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Original Article

INHIBITORY EFFECTS OF *ANCHUSA AZUREA* EXTRACTS ON XANTHINE OXIDASE ACTIVITY AND ITS HYPOURICEMIC EFFECTS ON MICE

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ABSTRACT

Objective: The aim of the present study was to determine the effects of the polyphenols and flavonoids from *Anchusa azurea* on XO activities *in vitro* and on serum and liver uric acid levels in normal and potassium oxonate-induced hyper uricemic mice. In addition, the renal function of the mice after flavonoid administration was estimated by the determination of blood urea and creatinine analysis.

Methods: In the present study, *Anchusa azurea* were extracted with solvent of varying polarity allowed its separation into four subfractions: crude extract (Cr) chloroform extract (ChE), ethyl acetate extract (AcE), and aqueous extracts (AqE). Total polyphenol and flavonoids contents of *Anchusa azurea* extracts were determined. The inhibitory activity of the extracts on the XO was evaluated and the type of inhibition was determined. Hyperuricemia is induced by intraperitoneally injection of potassium oxonate, the uric acid, urea and creatinine were measured in serum and supernatant of the liver. The effect of the extracts on renal function was evaluated. The rate of urea and creatinine levels can be indicators for the assessment of renal function.

Results: AcE were the richest in polyphenols and ChE was the richest fraction in flavonoids. The inhibitory activity of the extracts on the XO was evaluated, the results obtained showed that the inhibition is dose-dependent and ChE and AcE have the best inhibitory effect (IC_{50}= 0.334±0.006 and 0.263±0.002 mg/ml, respectively), and both showed a noncompetitive type of inhibition. For antihyperuricemic effect, AqE and CrE caused a decrease in serum uric acid (a decrease of 66%) followed by ChE with a percentage of 29.22 %. The AcE keeps almost the same value of uric acid of "PO" group. For the supernatant, only CrE caused a significant decrease of liver uric acid (18.5±4.83 mg/l). This decrease can be explained by the significant inhibition of the XO by inhibition of the synthesis pathways of uric acid. Comparing the urea level of "OP" group (0.48 g/l), only extracts CrE-AA, AqE-AA (0.41g/l, 0.39 g/l) decreased the level of urea significantly ($P \leq 0.05$) to the normal values of urea (0.34 g/l), we can conclude that the rate of urea and creatinine after treatment with plant extracts are normal and that the results of this study indicate the absence of renal damage in mice

Conclusion: *Anchusa azurea* fractions have a strong inhibitory effect on xanthine oxidase and also have a significant lowering effect on serum and liver creatinine and urea levels in hyper uricemic mice.

Keywords: *Anchusa azurea*, Polyphenols, Flavonoids, Hyperuricemia, XO.

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 [1]. 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 [2, 3]. Allopurinol is the most commonly used xanthine oxidase inhibitor prescribed clinically for the treatment of gout [4, 5]. However, the use of these agents is limited because of its undesirable effects [6, 7]. The search for better agents anti hyperuricemic is highly requested, this study represents an effort to find a new molecule hypouricemic.

The use of natural products isolated from medicinal plants represents a good source of novel and clinically important drugs in connection with the treatment of some kinds of clinical disorders as hyperuricemia and gout. Flavonoids are widely found in plants and are known to possess biological and pharmacological activities, including anti-oxidative, anti-bacterial, anti-viral and anti-cancer activities. Flavonoids are also known to be potent inhibitors of several enzymes, including xanthine oxidase, cyclooxygenase and lipooxigenase [8].

As part of our effort to find antihyperuricemic agents from herbs: *Anchusa azurea* is used which belongs to the Borraginacea commonly known as 'Hamham', It is widely distributed in the Mediterranean region. *Anchusa azurea* is one of the medicinal plants largely used in Algeria. It shows various healthcare properties, especially antitussives, diuretic, anti inflammatory and as a cataplasm. Moreover, *Anchusa azurea* is used as a pomade for external use to treat wounds and burnings. The aim of the present study was to determine the effects of phenolic fractions from *Anchusa azurea* on XO activities and on serum and liver uric acid levels in normal and potassium oxonate-induced hyper uricemic mice. In addition, the renal function of the mice after the administration of plant extracts was estimated by the determination of blood urea and creatinine levels.

MATERIALS AND METHODS

Materials

Anchusa azurea L. was collected from Borj Bouaririj A, Algeria in May 2010 and identified by Pr. Laouer H. (Department of vegetal biology, Setif 1 University). All other reagents were purchased from Sigma Chemicals (Germany), Fluka and Prolab.

