

Original Research Article

Kinetics of Inhibition of Xanthine Oxidase by *Lycium arabicum* and its Protective Effect against Oxonate-Induced Hyperuricemia and Renal Dysfunction in Mice

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Abstract

Purpose: To evaluate the in-vitro inhibition of xanthine oxidase (purified from bovine milk) by extracts of *Lycium arabicum*, as well as its in vivo hypouricemic and renal protective effects.

Methods: Four extracts of *Lycium arabicum*, methanol (CrE), chloroform (ChE), ethyl acetate (EaE) and aqueous (AqE) extracts, were screened for their total phenolics and potential inhibitory effects on purified bovine milk xanthine oxidase (XO) activity by measuring the formation of uric acid or superoxide radical. The mode of inhibition was investigated and compared with the standard drugs, allopurinol, quercetin and catechin. To evaluate their hypouricemic effect, the extracts were administered to potassium oxonate-induced hyperuricemic mice at a dose of 50 mg/kg body weight.

Results: The results showed that EaE had the highest content of phenolic compounds and was the most potent inhibitor of uric acid formation ($IC_{50} = 0.017 \pm 0.001$ mg/mL) and formation of superoxide ($IC_{50} = 0.035 \pm 0.001$ mg/ml). Lineweaver-Burk analysis showed that CrE and EaE inhibited XO competitively, whereas the inhibitory activities exerted by ChE and AqE were of a mixed type. Intraperitoneal injection of *L. arabicum* extracts (50 mg/kg) elicited hypouricemic actions in hyperuricemic mice. Hyperuricemic mice presented a serum uric acid concentration of 4.71 ± 0.29 mg/L but this was reduced to 1.78 ± 0.11 mg/L by EaE, which was the most potent hypouricemic extract.

Conclusion: *L. arabicum* fractions have a strong inhibitory effect on xanthine oxidase and also have a significantly lowering effect on serum and liver creatinine and urea levels in hyperuricemic mice.

Keywords: *Lycium arabicum*, Uric acid, Creatinine, Superoxide, Phenolic compounds, Flavonoids, Hyperuricemia

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INTRODUCTION

Xanthine oxidoreductase (XOR) is part of a group of enzymes known as molybdenum iron-sulfur flavin hydroxylases. Animal XORs are homodimers with a molecular mass of about 300 kDa. Each subunit contains a single peptide chain, which binds one molybdopterin cofactor (Mo-pt), two non identical 2Fe–2S centers, and

one FAD cofactor. It exists in two interconvertible forms known as xanthine oxidase (XO) and xanthine dehydrogenase (XDH). XOR is involved in the oxidation of hypoxanthine to xanthine and xanthine to urate [1]. The lack of uricase in humans results in plasma uric acid concentrations that are much higher than in most mammals. When these concentrations exceed the solubility limit of about 7 mg/dL

(hyperuricemia) at physiological pH, uric acid may nucleate to form crystals in tissues and joints [2]. These crystals can induce an acute inflammatory response, leading to the clinical presentation of acute gouty arthritis [3].

Standard management of acute gout consists of urate-lowering therapy. Allopurinol, a xanthine oxidase inhibitor, is the most commonly prescribed of these agents. However, a prolonged use of allopurinol leads to many of side effects e.g. hepatitis, nephropathy, allergic reaction and 6-mercaptopurine toxicity [4].

Therefore, there is urgent need to develop new XO inhibitors from natural sources. *Lycium arabicum* is a medicinal plant that grows wild in the mountains of Oures, Algeria. It belongs to Solanaceae family, used in traditional medicine to treat skin inflammation and rheumatism. The aim of this study was to evaluate the *in vitro* inhibition of xanthine oxidase as well as the *in vivo* hypouricemic effect of *L. arabicum* extracts in mice.

