Antioxidant Properties and Xanthine Oxidase Inhibitory Effects of *Tamus communis* L. Root Extracts

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This study was conducted to search for xanthine oxidase (XO) inhibitors from the root extracts of *Tamus communis* L. traditionally used in folk medicine in Algeria. Root extracts with different solvents were screened for purified milk xanthine oxidase inhibition. The root extracts (methanol, chloroform and ethyl acetate) and proteins, obtained in distilled water, inhibited bovine, sheep and human milk XO from three species in a concentration-dependent manner, with an additional superoxide scavenging capacity, which reached its highest level with ethyl acetate extract (IC₅₀ = 0.15, 0.04 and 0.09 g/L) for bovine XO, sheep XO and human XO, respectively. The antioxidant potential was confirmed with the non-enzymatic method, total radical-trapping antioxidant parameter (TRAP) assay, which showed that the *Tamus communis* L. extracts have a potential antioxidant activity in the same order obtained by using the reduction of cytochrome c, an enzymatic method, in which the antioxidant activity followed a decreasing order: ethyl acetate extract > chloroform extract > protein. Copyright © 2008 John Wiley & Sons, Ltd.

**Keywords:** antioxidant; medicinal plant; xanthine oxidase; superoxide scavenger; TRAP.

**INTRODUCTION**

Xanthine oxidoreductase (XOR) is a complex molybdenum iron–sulphur flavoprotein that is generally recognized as the terminal enzyme of purine catabolism in man and in a few other uricotelic species. XOR catalyses the hydroxylation of hypoxanthine to xanthine and xanthine to uric acid. The mammalian enzyme exists in two interconvertible forms, xanthine dehydrogenase (XDH; EC 1.1.1.204), which predominates *in vivo*, and xanthine oxidase (XO; EC 1.1.3.22) (Bray, 1975). The overproduction of uric acid can lead to hyperuricaemia, which can be linked to gout, due to the deposition of uric acid in joints leading to painful inflammation (Harris et al., 1999). The use of an XO inhibitor that blocks the synthesis of uric acid in the body can be considered as a therapeutic approach for the treatment of hyperuricaemia (Emmerson, 1996). The purine analogue, allopurinol, is the only XO inhibitor administrated clinically, and has been used in the therapy of both primary hyperuricaemia of gout and secondly in haematological disorders or antineoplastic therapy (Ishibuchi et al., 2001). Unfortunately, this drug has been reported to be associated with an infrequent but severe hypersensitivity (Bradlow, 1997).

The capacity of XO to generate reactive oxygen species (ROS) has led to widespread interest in the enzyme as an initiator of tissue damage in a range of pathological states. Hydrogen peroxide (H₂O₂) and superoxide anion radicals (O₂⁻) can interact in the presence of certain transition metal ions to yield a highly reactive oxidizing species, the hydroxyl radical (·OH). Continuous exposure to chemicals and contaminants may lead to an increase in the amount of free radicals in the body beyond its capacity to control them and cause irreversible oxidative damage to biomolecules (e.g. lipids, proteins, DNA), eventually leading to many chronic diseases, such as atherosclerosis, cancer, diabetes, aging and other degenerative diseases in humans (Halliwell, 1994).

Antioxidants are of great importance in terms of preventing oxidative stress that may cause several degenerative diseases. Many fruits, herbs and vegetables are potentially useful for decreasing the risks of several chronic diseases (Lampe, 1999).

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. In the past two decades, the potentials of free radical scavenging and XOR inhibitors were explored from a wide variety of traditionally used herbal plants (Montoro et al., 2005).

*Tamus communis* L. (fam. Dioscoreaceae), called El-Karma Saouda, is a herbal species, widely distributed in the north of Algeria and used in folk medicine to lower inflammatory pain. Rhizomes and berries have been reported to be used in folk medicine for rheumatism, arthrosis, lumbago and dermatosis (Duke, 2002). More recent phytochemical analysis of this plant revealed the presence of some compounds with cytotoxic activities...
such as hydroxyl/alkoxy-substituted phenanthrenes (Kovacs et al., 2007).

