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Original Article

Study of the Antioxidant Property and Xanthine Oxidase Inhibitory Activity of Various Extracts from the Algerian Medicinal Plant *Paronychia argentea* L.

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ABSTRACT

Introduction: *Paronychia argentea* L. is used in traditional medicine to treat various diseases in Algeria especially kidney stones. This study was focused on the antioxidant and xanthine oxidase inhibitory activities of these plant extracts. **Methods:** The different extracts were prepared using solvents of increasing polarity, and their total polyphenols and flavonoids contents were determined using spectrophotometric methods. The antioxidant activities of *Paronychia argentea* extracts (PAE) were assessed by their inhibitory effect on xanthine oxidase (XO) and their scavenger ability on superoxide radical ($O_2^{\bullet-}$) generated by this enzyme. **Results:** The analysis PAE showed that ethyl-acetate extract (EaE) has the highest concentration of total phenolics and flavonoids compared with the chloroform extract (ChE) and the crude extract (CE). Results revealed that all PAE were effective in both XO inhibiting and superoxide radical scavenging, with the following order for the two assays: ChE > EaE > CE. **Conclusion:** This study provided evidence that PAE had interesting antioxidant potential which was confirmed using two enzymatic methods. Therefore, this plant could be used to treat gout and lot of diseases, where inhibition of XO and scavenging of superoxide radical are necessary.

Key words: Flavonoids, Oxidative stress, *Paronychia argentea* L, Superoxide anion radical, Traditional medicine, Xanthine oxidase.

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INTRODUCTION

Oxidative stress occurs when the balance between the production of reactive oxygen species (ROS) and the quantity of antioxidants is interrupted. This can lead to damage of biomolecules and ultimately leads to cell death causing physiological disorders such as cancer, diabetes, asthma, premature aging, cardiovascular, neurodegenerative and inflammatory diseases.[1] ROS can be synthesized by a variety of enzymes, including xanthine oxidase (XO),[2] which catalyse the metabolism of purines, converting hypoxanthine to xanthine and xanthine to uric acid with reduction of molecular oxygen to hydrogen peroxide and superoxide anion radical ($O_2^{\bullet-}$).[3,4] XO causes gout; an inflammatory disease due to elevated levels of uric acid crystals in the serum.[5,6] In addition, oxidizing products of XO involved in the development of cardiovascular and metabolic diseases, leading to atherosclerosis.[7] The inhibitors of XO may be used for the treatment of gout or other XO induced diseases.[8] Several works have shown that phenolic compounds and flavonoids play a role as antioxidants including: inhibition of oxidative enzymes such as XO,[9] and scavenging of free radicals.[10]

Plants are a rich source of antioxidants such as phenolic compounds, anthocyanins and flavonoïds. Herbal antioxidants can reduce the developement of several human diseases related to oxidative stress.[11] *Paronychia argentea* L. Caryophyllaceae is one of the most used plants in folk medicine in Algeria, and popularly known: Arabic tea (Fettatet lahdjer, Bissat elmoulouk). The aerial parts are used as diuretic and to treat renal diseases as antiurolithiasis.[12] This plant was reported to contain the flavonoids isorhamnetin, quercetin, and luteolin.[13] It was reported to have digestive,[14] hypoglycemic,[15] and antimicrobial activities.[16] Furthermore, Dafni *et al.*[17] reported the use of leaf decoction of this plant as diuretic, to treat kidney stones, diabetes and heart ailments. It was also used as a blood purifier.[18] In Portugal, *Paronychia argentea* is used as analgesic, for stomach ulcer, anorexia, and flatulence.[19] This paper was aimed at studying the XO inhibitory activity and radical scavenging property of *Paronychia argentea* extracts by applying *in vitro* enzymatic assays.

MATERIALS AND METHODS

Plant material and chemicals

Aerial parts of *Paronychia argentea* were collected, at the flowering stage (April - May 2011), from Ouled Rahmoune, Constantine, Algeria, and air-dried at room temperature. The plant was identified and authenticated by Prof. Oudjehih Bachir, a botanist at University El Hadj Lakhdar, Batna, Algeria. All chemicals were purchased from Sigma (Germany), Pfizer Health AB (Sweden), Prolabo, Aldrich and Fluka.