EXPERIMENTAL

Materials

The leaves of *L. arabicum* were obtained from N'Gaous, Batna, Algeria, at the end of March 2012 (between 11.00 - 14.00 h). The plant materials were identified and authenticated by Prof Oudjih Bachir, a taxonomist at Department of Agronomy, Batna University, Batna, Algeria. A voucher specimen (I.A.B./950) was kept in the herbarium of the same department for future reference. Bovine milk was obtained from a local farm Batna, Algeria. All reagents were purchased from Sigma Chemicals, Fluka (Germany) and ProLab (Germany). The standard spectrophotometric diagnostic kits (uric acid, urea and creatinine) purchased from Spinreact (Spain).

Adult male mice (weighting 25 - 30 g) were purchased from the Pasteur Institute of Algeria were kept under standardized conditions (21 - 24 °C and a 12 h light/dark cycle) and fed a normal laboratory diet. Animal experiments were performed in accordance with National Guidelines for the Use and Care of Laboratory Animals [5]. Ethical approval, for the animal experimentation, was obtained from Ethics Review Committee of Faculty of Exact Sciences and Nature and Life Sciences, Biskra University, Biskra, Algeria (ref no. 307/V.D.P.G.). The animals were transferred to the laboratory at least 1 week before the experiments.

Extraction of phenolic compounds

The extractions were carried out according to the method of Markham [6], *Lycium arabicum* powder was soaked in 85 % aqueous-methanol with a ratio of plant material and extracting solvent of 1:10 w/v, under agitation overnight at 4 °C. The extract was filtered on filter paper then on sintered glass to obtain the first filtrate. This procedure was repeated on the residue using 50 % aqueous-methanol under agitation for 4 h to obtain the last filtrate. The first and the last filtrates were combined then the methanol was removed under reduced pressure on a rotavapor (Germany, bÜchi461) below 45 °C. The methanol extract was coded as CrE. A defined portion (30 %) of total CrE volume was lyophilized and stored at -20 °C until its use. CrE was subjected to fractionation using liquid-liquid extraction, it was successively extracted with different solvents of increasing polarity: hexane for defatting, chloroform for aglycone flavonoids extraction and ethyl acetate for glycoside flavonoids extraction. The obtained organic layer of each partition was evaporated under reduced pressure on a rotavapor below 45 °C to dryness and to afford hexane, chloroform, ethyl acetate and aqueous fractions coded as HxE, ChE, EaE and AqE, respectively. All of these fractions were stored at -20 °C prior to use.

Determination of total phenolic content

Total phenolic content was determined using Folin-Ciocalteu reagent [7]. Two hundred microliters of diluted sample were added to 1 mL of 1:10 (v/v) Folin-Ciocalteu reagent. After 4 min, 800 mL of saturated sodium carbonate solution (75 g/L) was added. After 1 h of incubation at room temperature (25 °C), the absorbance at 765 nm was measured. Gallic acid (0 - 160 µg/mL) was used for calibration of standard curve. The results were expressed as microgram gallic acid equivalent (µg GAE)/mg of extract.

Purification of milk xanthine oxidase (XO)

XO was purified from mammalian milk in the presence of 10 mM of dithiothreitol by ammonium sulphate fractionation followed by affinity chromatography on heparin-agarose. XOR concentration was determined by UV-visible spectrum using an absorption coefficient of 36000 M⁻¹ cm⁻¹ at 450 nm. The purity of enzyme was assessed on protein/flavin ratio (PFR = A₂₈₀/A₄₅₀) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (10 %) [8].

Evaluation of inhibitory activity of XO - formation of uric acid

The effect of *L. arabicum* extracts on XO was examined spectrophotometrically at 295 nm by following the production of uric acid using an absorption coefficient of $9600 \text{ M}^{-1} \text{ cm}^{-1}$ [9]. Different concentrations of tested compounds and extracts were added to and their effect on the generation of uric acid was used to calculate regression lines. The reaction was initiated by the addition of enzyme and inhibition was evaluated after 1 min. The absorption increments at 295 nm indicating the formation of uric acid were measured at room temperature. Inhibition of XO was calculated Eq 1.

$$\text{Inhibition (\%)} = \left\{ \frac{\text{Ac} - \text{As}}{\text{Ac}} \right\} 100 \dots\dots\dots (1)$$

where Ac is XO activity without extract and AS is XO activity with extract. The results are expressed as extract concentration that inhibited 50 % of enzyme activity (IC_{50}).

