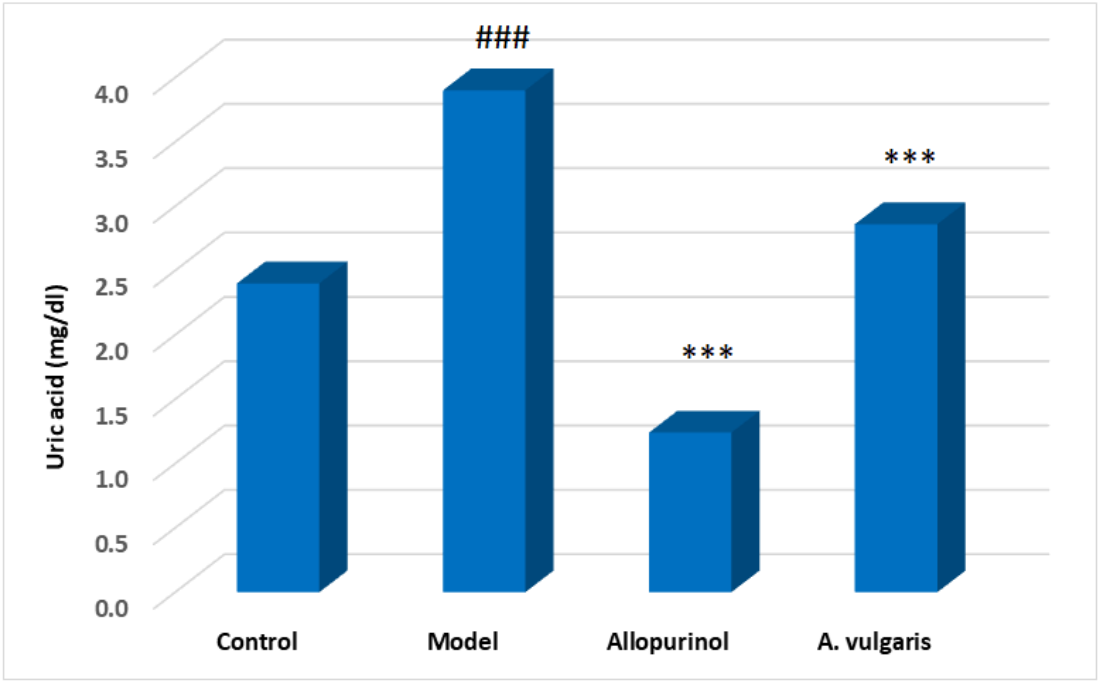

Fig. 1: The effect of *Artemisia vulgaris*, allopurinol on serum uric acid level in hyperuricemic mice. Data represent mean value ± standard deviation of serum uric acid (mg/dl). ### denotes *P*≤0.001 *vs* control group; * denotes *P*≤0.001 *vs* model group.**