METHODS

Purification of bovine milk xanthine oxidoreductase (XOR)

XOR was purified from bovine milk in the presence of 10 mM of dithiothreitol, by ammonium sulfate fractionation, followed by affinity chromatography on heparin agarose, according to Baghiani *et al.*[20] The concentration of XOR was determined *via* UV-visible spectroscopy using an absorption coefficient of 36000 $M^{-1}cm^{-1}$ at 450 nm. Estimation of the purity of the enzyme was based on protein/flavin ratio (PFR = A_{280}/A_{450}) and 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% SDS-PAGE), whereas XOR activity was assessed specrophotometrically by measuring the production of uric acid obtained from xanthine (100 μM, final concentration) at 295 nm using an absorption coefficient of 9600 $M^{-1}cm^{-1}$. Assays were performed at room temperature in air-saturated 50 mM phosphate buffer, pH 7.4, supplemented with 0.1 mM EDTA.

Extraction of phenolic compounds

The extraction was carried out using polar and non-polar solvents according to a procedure outlined by Baghiani *et al.*[21] One hundred g of dried and powdered plant material was soaked in 1 L of 85 % aqueous methanol at 4°C for 16 h. Then, the residue obtained after filtration was re-extracted with 1 L of 50 % aqueous methanol for 4 h. The resulting solutions from the first and the second extractions were concentrated by a rotary evaporator to obtain the crude extract (CE). The CE was then fractioned with *n*-hexane, chloroform, and ethyl acetate to obtain these solvent extracts (HxE, ChE and EaE, respectively), the remaining aqueous extract was labeled AqE. Solvents were removed by evaporation under reduced pressure, and dried extracts thus obtained were stored until use.

Total polyphenols contents determination

Total polyphenols contents of *Paronychia argentea* extracts (PAE) were determined with the Folin-Ciocalteau reagent using gallic acid as a standard according to Li *et al.*[22] In brief, 0.1 mL of PAE was mixed with 2.5 mL of distilled water and 0.5 mL of Folin-Ciocalteu stock reagent. After 5 min, 1.0 mL of 20% aqueous Na_2CO_3 solution was added to the mixture. The mixture was then incubated at room temperature for 1h and its absorbance was measured at 760 nm. The amount of total polyphenols in different extracts was determined from standard curve of gallic acid, and results are expressed in milligrams of gallic acid equivalents per g of dried PAE (mgGA-Eq/gE).

Total flavonoid contents determination

Total flavonoid contents in PAE were determined according to the method of Bahorun *et al*.[23] Briefly, 1 mL of each sample was mixed with 1 mL of aluminium chloride ($AlCl_3$) solution (2%, in methanol). After incubation for 10 min, absorbance of mixture was measured at 430 nm versus a prepared methanol blank. Quercetin and rutin were used as standards. Results are expressed as milligrams of quercetin and rutine equivalents per g of dried PAE (mgQ-Eq/gE and mgR-Eq/gE, respectively).

Effect of Paronychia argentea extracts on superoxide anion radicals generated by xanthine oxidase

Superoxide radicals generated by XO are able to reduce cytochrome c. Free radical scavenging activity of PAE was evaluated according to the method outlined by Boumerfeg *et al*.[24] In this method, a mixture containing xanthine (100 µM), horse heart cytochrome c (25 µM), in air-saturated sodium phosphate buffer (50 mM, pH 7.4), supplemented with 0.1 mM EDTA and various concentrations of PAE was obtained. The reaction started upon addition of XO, and the extent of cytochrome c reduction was determined at 550 nm against enzyme free mixture using an absorption coefficient of 21.100 M^{-1} cm^{-1}. Bovine erythrocytes superoxide dismutase (SOD), (330 U/mL final concentration) was employed to assess the sensitivity of the reaction. The inhibitory activity of cytochrome c reduction by PAE was expressed as percent inhibition (I %) calculated as follows:

$$I\ (\%) = [(A_{control} - A_{sample})/A_{control}] \times 100$$

Where $A_{control}$ is the absorbance of the control reaction (containing all reagents except the test solutions), and A_{sample} is the absorbance of the test compound. Extract concentration providing 50% inhibition (IC_{50}) was calculated from the plot of inhibition percentage against extract concentration.