Evaluation of inhibitory activity of XO - formation of superoxide

Effects of *L. arabicum* extracts on the generation of O_2^- were determined using the cytochrome c method [10] which is based on the ability of superoxide produced by xanthine/XO system to reduce cytochrome c. The reaction mixture contained xanthine ($100 \mu\text{M}$) and cytochrome c ($25 \mu\text{M}$) in air-saturated phosphate buffer (50 mM , pH 7.4) supplemented with 0.1 mM EDTA. The reaction was started by the addition of the enzyme. Within 1 min, reduced cytochrome c was calorimetrically determined at 550 nm against enzyme-free mixture using. The cytochrome c activity was calculated using an absorption coefficient of $21100 \text{ M}^{-1} \cdot \text{cm}^{-1}$. The results, expressed as extract concentration that inhibited the reduction of half-amount of cytochrome c (IC_{50}).

Determination of enzyme inhibition mechanism

To determine the mode of inhibition by *L. arabicum* fractions [11], Lineweaver-Burk plot analysis was performed using Sigma Plot 12.0. The assay was carried out in the presence and absence of *L. arabicum* extracts with varying concentrations of xanthine (10, 20, 40, 6, 80, 100, 120 μM) as the substrate, using the XO assay methodology. The mode of inhibition was compared with that of the positive control compounds: allopurinol, quercetin and catechin. The XO used was 0.02 U/mL to allow a more accurate determination of enzyme activity at low

substrate concentrations. The Lineweaver-Burk transformed values were plotted to determine the mode of inhibition.

Animal model of hyperuricemia

The method of Mo *et al* [12], with slight modifications, was used. Potassium oxonate (PO) is a uricase inhibitor used to induce hyperuricemia in mice. 1 h before intraperitoneal injection of *L. arabicum* extracts (50 mg/kg) extracts, the mice were intraperitoneally injected with PO (250 mg/kg). Blood samples were then collected in heparinized tubes 1 h after the administration of *L. arabicum* extracts and allopurinol (10 mg/kg). Two control groups were used: normal group (without PO treatment) and PO groups (treated with PO only). The blood was centrifuged at 3500 rpm for 5 min to obtain the serum. The serum was stored at $-20 \text{ }^\circ\text{C}$ until assayed. Livers were surgically excised from the mice that had been sacrificed by decapitation. The excised liver was rinsed in a saline buffer and was homogenized in an ice-cold 50 mM potassium phosphate buffer, pH 7.8. The homogenate was centrifuged at 1500 g for 10 min at $4 \text{ }^\circ\text{C}$ and the pellet was discarded. The supernatant fraction was used to measure uric acid concentration.

Assessment of renal function

Blood and liver samples were prepared in the same way as for the uric acid measurements. For renal function assessment, urea and creatinine levels were measured using a semi-automatic Biochemistry Analyzer (Bechman) with standard spectrophotometric diagnostic kits purchased from Spinreact (Spain).

Statistical analysis

The results are expressed as mean \pm SD or SEM. Where applicable, the data were subjected to one-way analysis of variance (ANOVA), where the differences between extracts were determined by Tukey's multiple comparison test, Dunnett's and multiple comparison test for comparison between extracts and standards, using GraphPad program. $P \leq 0.05$ was regarded as significant.

RESULTS

Total phenolic content

The content of polyphenols in the extracts was in the following rank order: EaE > CrE > ChE > AqE (Table 1).