Extraction procedure

The extractions were carried out using various polar and non-polar solvents. According to the method 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 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 calorimetric method as described by Bahrain *et al.* (1996). Each sample (1 ml) was mixed with 1 ml of aluminum chloride ($AlCl_3$) solution (2%) and allowed to stand for 15 min. The 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 [11]. 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 the 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 purchased from Sigma, the effect of extracts on xanthine oxidation was examined spectro photometrically at 295 nm following the production of uric acid using an absorption coefficient of 9600 M^{-1} cm^{-1}. Different concentrations of testing compounds were added to the 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 [12]. 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. Animal experiments in mice were conducted after the experiment procedures were revised and approved by the ethics committee of the university Ferhat Abbas SETIF1, Algeria. 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 was 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 [13]. 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 10 mg/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 mice on 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 [14, 15]. The concentration of uric acid in the serum and supernatant of the liver is determined by an enzymatic method.

Statistical analysis

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

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 [11]. 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 [10] 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 are shown in table 1, showed that the extract (AcE) is the richest in polyphenols followed by ChE, whereas ChE is the richest in flavonoids followed by AcE.

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

Extracts	Polyphenols [a]	Flavonoïds [b]
CrE	9.94±0.008	1.88±0.43
ChE	49.11±0.17	8.98±1.74
AcE	60.31±0.70	3.78±0.78
AqE	32.77±0.59	1.32±0.33

[b] µg of Gallic acid equivalent per mg of extract

[a] µg of quercetin equivalent per mg extract.

Values represent the average of 3 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 (Fig.1). 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.

ChE and AcE inhibited XO noncompetitively, while the CrE and AqE are uncompetitive inhibitors. 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. The position of the hydroxyl (-OH) is probably responsible for the type of inhibition.

Several studies have evaluated the inhibitory effect of different plants on the XO and XOR activity [16-18, 7]. The inhibition of XO activity has been attributed to various compounds such as polyphenols and flavonoids [16, 19, 12, 20].

There is a linear correlation between the content of polyphenols and XO Inhibition (R^2 = 0.9034), AcE extract is the richest in poly phenols, this extract exhibited the best inhibitory effect followed by ECh which is the richest in flavonoids. Based on the correlation between the concentration of polyphenols and flavonoids and XO inhibition by these extracts, our results are confirmed by those obtained by Boumerfeg *et al.,* (2009) on the roots of *Tamus communis* [12], Wu *et al.,* (2010) on *Geranium sibiricum* [19] and Baghiani *et al.,* 2010 [21] on *Carthamus caurulis, and* Widyarini *et al.,* (2015) on *Cordifolia (ten) Steenis* and *Sonchus Arvensis L* [22]. They found that the richest extracts phenolic compounds are the most active XO. Cos and his collaborators have shown in a study on a thirty flavonoids, inhibition of XO may be related not only on the content of polyphenols and flavonoids, but also the nature

(structure) of these compounds, glycoside extracts (AcE) and non glucoside (ChE) showed the highest inhibitory effect. CrE and AqE inhibited XO with a non-competitive manner, the same case of catechin, This is consistent [23]. Indicating that these extracts act differently on the enzyme, in this type of inhibition, the molecule reacts with the enzyme substrate after attaching, giving a non-functional complex.

Berboucha and his collaborator [24] tested the XO inhibitory activity of a mixture of quercetin and catechin, in order to determine the effect of the simultaneous interaction of two potential inhibitors, the results showed a mixed type of inhibition. This means that the type of inhibition can be altered by the presence of other molecules present in the extract of plants by non-specific interactions.