The aim of the present study was to investigate the potential of *T. communis* root extracts to inhibit XOR and to act as an antioxidant and free radical scavenging material.

**MATERIAL AND METHODS**

**Collection of plant material.** The roots of *T. communis* L. were collected from Bouandas, Setif, Algeria, authenticated by Dr Laouar Hocine, Setif University. A voucher specimen was deposited at the Laboratory of Botany, Department of Biology, Faculty of Sciences, University Ferhat Abbas of Setif, Algeria. Milk was kindly donated by volunteer mothers in Setif city and surrounding area and stored at −20 °C before use. Bovine and sheep milks were obtained from local farms. All other reagents were purchased from Sigma Chemicals (Germany).

**Purification of milk xanthine oxidoreductase.** XOR was purified routinely in our laboratory from mammalian milk, in the presence of 10 mM of dithiothreitol, by ammonium sulphate fractionation, followed by affinity chromatography on heparin-agarose, as previously described for human, bovine, sheep and camel (Baghiani et al., 2002, 2003). The XOR concentration was determined from the UV-visible spectrum by using an absorption coefficient of 36 000 M$^{-1}$ cm$^{-1}$ at 295 nm using an absorption coefficient of 36 000 M$^{-1}$ cm$^{-1}$ at 295 nm. The purity of the enzyme was assessed on protein/flavin ratio (PFR = $A_{280}/A_{450}$) (Bray, 1975) and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (10%) (Laemmli, 1970). The activity of XOR was spectrophotometrically determined by measuring the production of uric acid from xanthine (100 μM, final concentration) at 295 nm using an absorption coefficient of 9600 M$^{-1}$ cm$^{-1}$ (Avis et al., 1956). 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 extractions were carried out using various polar and non-polar solvents (Markham, 1982). According to the method, dried plant material was ground in a Waring blender. It was mixed with a 10–20 volume of 85% aqueous methanol. The slurry was placed on a shaker for 24 h. The extract was filtered through a Buchner funnel and the methanol removed on a rotary evaporator to give crude extract (fraction labelled CE). The aqueous solution was extracted with hexane several times to eliminate lipids. The water fraction was partitioned against chloroform labelled CHE. The remaining aqueous phase was extracted exhaustively with ethyl acetate until the final extract was colourless (fraction labelled EAE). All the solvents were removed by evaporation under reduced pressure and the extracts were stored at −20 °C until use.

**Determination of polyphenol contents.** Total polyphenols were measured using Prussian blue assay methods described by Price and Butler (1977) modified by Graham (1992). Phenolics were expressed as gallic acid equivalents. Briefly 0.1 mL of *T. communis*, root extract (TCRE) samples were dissolved in methanol, 3 mL distilled water was added and mixed, then 1 mL of K$_3$Fe(CN)$_6$ (0.016 m) was added to each sample followed by the addition of 1 mL of FeCl$_3$ (0.02 mM dissolved in 0.1 M HCl). It was immediately mixed using a vortex, and 5 mL stabilizer (30 mL gum Arabic, 1%; 30 mL H$_2$PO$_4$, 85% and 90 mL of distilled water) were added to the sample and mixed. The absorbance was measured at 700 nm using a UV/VIS-8500 Techom spectrophotometer. The amount of total polyphenols in different extracts was determined from a standard curve of gallic acid ranging from 0.00 to 200 μg/mL.

**Determination of flavonoid contents.** Flavonoids were quantified using aluminium chloride reagent AlCl$_3$, Bahorun et al. (1996). Flavonoids were measured as quercetin equivalents. 1 mL of TCRE samples was dissolved in methanol, 1 mL of AlCl$_3$ (2%) in methanol was added, and after incubation for 10 min, the absorbance was measured at 430 nm.

**Protein extraction.** The extraction of proteins was carried out following the Mahmood et al. (2003) method with slight modification. The TCRE were dried at 37 °C for several days then crushed manually in a mortar with a pestle. A volume of 90 mL of distilled water was added to 10 g of dry powder, and mixed continuously until there was no further change in the colour of the solution. This solution was centrifuged at 800 × g for 15 min, using a Sigma 3K30 C centrifuge. The supernatant (brownish-black) was filtered through Whatman filter, dialysed overnight against the same buffer and stored at −20 °C. Protein determination was carried out according to Macart and Gerbaut (1982), using a standard curve of bovine serum albumin (BSA) ranging between (0.0 and 1.5 mg/mL) and UV/VIS-8500 Techom spectrophotometer.