Table 1: Total polyphenols content of *L. arabicum* extracts

Extract	Total phenols ($\mu\text{g AGE/mg}$)
CrE	276.16 \pm 3.71
ChE	234.86 \pm 0.81
EaE	278.55 \pm 2.02
AqE	163.37 \pm 4.96

Values are mean \pm SD (n = 3)

Milk xanthine oxidase (XO)

The results of the freshly purified milk XOR from different species showed an ultraviolet/visible spectrum with three major peaks (280, 325, 450 nm) with A_{280}/A_{450} (protein to flavin ratio, PFR) of 5.15 indicating a high degree of purity, the specific activity of the purified enzyme was of 2055.05 nmol/min/mg protein. Run on SDS-PAGE, purified enzyme showed quite similar patterns with one major band of approximately 150 KDa.

Inhibitory activity of XO

All fractions of *L. arabicum* elicited a dose-dependent inhibition of XO enzyme activity. The IC_{50} results showed that the highest activity was observed with the EaE (17.92 \pm 0.16 $\mu\text{g/mL}$) followed by ChE (39.11 \pm 0.28 $\mu\text{g/mL}$), AqE (63.77 \pm 0.16 $\mu\text{g/mL}$) and CrE (85.38 \pm 6.36 $\mu\text{g/mL}$) (Figure 1). The standard drug, allopurinol, had an IC_{50} of 8.57 \pm 0.30 $\mu\text{g/mL}$.

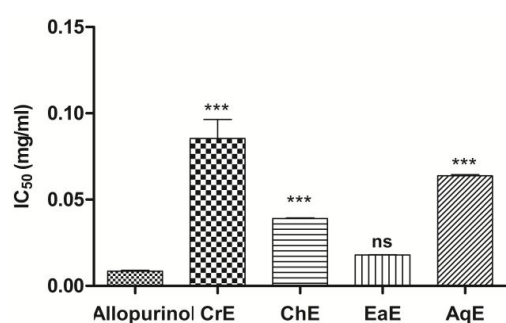


Figure 1: Inhibition of uric acid production by *L. arabicum* extracts. Values are mean \pm SD (n = 3); *** p \leq 0.001, ns = not significance, compared to allopurinol

The results shown in Figure 2 indicate that EaE presents the most potent scavenging effect of $O_2^{\cdot-}$ with an IC_{50} of 0.035 \pm 0.001 mg/mL, which is approximately 2-fold higher than that of AqE (0.062 \pm 0.001 mg/mL). On the other hand, ChE and CrE presented approximately the same

scavenger activity (0.240 \pm 0.008 and 0.282 \pm 0.012 mg/mL, respectively).

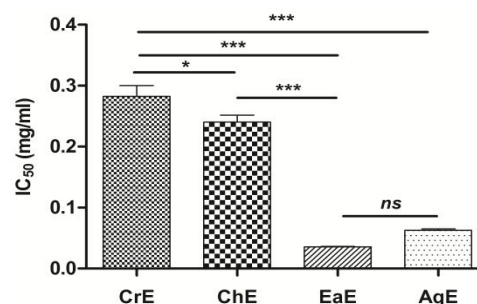


Figure 2: Effect of *L. arabicum* on the formation of $O_2^{\cdot-}$. Values are mean \pm SD (n = 3); * p \leq 0.05, *** p \leq 0.001, and ns = not significance

Enzyme inhibition mechanism

The data indicates that the EaE (18 $\mu\text{g/mL}$), which is a potent inhibitor of xanthine oxidase, showed a competitive inhibition pattern (Figure 3), as did the CrE (93 $\mu\text{g/mL}$). The same type of inhibition was found in the case allopurinol (8 $\mu\text{g/mL}$) (Figure 4).

However, ChE and AqE showed mixed-type of inhibition (Figure 5). Mixed inhibition was similarly observed for quercetin (50 $\mu\text{g/mL}$). Our results showed that glycosidic flavonoids (EaE) of *L. arabicum* present the highest inhibition of XO and in a competitive type and the non-glycosidic flavonoids (ChE) showed a mixed inhibition.

The results confirmed that catechin and quercetin showed a noncompetitive and mixed inhibition, respectively, at a dose of 50 $\mu\text{g/mL}$ (Figure 6).