Effect of Paronychia argentea extracts on xanthine oxidase activity

The effect of PAE on XO activity was evaluated spectrophotometrically at 295 nm by measuring the formation of uric acid from xanthine at room temperature following the procedure published by Boumerfeg *et al.*[25] Mixtures containing a final concentration of 100 µM of xanthine, and various amounts of PAE were made. Addition of 1176 nmol of urate / min/mg XOR protein to each mixture initiated the reaction; enzyme activity of the control was set as 100 % activity. Allopurinol, a clinical drug for XO inhibition, was used as standard inhibitor. The percent inhibition was calculated by using the following formula:

$$I\ (\%) = [(A_{control} - A_{sample})/A_{control}] \times 100$$

Where $A_{control}$ is the absorbance of the control reaction (containing all reagents except the test solutions), and A_{sample} is the absorbance of the test compound. Extract concentration providing 50% inhibition (IC_{50}) was calculated from the plot of inhibition percentage against extract concentration.

Statical analysis

All determinations were conducted in triplicate or more and all results were calculated as mean ± standard deviation (SD) and as mean ± standard error of the mean (SEM). Statistical analysis was performed using Student's *t*-test for significance and analysis of variance (ANOVA) followed by Dunnet's test for the multiple effects comparison of the different extracts. The p values less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Purification of bovine milk xanthine oxidoreductase

Xanthine oxidoreductase purified from fresh bovine milk yielded 23.21 mg of XOR protein per liter, which is comparable to the amounts reported by Baghiani *et al.*[20] The obtained enzyme was largely (more than 90%) under the oxidase form. The freshly purified bovine milk XOR exhibit an ultraviolet/visible spectrum with three major peaks at 280, 330, and 450 nm (Figure 1A), with A280/A450 (protein to flavin ratio, PFR) of 5.15 which indicates a high degree of purity. These results agree with those reported in the literature.[21,26] The purified enzyme showed one major band of an approximate molecular weight of 150 KDa when run on SDS/PAGE (Figure 1B). The oxidase form of the purified enzyme showed an activity of 1988.55 nmol of urate/min/mg protein. These results are similar to those reported by Baghiani *et al.*[9,20,21] and Atmani *et al.*[27]

Extraction of phenolic compounds and determination of total polyphenol and flavonoid contents

In Table 1 are listed the yields, percentages, and amounts of total polyphenols and flavonoids. Results show that aqueous extract (AqE) has the highest yield, followed by the crude extract (CE), whereas the other extracts displayed lower yields. In the other hand, results also show that ethyl acetate (EaE) extract contained the highest total polyphenols and flavonoids contents.

Effect of Paronychia argentea extracts on the generation of superoxide anion radicals by XO system

The ability of PAE to scavenge superoxide anion ($O_2^{\bullet-}$) radicals was determined by following reduction of cytochrome c (cyt c) at 550 nm. Results revealed that SOD (330 U/mL) has totally inhibited the reduction of cyt c (I% = 100%), and show that all PAE significantly inhibit the cyt c reduction in a concentration-dependent manner ($p < 0.0001$) (Figure 2A). Comparison between these extracts reveal that ChE has the highest ability to scavenge superoxide anion radicals followed by EaE and CE, with IC_{50} (mg/mL) values of 0.092 ± 0.00014, 0.098 ± 0.0002, and 0.277 ± 0.0015, respectively (Figure 2B).

Superoxide anion formed during XO reaction can be quantified using cytochrome c.[28] It causes damage to biomolecules by forming H_2O_2, $OH^{\bullet}$ that can initiate lipid peroxidation.[29] Since the presence of proteins, sugars, and polyphenols in the extracts may affect their antioxidant activity,[30] it is possible that the antioxidative properties of PAE are caused by the presence of polyphenols and flavonoids. Moreover, the antioxidant properties of polyphenols are directly linked to their structure.[31] Phenols are made up of one or more aromatic rings with one or more hydroxyl groups; these phenols therefore are potentially able to quench free radicals by forming resonance-stabilized phenoxyl radicals.[32] On the other hand, structure-activity relationships of flavonoids in scavenging superoxide anion were studied by Cos *et al.*[33] and Dugas *et al.*[34] They

Table 1: The yields and the amounts of total polyphenols and flavonoids compounds in different extracts of *Paronychia argentea*.