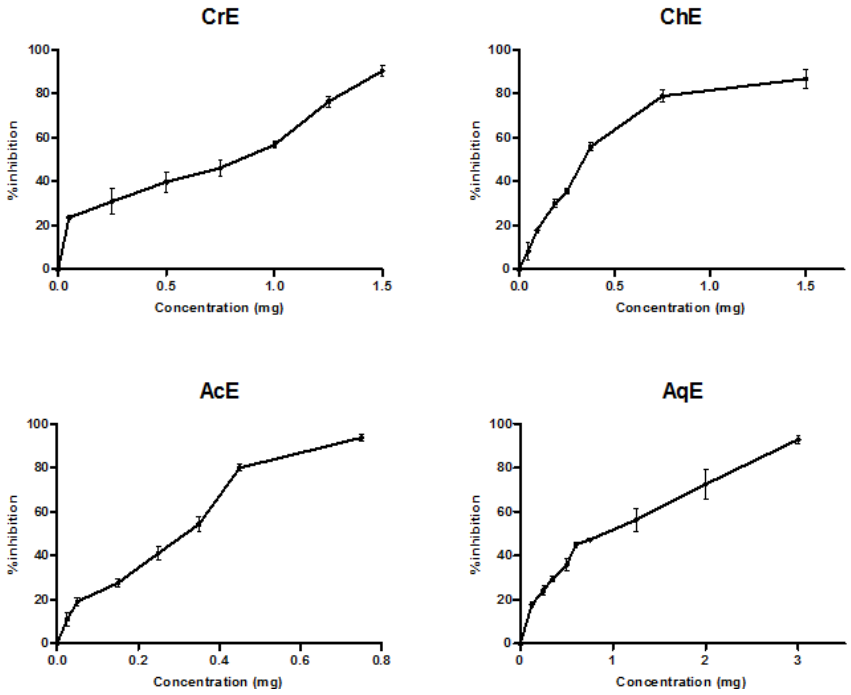

Fig. 1: Inhibition of XO by the extracts of *Anchusa Azurea*

ChE and AcE have an inhibitory effect more or less significant (IC_{50}= 0.334±0.006 and 0.263±0.002 mg/ml, respectively). CrE and AqE weakly inhibit the XO activity, a concentration of 700 µg/ml and 800 µg/ml reduced the activity of XO by 50%. XO inhibition by AcE and ChE was 134-times and 167-times, respectively, comparing to the IC_{50} of allopurinol (table. 2).

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

Extracts	IC_{50} (mg/ml)	inhibition type
CrE	0.828±0.108***	Uncompetitive
ChE	0.334±0.006***	Noncompetitive
AcE	0.263±0.002**	Noncompetitive
AqE	0.726±0.016***	Uncompetitive
Allopurinol	0.002±0.00002	Competitive
Quercetin	Nd	Competitive
Catechin	Nd	Uncompetitive

Values are an average of 3 trials±SD. The comparison was realized against allopurinol.

**: $p \leq 0.01$, ***: $p \leq 0.001$, and Nd: not determined.

Cos and his collaborator (1998) [16] measured the activity of a thirty flavonoids on the production of uric acid 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 C_2 and C_3 carbons of the ring A flavonoid is very important for the inhibition. With a double bond between C_2 and C_3, ring B will be coplanar with rings A and C due to the conjugation. Saturation of this double bond will destroy conjugation and coplanarity. This suggests that a planar flavonoid structure is important for inhibition of xanthine oxidase.

In conclusion, to obtain an overall view of the inhibitory activity of XO extracts AA, an *in vivo* study is desirable on hyper urecemic models, based on the fact that a substance may be very active *in vitro* may lose this activity once penetrated into the body, and vice versa.

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 increases the levels of uric acid in the mice serum giving an animal model hyper uremic [13, 15]. The uric acid, urea and creatinine were measured in serum and supernatant of the liver.

We tried to verify the inhibitory effect of extracts of *Anchusa azurea* 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*.

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 (table. 3).

From the results obtained (fig. 2), it appears that treatment with all extracts decreased uric acid value compared to the control (1.48±0.07 mg/l). In the "PO" group, the uric acid levels increased 4 times in the serum (6.33±1.22) and to almost two times for the liver supernatant (31.36±5.4 mg/l), the administration of 10 mg/kg of allopurinol decreased the rate of uric acid to the normal (1.89±0.32 mg/l, 16.36±2.31 mg/l, respectively for serum and liver supernatant).

AqE and CrE caused a decrease in serum uric acid (a decrease of 66%) followed by ChE with a percentage of 29.22 %. The AcE keeps almost the same value of uric acid of "PO" group. For the supernatant, only CrE caused a significant decrease liver uric acid (18.5±4.8 mg/l). This decrease can be explained by the significant inhibition of the XO by inhibition of the synthesis pathways of uric acid [7]. The hypouricemic effect may be due to the inhibitory effect of these extracts on XO (IC_{50} (CrE) = 0.828±0.108 mg/ml, IC_{50} (AqE) = 0.726±0.016 mg/ml).

These results are in contradiction with the results obtained *in vitro*, AcE and ChE present the best effect *in vitro* (IC_{50}=0. 263±0.002 mg/ml; IC_{50}=0. 334±0.006 mg/ml), these extracts keep the same value of uric acid of PO group. However, CrE and AqE caused a similar decrease of uric acid (2.1±0.47 mg/l; 2.04±0.45 mg/l) that allopurinol. This incoherence may be due to the difference in the bioavailability of flavonoids or polyphenols and metabolism in mice [15].