In vivo hypouricemic effect of L. arabicum extracts

Mice treated with 250 mg/kg potassium oxonate exhibited a significant elevation of serum uric acid levels in comparison to normal-vehicle group (Table 2). The administration of *L. arabicum* extracts significantly decreased serum and liver uric acid levels. However, AqE present a strong *in vitro* XO inhibitory effect but not a strong hypouricemic activity.

The results showed that the administration of PO to the increase levels of urea and creatinine in serum and liver (Table 3). The results showed that the administration of *L. arabicum* extracts and allopurinol decrease urea and creatinine levels.

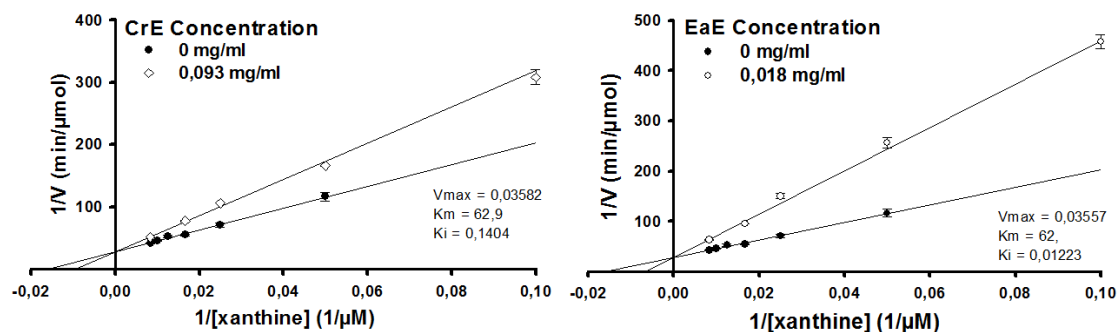


Figure 3: Lineweaver-Burk plot of inhibition of XO by CrE and EaE of *L. arabicum*. Values are mean \pm SD (n = 3)

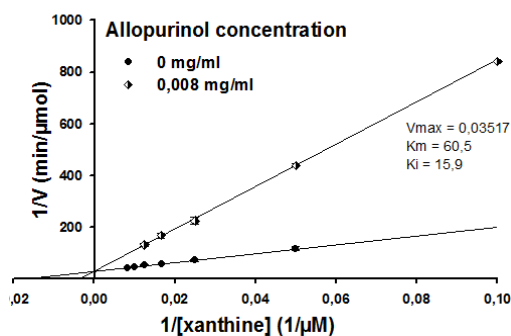


Figure 4: Lineweaver-Burk plot of inhibition of XO by allopurinol. Values are mean \pm SD (n = 3)

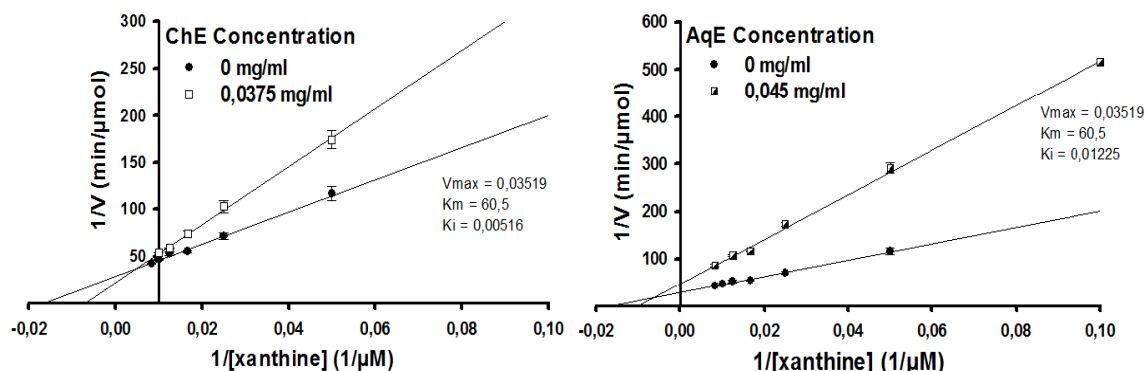


Figure 5: Lineweaver-Burk plot of mixed inhibition of XO by ChE and AqE. Values were expressed as mean \pm SD (n = 3)