**Effects of TCRE on XO activity.** The effect of TCRE on xanthine oxidation was examined spectrophotometrically at 295 nm following the production of uric acid using an absorption coefficient of 9600 M$^{-1}$ cm$^{-1}$ (Avis et al., 1956). Assays were performed at room temperature, in the presence of a final concentration of 100 μM of xanthine, in air-saturated sodium phosphate buffer (50 mM, pH 7.4), supplemented with 0.1 mM EDTA, with various amounts of plant extracts, and dissolved in dimethylsulphoxide (DMSO). Control experiments revealed that the solvent did not influence the activity of XO at this concentration. The reaction was started by the addition of XO (73.8, 121.9, 1227 nmol of urate/min/mg protein for HXO, SXO and BXO, respectively). The enzyme activity of the control sample was set to 100% activity.

**Determination of antiradical power of Tamus communis L. root extracts.** The antiradical activity was determined spectrophotometrically according to Robak and Gryglewski (1988), by monitoring the effect of TCRE on superoxide anion radicals produced by the XO system. These radicals are able to reduce cytochrome c. The reaction mixture contained 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 TCRE. All concentrations indicated are final ones. The reactions were started by the addition of XO. After 2 min, reduced cytochrome c

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was spectrometrically determined at 550 nm against enzyme-free mixture. The cytochrome c activity was calculated using an absorption coefficient of 21.100 M⁻¹ cm⁻¹, and the sensitivity of the reaction was determined using bovine erythrocyte superoxide dismutase (SOD) (330 U/mL final concentration).

**Determination of total radical-trapping antioxidant parameter (TRAP).** Evaluation of the effect of *T. communis* L. root extracts was carried out following the non-enzymatic method TRAP (Bartosz et al., 1998), which is a simple spectrophotometric method for the determination of the peroxyl radical trapping capacity of body fluids and food products or plant extracts. In this method, decomposition of 2,2′-azobis(2-amidopropane) hydrochloride (ABAP) is the source of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) oxidation to green cation radical. The assays were performed as follows, 0.1 m sodium-phosphate buffer pH 7.4 was prewarmed to 37 °C and added to a cuvette to obtain 3 mL of the final reaction volume, then 90 μL of 5 mM ABTS solution and sample studied or standard antioxidants solutions were added followed by 300 μL of 200 μM ABAP solution. The cuvettes were placed in a thermostated recording spectrophotometer adjusted to provide a temperature of 37 °C (inside the cuvettes) and the absorbance at 414 nm was measured. The results represent mean values of at least three determinations.

**Statistical analysis.** All determinations were conducted in triplicate and all the results were calculated as mean ± standard deviation (SD). Statistical analysis was performed using Student’s *t*-test for significance and analysis of variance (ANOVA) followed by Dunnett’s test were done for the multiple comparison of the effect of different extracts. Values of *p* < 0.05 were considered statistically significant.

**RESULTS AND DISCUSSION**

**Xanthine oxidase purification**

The freshly purified milk XO from different species showed an ultraviolet/visible spectrum with three major peaks at 280, 325, 450 nm, with A₂₈₀/A₄₅₀ (protein to flavin, PFR) ratio of 5.3, 5.2 and 5.1 for bovine, sheep and human, respectively, indicating a high degree of purity (Bray, 1975). Run on SDS-PAGE, purified enzymes showed quite similar patterns with one major band of approximately M, 150 KDa. Traces of degradation bands appeared on storage (Abadeh et al., 1992). This is analogous to the well-studied bovine (Bray, 1975), human, (Abadeh et al., 1992) and camel (Baghiani et al., 2003) enzymes.

