Kinetics of Inhibition of Xanthine Oxidase by Globularia alypum and its Protective Effect against Oxonate-Induced Hyperuricemia and Renal Dysfunction in Mice

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ARTICLE INFO

ABSTRACT

In the present study, *Globularia alypum* (GA) was extracted with solvent of varying polarity allowed its separation into four subtractions: crude extract (CrE) chloroform extract (ChE), ethyl acetate extract (AcE) and aqueous extracts (AqE). The results showed that AcE had the highest content of phenolic compounds. The inhibitory activity of the extracts on xanthine oxidase (XO) was evaluated, the results obtained showed that the inhibition is dose-dependent and the AcE had a very significant inhibitory effect (0.069 ± 0.003 mg/ml) followed by CrE and AqE (0.081 ± 0.000 et 0.088 ± 0.002 mg/ml, respectively). The CrE, ChE and AqE inhibit competitively XO, whereas AcE is a non-competitive inhibitor. Hyperuricemia was induced by intraperitoneally injection of potassium oxonate, the uric acid, urea and creatinine were measured in serum and liver supernatant. The ChE, AcE, and AqE extracts had reduced significantly the plasma and liver uric acid levels (decreased 44.76%, 43.03% and 31.31%, respectively). Comparing these results with those obtained *in vitro*, the ChE extract with the lowest inhibitory effect *in vitro* ($IC_{50} = 0,123$ mg / ml) was the most effective extract *in vivo*. In summary, there was a contradiction between inhibition *in vitro* and *in vivo*, this inconsistency or difference may be due to the difference in the bioavailability of flavonoids or natural substances and their extensive metabolism in mice.

INTRODUCTION

Hyperuricemia is the most cited pathology involving XO. It is a pathological state that arises from overproduction (by XO) or under excretion (renal tubule disorders) of uric acid. As a result of hyperuricemia, insoluble uric acid forms microscopic crystals in the capillary vessels of joints. These crystals cause inflammation and sharp pain, which is termed acute gouty arthritis or acute gout (Sachs *et al.,* 2009). Xanthine oxidase (XO) catalyses the oxidation of hypoxanthine and xanthine to uric acid. Therefore, XO inhibitors have been proposed as potential therapeutic agents for treating hyperuricemia as they could be used to block the biosynthesis of uric acid (Unno *et al.,*

2004; Newcombe, 2013). Allopurinol is the most commonly used xanthine oxidase inhibitor prescribed clinically for the treatment of gout (Hille, 2010; Chen *et al.,* 2011). However, the use of this agent is limited because of its undesirable effects (Bomalaski and Clark, 2004; Yu *et al.,* 2006). Therefore, there is an urgent need to develop new XO inhibitors from natural sources.

The use of natural products isolated from medicinal plants represented a good source of novel and clinically important drugs in connection with the treatment of some kinds of clinical disorders as hyperuricemia and gout. A potential source of such compounds can be obtained from medicinal plants. Flavonoids and polyphenolic have been reported to possess xanthine oxidase inhibitory activity (Lin *et al.,* 2002). As part of our efforts to find antihyperuricemic agents from herbs*: Globularia alypum* belongs to Globulariaceae commonly known as 'Tasalgha, It is widely distributed in the Mediterranean region.

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It is one of the most traditional plant remedies. The plant is known for its uses in the indigenous system of medicine for a variety of purposes such as hypoglycaemic agent, laxative, cholagogue, stomachic, purgative, sudorific and also in the treatment of cardio-vascular and renal diseases (Fehri and Aiache, 2010; Balansard and Delphant, 1948), rhumatism, gout, typhoid, intermittent fever and diabetes (Skim *et al.,* 1999; Jouad *et al.,* 2002).

The aim of this study was to evaluate the *in vitro* inhibition of xanthine oxidase as well as the *in vivo* hypourecimic effect of GA extracts in mice.

MATERIALS AND METHODS

Materials

Globulaia alypum was collected from SETIF, Algeria in May 2010. All other reagents were purchased from Sigma Chemicals, Fluka (Germany) and Prolab (France).

Extraction procedure

The extractions were carried out using various polar and non-polar solvents. According to the method Markham (Markham, 1982), the powdered plant material (100 g) was extracted with methanol, at room temperature for overnight. The MeOH extracts were combined and concentrated under reduced pressure on a rotary evaporator. MeOH extract (CrE) successively extracted with hexane, chloroform and ethyl acetate. Each fraction was evaporated at a temperature of 40°C to dryness under reduced pressure to give hexane (HE), chloroform (ChE), ethyl acetate (AcE), and the remaining aqueous (AqE) extracts.