Table 3: Hypouricemic effects of extracts of *Anchusa azurea*

	Serum uric acid (mg/l)	Liver uric acid (mg/l)
Control	1.48±0.07^{ns}	17.9±1.591^{ns}
Allopurinol	1.89±0.1	16.36±1.033
OP	6.33±0.46***	30.18±2.141***
CrE	2.1±0.21^{ns}	18.5±1.854^{ns}
ChE	4.48±0.33***	21.45±1.854^{ns}
AcE	5.7±0.33***	20.47±1.150^{ns}
AqE	2.04±0.20^{ns}	20.08±1.028^{ns}

Comparison against the Allopurinol group, *** p ≤ 0.001, ns: not significant.

Several researchers study on gout disease, they have shown that flavonoids can influence the activity of XO and thus can reverse the disease of gout by reducing both the concentrations of uric acid and inhibiting XO [25, 15]. Huang et his collaborators (2011) evaluated the effect of some flavonoid on the activity of XO *in vitro* and *in vivo* in serum and liver. They found that these flavonoids have not a significant effect *in vitro*. However, a slight decrease in serum uric acid levels between 8.6-16.5% was observed, they also found that there is no correlation between the activity of XO *in vivo* and *in vitro* [15].

Renal function 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 hyper uricemic mice, Allopurinol (10 mg/kg) increased the rate of creatinine and the urea level compared to normal control mice. All extracts of *Anchusa azurea* increased the urea but this increase is not significant, but they caused a very significant increase (P ≤0.001) of creatinine. The group "PO" have very significant increase (P ≤0.001) of creatinine and urea levels (p ≤0.01). Comparing the urea level of "OP" group (0.48 g/l), only extracts CrE-AA, AqE-AA (0.41g/l; 0.39g/l) decreased the level of urea (P ≤0.05) by to the normal urea (0.34 g/l) (fig. 3).

Alteration of the level of urea and creatinine can be caused by a number of factors other than renal disease, including the protein degradation, poor hydration, and liver failure. A decrease in the urea in the animals treated with the flavonoids may be inefficient due to the production of urea in the liver. The rate of formation of urea depends on the rate of protein catabolism, an increase of urea reflects an acceleration of the rate of protein catabolism and decrease the urinary excretion of urea [15].

Therefore, we can conclude that the rate of urea and creatinine after treatment with plant extracts are normal and that the results of this study indicate the absence of renal damage in mice.

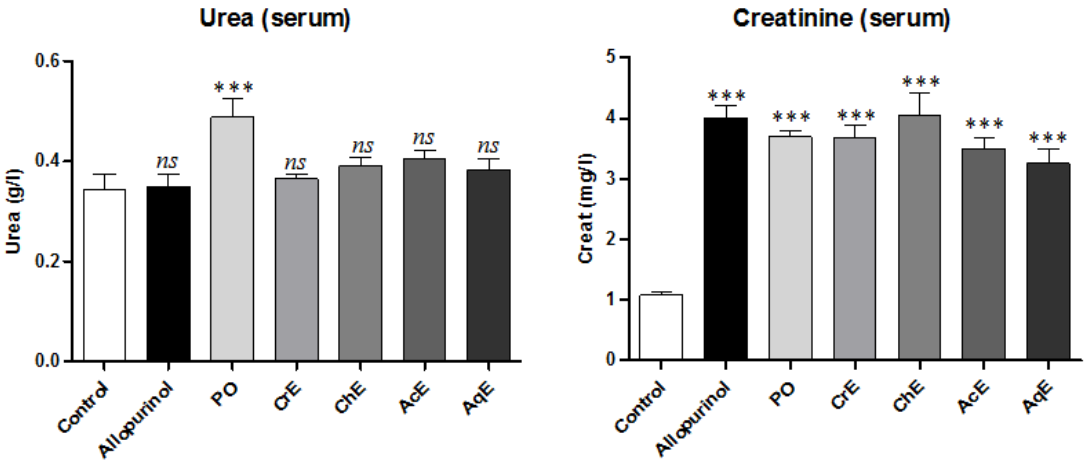

Fig. 3: Renal function evaluation by the determination of urea and creatinine. The comparison against the control group, *** p ≤ 0.001, ns: not significant

CONCLUSION

The medicinal plant *Anchusa azurea* is among the plants widely used today in traditional medicine in Algeria, In tests conducted in this study, a significant correlation between polyphenols content and inhibition activity of XO was observed. In our study, CrE and AqE extracts of *Anchusa azurea* did not show any significant effect on XO activity *in vitro*, but did have a significant effect on XO activities *in vivo*. Serum uric acid levels in mice treated with the flavonoids from CrE and AqE tested here were almost the same than those in normal control mice and those treated with allopurinol. Therefore, the extracts tested here can be a candidate for replacing allopurinol for the treatment of gout.

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.

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CONFLICT OF INTERESTS

The authors have no conflict of interest directly relevant to the content of this article

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