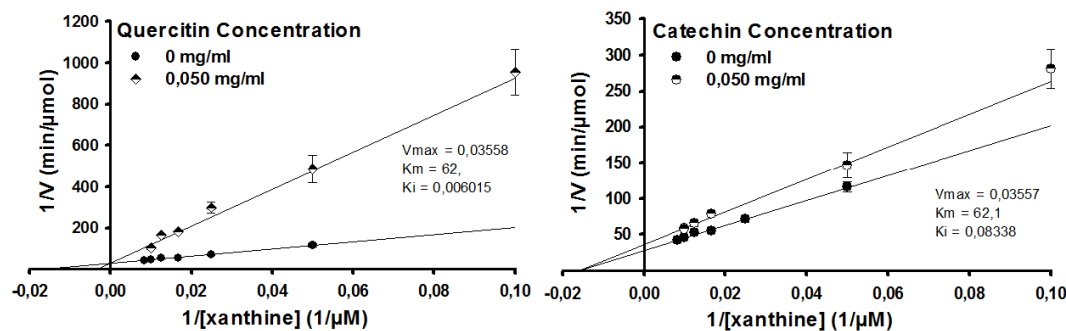


Figure 6: Lineweaver-Burk plot of inhibition of XO by quercetin and catechin. Values are mean \pm SD (n = 3)

Table 2: Serum and liver uric acid levels (mg/L) in normal hyperuricemic mice and treated with allopurinol and extracts

Group	Serum	Liver
Normal	1.55±0.12	4.18±0.21
PO	4.71±0.29	10.35±0.83
PO+allopurinol	1.89±0.10***	1.54±0.13***
PO+CrE	1.92±0.09***	4.16±0.39***
PO+ChE	1.88±0.16***	2.70±0.20***
PO+EaE	1.78±0.11***	1.74±0.18***
PO+AqE	3.24±0.21***	5.88±0.38***

Normal; Non treated group, PO; hyperuricemic mice, PO+allopurinol; hyperuricemic mice treated with allopurinol (10 mg/kg), PO+CrE; hyperuricemic mice treated with CrE (50 mg/kg), PO+ChE; hyperuricemic mice treated with ChE (50 mg/kg), PO+EaE; hyperuricemic mice treated with EaE (50 mg/kg) and PO+AqE; hyperuricemic mice treated with AqE (50 mg/kg). Values are mean ± SEM (n = 10), compared with PO; ***:p ≤ 0.001

Table 3: Urea (g/L) and creatinine (mg/L) levels in normal and hyperuricemic mice treated with allopurinol and extracts

Group	Serum		Liver	
	Urea	Creatinine	Urea	Creatinine
Normal	0.012±0.002	1.000±0.002	0.100±0.012	1.571±0.202
PO	0.070±0.004	3.570±0.012	0.178±0.002	4.375±0.263
PO+Allopurinol	0.014±0.002***	1.571±0.202***	0.138±0.014 ^{ns}	2.800±0.200***
PO+CrE	0.044±0.002***	1.800±0.200***	0.133±0.011 ^{ns}	3.000±0.189***
PO+ChE	0.017±0.001***	1.014±0.014***	0.104±0.011*	3.286±0.285**
PO+EaE	0.016±0.002***	1.500±0.223***	0.117±0.015*	2.714±0.184***
PO+AqE	0.057±0.002*	2.333±0.210***	0.122±0.020 ^{ns}	2.571±0.202***

Values were expressed as mean ± SEM (n = 10), compared with hyperuricemic group (PO); *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ns = non-significant

DISCUSSION

Phenolic compounds including phenolic acids and flavonoids are known to possess biological and pharmacological activities, including anti-oxidative, anti-bacterial, anti-viral and anti-mutagenic activities. Flavonoids are also known to be potent inhibitors of several enzymes, including xanthine oxidase, cyclooxygenase, 5-lipoxygenase and phosphoinositide 3-kinase [13].