Determination of total flavonoid contents

The total flavonoid content of each extract was determined by a colorimetric method as described by Bahorun *et al* (1996). Each sample (1 ml) was mixed with 1ml of aluminum chloride ($AlCl_3$) solution (2%) and allowed to stand for 15 min. Absorbance of the mixture was then determined at 430 nm versus prepared methanol blank. Results were expressed as equivalent quercetin and rutin (mg quercetin or rutin /g dried extract).

Determination of Total polyphenols

Total phenolic content was determined by the Folin-Ciocalteu method (Li *et al.,* 2007). 200 µl of diluted sample were added to 1 ml of 1:10 diluted Folin-Ciocalteu reagent. After 4 min, 800 ml of saturated sodium carbonate solution (75 g/l) was added. After incubation for 1h at room temperature, the absorbance at 765 nm was measured in triplicate. Gallic acid (0-160 µg/ml) was used for calibration of standard curve. The results were expressed as microgram gallic acid equivalent (mg GAE) / mg of extract.

Effect of extracts on XO

Bacterial xanthine oxidase was puchased from sigma, the effect of extracts on xanthine oxidation was examined spectrophotometrically at 295 nm (UV-visible Technicomp 8500)

following the production of uric acid using an absorption coefficient of 9600 $M^{-1}cm^{-1}$. Different concentrations of tested compounds were added to samples before the enzyme had been added and their effect on the generation of uric acid was used to calculate regression lines and IC_{50} values. The reaction was started by the addition of XO.

The enzyme activity of the control sample was set to 100% activity (Boumerfeg *et al.,* 2009). The type of inhibition of different extracts was determined by the Lineweaver-Burk plot using varying concentrations of xanthine.

Hypouricemic effects of extracts on potassium oxonate-induced hyperuricemia in mice

Experiments were performed on adult male mice from the Pasteur Institute of Algeria weighing 25-30 g. They were kept under standardized conditions (temperature 21-24 °C and a light/dark cycle of 12 hours/12 hours) and fed a normal laboratory diet. After 1 week of acclimatization, mice's were divided into groups of 9-10 animals. Induction of hyperuricemia is made by injection intraperitoneally of 200 mg / kg of potassium oxonate, which is an uricase inhibitor (Osada *et al.,* 1993). After 1 hour the extracts were administered intraperitoneally (100 mg / kg). The mice are divided into 7 groups:

- Group 1 (Control): untreated or injected with the vehicle (normal saline), normal group.
- Group 2 (PO): injected with potassium oxonate.
- Group 3 (PO + allopurinol): the group injected with potassium oxonate then treated with 10mg/kg of allopurinol.
- Group 4 (PO + CrE): injected with potassium oxonate then treated with 100 mg / kg of CrE.
- Group 5 (PO + ChE): injected with potassium oxonate then treated with 100 mg / kg on of ChE.
- Group 6 (PO + AcE): injected with potassium oxonate then treated with 100 mg / kg of AcE.
- Group 7 (PO + AqE): injected with potassium oxonate then treated with 100 mg / kg of AqE.

Whole blood samples were collected from anesthetized mice and centrifuged at 1500 g for 5 minutes. The serum obtained was stored at -20 °C until use. Livers from each group were collected, rinsed with saline and homogenized in a phosphate buffer (50 mM, pH 7.8) and centrifuged at 8000 g for 15 min at 4°C. The supernatant is used for the determination of uric acid (Shi-Fu *et al.,* 2007; Huang *et al.,* 2011). The concentration of uric acid in the serum and supernatant of the liver is determined by an enzymatic method.

To evaluate the effect of the extracts on renal function, the levels of urea and creatinine was determined. These assays were carried out at the Central Laboratory of the University, Hospital of SETIF using a Bechman device and kits purchased from Cypress Diagnostics.

Statistical analysis

All experiments were done in triplicate and results were reported as mean ± SD. Data were analyzed by one way ANOVA. Statistically significant effects were further analyzed and means were compared using Tukey test. Statistical significance was determined at p <0.05.

RESULTS AND DISCUSSION

Total phenolic and flavonoid content

In order to characterize the different extracts, a total dosage of flavonoids and polyphenols is performed. The assay method of total polyphenols is the Folin-Ciocalteau. Gallic acid was used as standard and total polyphenol content is expressed as micrograms of gallic acid equivalents per milligram of extract (EAG µg/mg extract).