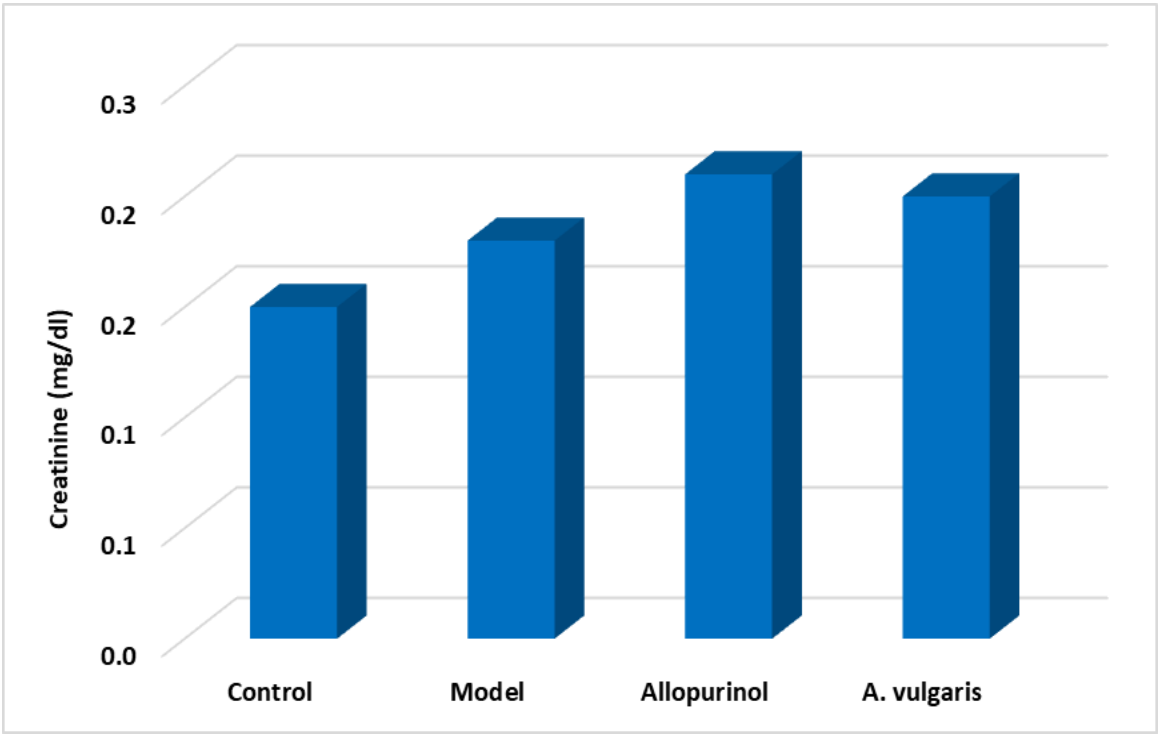

Fig. 2: Serum creatinine level among all study groups. Each value represents mean ± standard deviation of serum creatinine (mg/dl). Statistical analyses were performed by using unpaired t-test.

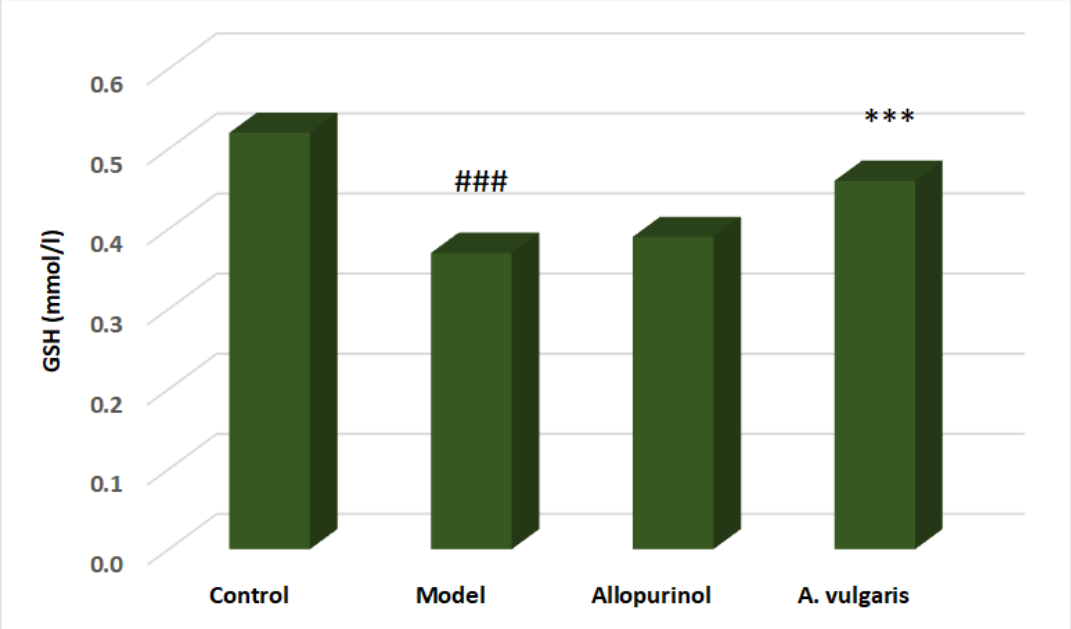

Fig. 3: Serum GSH level among all study groups. Each value represents mean ± SD of GSH (mmol/l). Statistical analyses were performed by using unpaired t-test, ### denotes *P*≤0.001 *vs* control group; * denotes *P*≤0.001 *vs* model group.**

Effects of A.vulgaris on body weights

Results revealed that 20mg/kg allopurinol orally decreased the body weight significantly while the model group and *A.vulgaris* showed no change in body weight of mice from day 1st till 7thday of the study as shown in table 2.

Table 2: Effect of *A.vulgaris* flavonoids fraction and allopurinol on body weights from day 1 (D1) till day 7 (D7). Each value represents mean ±SD of body weight (g). Statistical analyses were performed by using unpaired t-test, ***$P < 0.001$ *vs* model group.

Group (n=10)	Diff. in D1& D7 weight (g)
Control	1.32±0.89
Model	1.02±0.75
Allopurinol	-0.74±1.17***
A.vulgaris	1.86±1.1

Histopathological observations

Section of kidney of control group shows normal renal tissue with unremarkable histopathological changes of tubules and glomeruli. Scattered dilated congested interstisial blood vessels and mild dilatation of some renal tubules are noticed in section of kidney of hyperuricemic model group**.** In allopurinol treated group, histopathological examination

shows renal parenchymal tissue with multifocal areas of variably dilated tubules with scattered congested interstitial blood vessels and interglomerular congested capillaries and focal hemorrhage. While in *A.vulgaris* group, Section shows renal tissues with unremarkable histopathological changes of renal tubules and glomeruli apart from scattered dilated congested interstisial blood vessels and mild dilitation of some renal tubules as shown in Fig.4.