The most potent XO inhibitor observed was EaE which was 2-folds lower than allopurinol, its activity could be explained by its high polyphenolic content. However, the IC₅₀ of ECh is lower than that of CrE, the total polyphenols contents of CrE was found higher than that of ChE. Our results showed that the XO inhibition could be linked not only on the polyphenols and flavonoids contents of the extract, but also to the nature (structure) of these compounds.

The scavenging effect of free radicals by various constituents of plants may be due to phenolic acids and flavonoids [9]. The different extracts of *L. arabicum* inhibitory effects on XO, which makes it difficult to know, in the test of Cyt-c, if these extracts have a scavenger effect on O₂^{•-} or an inhibitory effect on the enzyme. The IC₅₀ of

EaE, CrE and ChE for the production of uric acid from the oxidation of xanthine by XO is nearly 2, 3 and 6 times lower than that inhibiting the reduction of Cyt c, respectively. The AqE present nearly the same IC₅₀.

Both allopurinol and oxypurinol, (isosteres of hypoxanthine and xanthine, respectively), inhibit xanthine oxidase, thereby limiting the biosynthesis of uric acid and decreasing its serum level and urine excretion and promoting the renal clearance of hypoxanthine and xanthine. Allopurinol, a competitive inhibitor, is a substrate for xanthine oxidase, which strongly binds at the active site, with concomitant reduction of the molybdenum site. The binding of oxypurinol to the reduced molybdenum site of the enzyme has been shown to be reversible and released after re-oxidation of the enzyme. Although the inhibitor binds very tightly to the enzyme, the inhibition is time-dependent and, therefore, it takes some time to inhibit the enzyme completely [14].

Tea catechins have been shown to exhibit moderate XO inhibition at low concentrations [15], and to inhibit XO non-competitively [16]. Takahama *et al* (2011) reported that the quercetin-dependent inhibition of XO activity was a mixed-type namely, competitive/non-

competitive inhibition [17]. Our results confirm these findings, where catechin and quercetin showed a noncompetitive and mixed inhibition, respectively, at a dose of 50 µg/mL.

Potassium oxonate, a selective uricase inhibitor, blocks the effect of hepatic uricase and produces hyper-uricemia [12]. The AqE exhibited a strong *in vitro* XO inhibitory effect but not a strong hypouricemic activity. It is possible that differences in the structure, absorption and metabolism of various tested extracts may lead to the formation of metabolites with different XO inhibitory activities. Renal failure is the cessation of renal function and it can be acute or chronic. In acute renal failure there is rapid loss of renal function within hours or days, although the condition is potentially reversible and normal renal function can be regained. The deterioration is sudden, with increases in the concentrations of urea and creatinine [18].

Oxonate-treated mice consistently produced increased plasma creatinine and urea levels which indicates renal dysfunction [19]. Studies showed that allopurinol therapy significantly decreases serum uric acid levels in hyperuricemic patients with mild to moderate chronic kidney disease. Its use is safe and helps preserve kidney function during 12 months of therapy [20]. Which explain the normal level of creatinine and urea in hyperuricemic mice treated with allopurinol (10 mg/Kg). *L. arabicum* extracts decrease significantly urea and creatinine serum levels, EaE, the best hypouricemic extract, presented a protective effect higher than that of allopurinol. However, AqE did not show a strong hypouricemic activity but it has a strong protective effect by decreasing of urea and creatinine levels.

CONCLUSION

L. arabicum extracts have significant inhibition effect on XO activity *in vitro* as well as a significant hypouricemic effect in hyperuricemic mice. Inconsistencies between the *in vitro* and *in vivo* data may be due to differences in the bioavailability of extracts and their extensive metabolism in mice. These results suggest that *L. arabicum* may be helpful in preventing or slowing the progress of several diseases that are related to XO.

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