The dosage of flavonoids is formed according to the method of aluminum trichloride using as a standard the quercetin. The flavonoid content is expressed as micrograms of quercetin equivalents per milligram of extract (EQ µg / mg extract). The results showed that the extract (AcE) is the richest in polyphenols and flavonoids followed by CrE and AqE, whereas ChE is the poorest (Table 1).

Table 1: Determination of total polyphenols and flavonoids in the extract of GA.

Extracts	Polyphenols [a]	Flavonoïds [b]
CrE	140.24 ± 4.18	2.64 ± 0.08
ChE	81.01 ± 1.91	1.12 ± 0.15
AcE	157.74 ± 5.27	8.56 ± 0.22
AqE	100.86 ± 0.76	2.20 ± 0.12

[b] µg of gallic acid equivalent per mg of extract
[a] µg of quercetin equivalent per mg extract.
Values represent the average of 3 to 4 measurements ± SD.

Effect of extracts on XO

The inhibitory activity of the extracts on the XO was evaluated spectrophotometrically by following the decrease of the production of uric acid at 295 nm (XO activity). The results obtained showed that the inhibition is dose-dependent (Figure.1).

The AcE has a very significant inhibitory effect (IC_{50} = 0.069 ± 0.003 mg/ml) followed by CrE and AqE (0.081 ± 0.000 et 0.088 ± 0.002 mg/ml, respectively). IC_{50} values are 34, 40 and 44 times higher comparing to allopurinol which is a specific inhibitor of XO, respectively for AcE, CrE and AqE (Table 2).

A significant correlation (R^2 = 0.631) was observed between the polyphenol content and their inhibitory effect. The most important inhibitory effect EAc extract on the XO can be explained by the presence of relatively high in polyphenols and flavonoids rate.

To determine the relationship between the chemical structures of flavonoids and their inhibitory activity, we determined the type of inhibition for each extract. The results are shown in Table 2 and Figure 2. The CrE, ChE and AqE inhibit competitively XO, The same type of inhibition was found in the case allopurinol and quercetin (Figure 3). Whereas AcE is a non-competitive inhibitor. Inhibition of XO activity by the extracts resulted in the presence of one or more compounds acting independently or synergistically on the active sites of the enzyme.
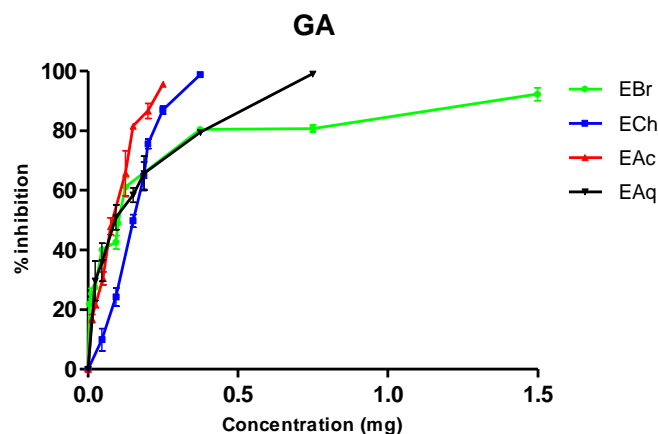

Fig. 1: Inhibition of XO by the extracts of *GA*.

Table 2: The determination of IC_{50} and the type of inhibition of extracts of *GA*.

Extraits	IC_{50} (mg/ml)	Type of inhibition
CrE	0.088 ± 0.000[***]	compétitif
ChE	0.123 ± 0.004[***]	compétitif
AcE	0.069 ± 0.003[***]	Non compétitif
AqE	0.081 ± 0.002[***]	compétitif
Allopurinol	0,002±0,00002	competitive
Quercetin	Nd	competitive
Catechin	Nd	uncompetitive

Values are an average of 3 to 4 trials ± SD. Comparison was realized against allopurinol. ***: $p \leq 0,001$, and ns: not significant.

Berboucha *et al* (2010) tested the XO inhibitory activity of a mixture of quercetin and catechin to determine the effect of simultaneous interaction of two potential inhibitors. The results showed that the type of inhibition is mixed, which means that the type of inhibition can be modified by the presence of other molecules present in the extract of plants by non-specific interactions.