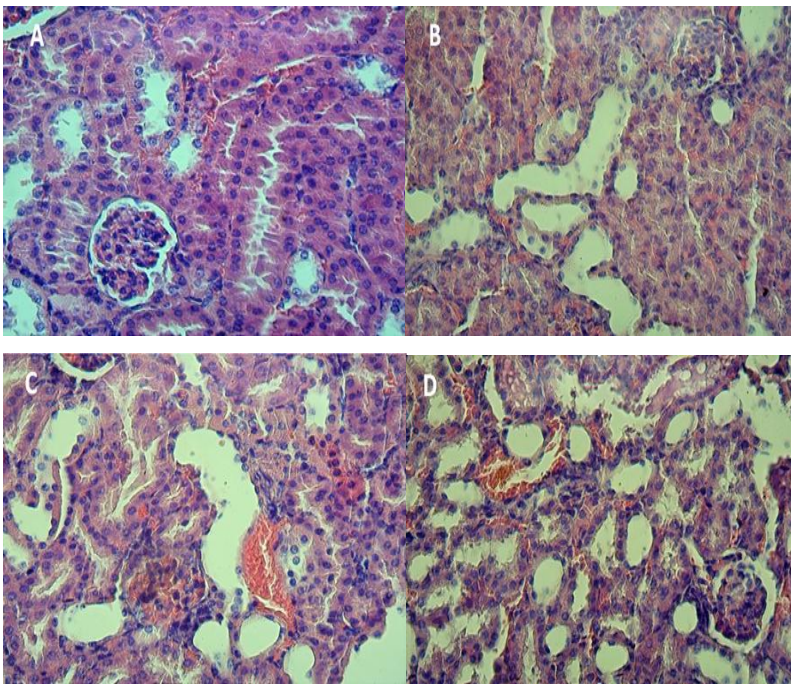

Fig. 4: Histopathological observations of kidney sections in all study groups (A) control group (B) hyperuricemic model group (C) allopurinol treated group (D) *A.vulgaris* treated group.

DISCUSSION

The inadequate management of hyperuricemia by the current drugs and the possible association between hyperuricemia, cardiovascular, and metabolic comorbidities have led to the development of newer agents.[11]

Artemisia vulgaris has been used in traditional medicine worldwide and has many pharmacological effects, so the present study was focused on discovering the capability of flavonoids from *A.vulgaris* to reduce serum uric acid.

In model group, the intraperitonial injection of uric acid lead to significant increase in serum uric acid. GSH level decreased significantly, despite that uric acid act as antioxidant, it

possess an antioxidant – prooxidant urate redox shuttle depending on many factors such as acidity of environment, availability of other antioxidants, supply of oxidant and oxidant enzyme.[26] Also the reaction of uric acid with oxidants can produce new free radicals. In cell culture systems, soluble uric acid has been found to stimulate pro-oxidants.[27]

Allopurinol significantly decreased the serum uric acid level when compared with model group, allopurinol is a well-known inhibitor of XOD .While it did not elicit a significant change in serum GSH level. A study of Haidari et al,[28] states that allopurinol could not significantly change oxidative stress biomarkers although the hypouricemic effect of allopurinol was much higher than that of *Allium cepa* L. and quercetin. The inhibition of XOD by allopurinol generate superoxide through reducing the enzyme and transferring of an electron to oxygen.[29] While there is a significant decrease in body weight which may be due to the side effects of allopurinol on gastrointestinal system , such as nausea and diarrhea.[30]

Flavonoids fraction of *A.vulgaris* significantly decreased the serum uric acid level when compared with model group, this can be explained by the structural similarity of flavonoids with XO substrate and the possible ability to inhibit enzyme activity.[31,32] Therefore the hypouricemic property of flavonoids observed in this study, could be attributed to the inhibitory effects on XOD activity. GSH decreased significantly by *A.vulgaris*, flavonoids have been considered as great antioxidants and could be more effective than known antioxidants like Vitamin C, E and carotenoids.[33] Different mechanisms involved in antioxidant effects of flavonoids such as conserving antioxidant defense ,inhibiting the enzymes involved in generation of free radicals and scavenging reactive oxygen species and reactive nitrogen species.[34]

Concerning histopathological observation neither allopurinol nor *A.vulgaris* could restore the normal renal histology. It is noteworthy that allopurinol exert nephrotoxicity at high doses in rodents, but it is not a serious issue in humans.[30]

CONCLUSION

The results of the current study demonstrate that flavonoids rich fraction of *A.vulgaris* have hypouricemic effect in hyperuricemic mice. Despite that its lowering effect is not efficient as allopurinol. However, the antioxidant role of flavonoids could be beneficial in reducing oxidative stress accompanied with hyperuricemia.

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