Extracts	% yield (w/w)	Total polyphenols (mg GA-Eq / g E)	Total flavonoids (mg Q-Eq / g E)	Total flavonoids (mg R-Eq / g E)
CE	10.971±0.637	217.463±0.87	13.349±0.562	26.765±0.837
HxE	0.314±0.097	nm	nm	nm
ChE	0.212±0.015	211.444±0.778	38.621±1.303	87.717±1.811
EaE	0.786±0.175	525.796±0.796	194.193±8.622	382.176±4.74
AqE	11.271±1.831	nm	nm	nm

nm: not mentioned. Values are expressed as mean ± SEM (*n* =3).

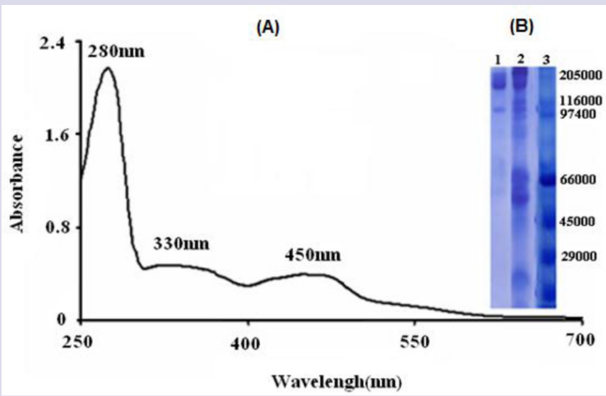

Figure 1: UV/Visible absorbance spectrum of Bovine XOR (A), 10% SDS-PAGE of XOR preparation (B). Lan1: purified bovine milk XOR; Lan2: crud bovine milk XOR and Lan3: molecular weight markers: Myosin 205 000; β-galactosidase 116 000; Phosphorylase 97 400; Serum albumin 66 000; Ovalbumin 45 000; Carbonic anhydrase 29 000.

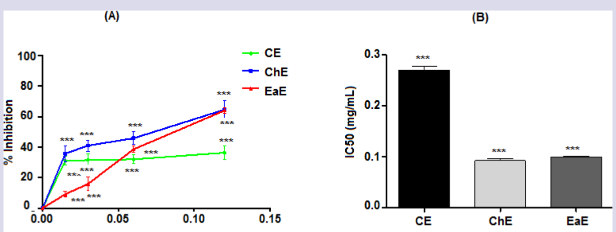

Figure 2: Inhibitory effect of *P. argentea* extracts on the reduction of cyt c by $O_2^{\bullet -}$ anion radical produced by XO (A), concentrations of *P. argentea* extracts which inhibit 50% reduction of cyt c by superoxide produced by XO (scavenger effect, IC_{50}) (B). CE: methanol extract; ChE: chloroform extract; EaE: ethyl acetate extract. Values are means ± SD (*n* = 3). *** *p* < 0.0001, comparing with the control in absence of extracts.

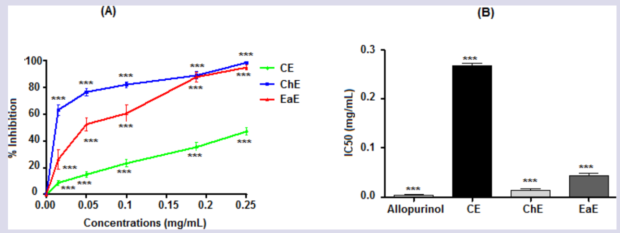

Figure 3: Inhibitory effect of *P. argentea* extracts on the activity of XO (A), concentrations of *P. argentea* extracts and allopurinol that inhibit 50% of XO activity (IC_{50}) (B). CE: methanol extract; ChE: chloroform extract; EaE: ethyl acetate extract. Values are means ± SD (*n* = 3). *** *p* < 0.0001, comparing with the control in absence of extracts.

discovered that the presence of hydroxyl groups and carbon-carbon double bonds in certain positions enhance flavonoids scavenging activity.