Cos *et al.* (1998) measured the activity of thirty flavonoids on uric acid production by XO. They have thus determined the relationship between the chemical structure of flavonoids and their inhibitory activity of this enzyme. The presence of the double bond between C2 and C3 carbons of the ring A flavonoid is very important for inhibition. This double bond and the conjugation it induces, causes the coplanarity of the ring B with the rings A and C.

A flat structure is in fact necessary for high inhibitory activity against the XO (Van Hoorn *et al.*, 2002). Finally, to get an overall view of the XO inhibitory activity of the extracts, an *in vivo* study is desirable on hyperuricemic models, starting from the fact that a substance can be active in vitro, can lose activity once penetrated into the body and vice versa.
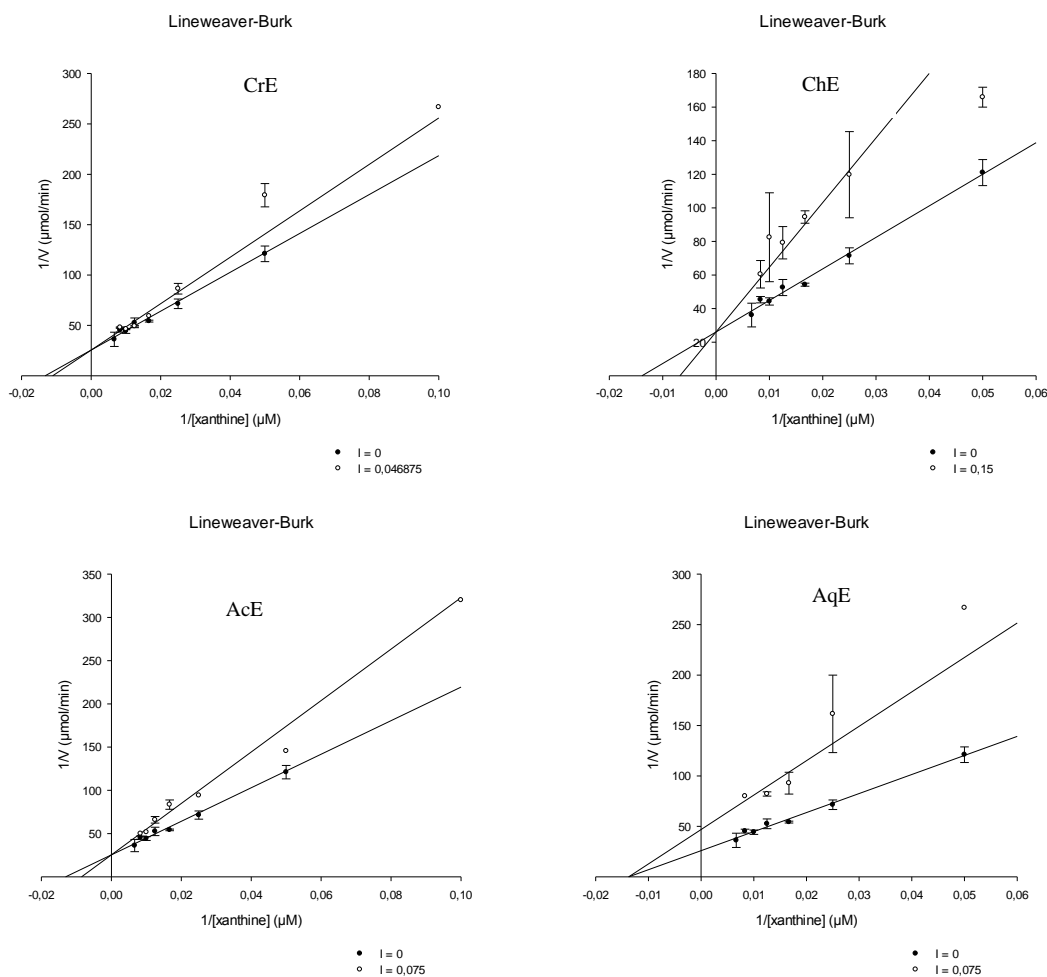
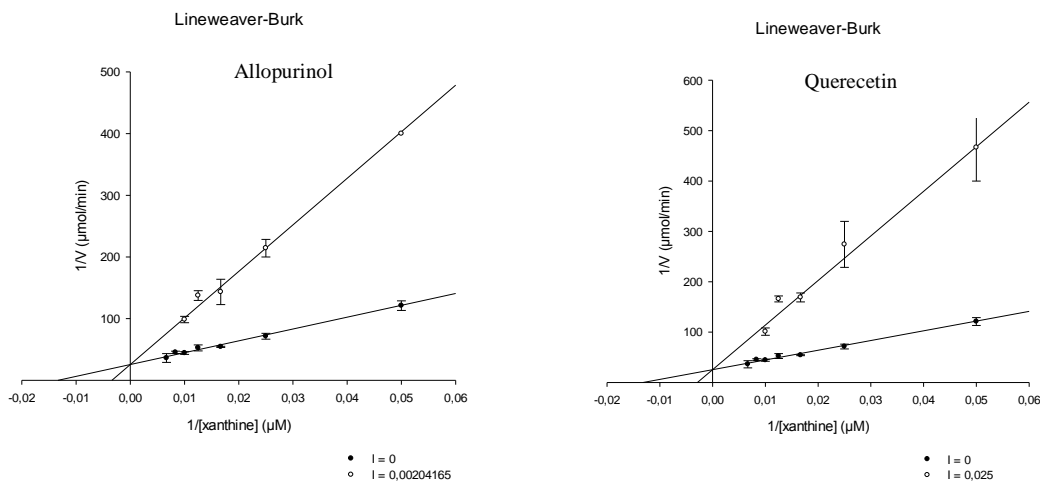Fig. 2: Lineweaver-Burk plot of inhibition of XO by extracts of GA. Values were expressed as mean ± SD (n = 3

Fig. 3: Lineweaver-Burk plot of inhibition of XO by quercetin and allopurinol. Values are mean ± SD (n = 3).

Hypouricemic effects of extracts on potassium oxonate-induced hyperuricemia in mice

Hyperuricemia is induced by intraperitoneally injection of potassium oxonate which is a competitive inhibitor of liver uricase, it partially blocks the conversion of uric acid to allantoin and therefore it increase the levels of uric acid in the mice serum giving an animal model hyperuremic (Osada *et al.*, 1993; Huang *et al.*, 2011). The uric acid, urea and creatinine were measured in serum and supernatant of liver.

We tried to verify the inhibitory effect of extracts of GA on the XO *in vivo* by measuring the uric acid level in serum and liver supernatant to confirm the *in vitro* inhibitory effect of the extracts and thus try to compare between the inhibitory effect *in vitro* and *in vivo*.
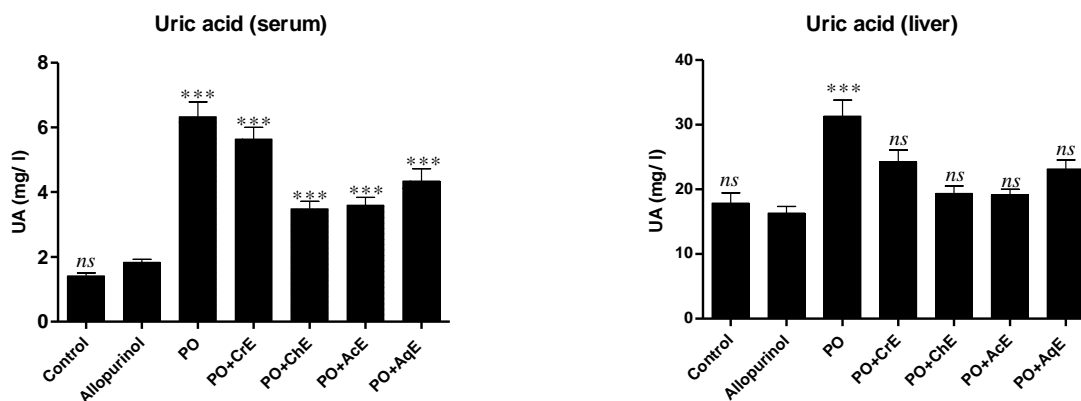
Uric acid (serum)

Uric acid (liver)

Fig. 4: Hypouricemic effects of extracts of GA. Comparison against the Allopurinol group, *** p ≤ 0,001, ns: not significant.