Effect of Paronychia argentea extracts on XO activity

Since an inhibitory effect on the enzyme itself would lead to a decrease in cyt c reduction,[35] the effect of PAE on the inhibition of XO was studied. Results demonstrated that all extracts exerted a very significant concentration-dependent inhibition of XO activity ($p < 0.0001$) (Figure 3A). The IC_{50} values revealed that the highest XO inhibitory effect was shown with ChE followed by EaE and CE (Figure 3B).

The presence of polyphenols and flavonoids in the extract can enhance XO inhibition.[36,37] Thus, the higher XO inhibitory effect of ChE and EaE can be due to their richness in flavonoids. XO causes hyperuricemia, gout and cardiovascular disease, and their inhibitors are considered effective drugs to control these uric acid-related problems.[38,39] Allopurinol is the most used XO inhibitor drug, but it have several adverse effects including: gastrointestinal irritation, hypersensitivity syndromes, fever, hepatitis, eosinophilia and worsening renal function.[40] Thus, and due to the limitations of currently available XO inhibitory drugs, the development of new ones with increased therapeutic activity and less side effects is an active field of research.[38] XO contains a molybdopterin (Mo) domain which is the active site of the enzyme.[41,42] The inhibitors of the enzyme; allopurinol and 3,4-dihydroxy-5-nitrobenzaldehyde (DHNB) inhibit the XO activity *via* interaction with its Mo center.[38,43] Therefore, it is possible that XO inhibitory activity of PAE is due to the presence of various compounds which are fixed to this active site.

On the other hand, inhibition of cyt c reduction is due to the XO inhibitory effect and/or the scavenger effect on $O_2^{\bullet -}$ produced by this enzyme.[44] Figure 4 show a comparison between IC_{50} values of cyt c reduction and XO inhibition effects of PAE. The ChE and EaE IC_{50} values of cyt c reduction are higher than those of XO inhibition, so according to Cos *et al.*[33] the inhibition of cyt c reduction by these extracts is due to their XO inhibitory effect and to a weak $O_2^{\bullet -}$ radical scavenger effect. CE has an IC_{50} of cyt c reduction almost identical to that of XO inhibition, therefore according to Cos *et al.*[33] it is considered as an antioxidant because of its ability to inhibit XO.

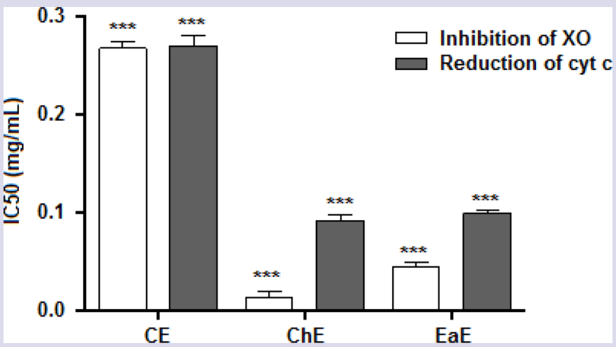

Figure 4: Comparison of IC_{50} values of cyt c reduction and XO inhibition effects of *Paronychia argentea* extracts. CE: methanol extract; ChE: chloroform extract; EaE: ethyl acetate extract. Values are expressed as mean ± SD (n = 3). *** $p< 0.0001$, comparing with the control in absence of extracts.

CONCLUSION

In conclusion, *Paronychia argentea* extracts contain significant amounts of flavonoids and polyphenols and have a strong antioxidant activity. Consequently, these plant extracts can be used as a source of bioactive compounds which may be useful natural antioxidants and therapeutic agents for hyperuricemia, gout and other related diseases, where inhibition of XO and scavenging of superoxide radicals are necessary. However, further investigations to isolate and identify the antioxidant compounds present in the plant extracts, and further studies on definitive mechanisms of their therapeutic activities *in-vivo* are needed.

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CONBFLICT OF INTEREST

The authors declare no conflict of interest.

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