Urea (serum)

Creatinine (serum)

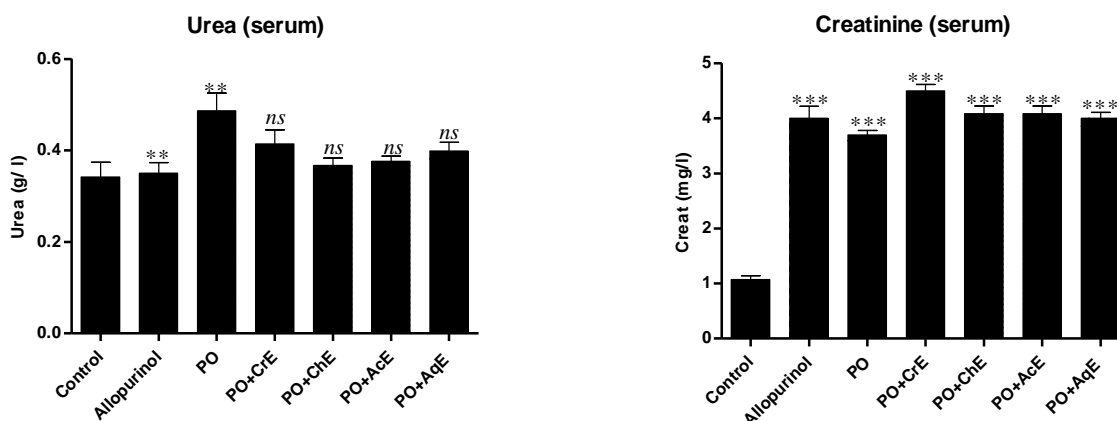**Fig. 5**: Renal function evaluation by the determination of urea and creatinine. Comparison against the control group, *** p ≤ 0.001, ns: not significant.

The value of uric acid in the control group: 1.48 ± 0.07 mg/l, for the "OP" group, the uric acid level is significantly increased: 6.33 ± 0.46 mg/l two hours after the intraperitoneal injection of potassium oxonate indicating that it has successfully induced hyperuricemia (Figure 4).

The ChE, AcE, and AqE extracts have reduced significantly the plasma and liver uric acid levels (decreased 44.76%, 43.03% and 31.31%, respectively). The ChE and AcE caused a very remarkable effect comparing to the positive control "allopurinol" especially the liver uric acid (allopurinol = 16.36 mg/l, ChE = 19.4 mg/l and AcE= 19.1 mg /l). Comparing these results with those obtained *in vitro*, the ChE extract with the lowest inhibitory effect *in vitro* (IC_{50} = 0.123 mg/ml) is the most effective effect *in vivo*. CrE reduced the uric acid levels but this reduction remains not significant (5.64 ± 0.36 mg/l) compared to allopurinol group (1.89 ± 0.1 mg/l). In contrast to *in vitro* results, the CrE has a remarkable inhibitory activity (IC_{50} = 0.088 mg/ ml). In summary, there is a big contradiction between inhibition *in vitro* and *in vivo*, this inconsistency or difference may be due to the difference in the bioavailability of flavonoids or natural substances and their extensive metabolism in mice (Huang *et al*., 2011).

Renal fonction evaluation

The objectives of this study is to evaluate the effect of the extracts on renal function. The rate of urea and creatinine

levels can be indicators for the assessment of renal function. Kidney injury may be accompanied by an increase of creatinine and urea.

In hyperuricemic mice, Allopurinol (10 mg / kg) increased the rate of creatinine and the urea level comparing to normal control mice (Figure 5). Our results are in agreement with the results obtained by Li and al (2011), they showed that the induction of the hyperuricemia by potassium oxonate caused renal dysfunction and thus it increased the rate of uric acid and creatinine . All extracts and allopurinol did not decrease the rate of creatinine (Figure 5). However, the level of urea decreased compared to normal control mice. By comparing to the "PO" group (0.48 g /l), only the AcE and ChE presented a protective effect by decreasing of urea levels.

CONCLUSION

The test for inhibition of XO activities revealed that the AcE is the most powerful extract. The CrE, ChE and AqE exhibited and showed a competitive type of inhibition. Inhibition of XO activity by the extracts has resulted in the presence of one or more compounds acting independently or synergistically on the active sites of the enzyme. The *in vitro* inhibitory effect of this plant on the XO is confirmed *in vivo* on an animal model hyperurecimic. The extracts ChE, AcE and AqE caused a

significant decrease in plasma uric acid. The hypouricemic effect could be due to inhibition of XO by these extracts. Indeed, comparison of the inhibition of XO *in vivo* and *in vitro*, according to a "contradiction"; the extracts are not effective *in vitro*, are highly effective in vivo, and vice versa. These results remain preliminary, it would be interesting to test the activity of fractions and molecules that underlie the various activities detected in the different extracts. In addition, extensive further studies concerning the identification of phenolic compounds by more efficient methods are needed and a toxicological study is very requested.

ACKNOWLEDGMENTS

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