The XO activity of purified sheep milk (121.9 nmol of urate/min/mg protein) was low relative to that of the bovine milk enzyme (1227 nmol of urate/min/mg protein), but higher than that of human XO (73.8 nmol of urate/min/mg protein). This large difference in activity between milk XO is due to the intrinsic nature of the enzyme from different species, especially the existence in a molybdenum-deficient form. Such an inactive form represents more than 97% of HXO, 81% of SXO and 44% of BXO (Baghiani et al., 2002, 2003).

**Phenolic compounds and protein content**

The values of total polyphenols, flavonoids and proteins in TCRE are shown in Table 1. There was a wide range of phenol concentrations in TCRE. The value varied from 2.97 ± 0.53 to 129.66 ± 1.56 mg equivalent gallic acid/g lyophilizate and from 1.34 ± 0.43 to 10.15 ± 0.14 mg equivalent quercetin/g lyophilizate for polyphenols and flavonoids, respectively. The highest level of polyphenols and flavonoids were recorded in the chloroform extract (CHE) followed by the ethyl acetate extract (EAE), while the total polyphenol levels were particularly low in the aqueous extract (AE). However, the protein content was limited to 0.15–0.29%. The protein extract (PE), analysed by SDS-PAGE 10%, contains several proteins with molecular weight ranging approximately from 40 to 90 KDa. It is well known that polyphenols are widely distributed in plants, they are sometimes present in surprisingly high concentrations (Harborne, 1993), especially in medicinal plant and many edible plants (Hagerman et al., 1998).

**Effect of *Tamus communis* L. root extracts on the generation of superoxide anion radicals by the xanthine/xanthine oxidase system**

The antioxidant activity of *T. communis* L. has been reported for the first time in the present study. Cytochrome c₃ has been used extensively for the detection of O₂⁻ produced in biological systems due to its fast superoxide-mediated reduction to cytochrome c⁵⁺ (McCord and Fridovich, 1968). The effects of three *T. communis* L. root extracts at different concentrations were studied for their ability to scavenge superoxide anion radicals (O₂⁻) generated by the xanthine/xanthine oxidase system. The amount of generated O₂⁻ was determined by measuring the reduction of cytochrome c. Under our experimental conditions, the activity of cytochrome c in the absence of extracts were 22.33, 453.07, 2135.91 nmol/
min/mg protein reduced by $O_2^-$ generated from HXO, SXO, and BXO, respectively. The reduction of cytochrome $c$ was almost totally inhibited by SOD (330 U/mL). All the extracts were able to inhibit cytochrome $c$ in a concentration-dependent manner (Fig. 1). The most potent scavenger of superoxide anion radical observed was EAE with an IC$_{50}$ of 0.15, 0.04 and 0.09 followed by the CHE extract 0.18, 0.13 and 0.12 for $O_2^-$ generating from BXO, SXO and HXO, respectively ($p < 0.05$) (Fig. 2). Since an inhibitory effect on the enzyme itself would also lead to a decrease in reducing cytochrome $c$, the effect of extracts on the XO activity was checked. In this regard, the effect of these extracts on the metabolic conversion of xanthine to uric acid were evaluated. In the xanthine/xanthine oxidase system, the extracts were effective in inhibiting the uric acid formation, which were demonstrated with the respective data (IC$_{50}$ g/L):

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\begin{align*}
\text{CHE} & : 0.138 \pm 0.0014, 0.09 \pm 0.001 \\
\text{EAE} & : 0.316 \pm 0.005, 0.172 \pm 0.0014 \\
\text{PE} & : 0.22 \pm 0.007, 0.2 \pm 0.0014
\end{align*}
\]

for BXO, SXO and HXO, respectively ($p < 0.05$). The previous results demonstrated that the extracts have an inhibitory effect on XO, thereby the inhibition of cytochrome c reduction is due to dual effect of the extracts. Firstly, these compounds inhibit the xanthine oxidase activity and secondly, some of them scavenge superoxide radical. Taking this fact into account, it was not possible to show a clear-cut scavenging effect on superoxide radicals. Several flavonoids and other phenolic compounds are considered as antioxidants not only because they act as free radical scavengers, but also because of their ability to inhibit XO (Cos et al., 1998). The antioxidant activity of quercetin and its derivatives has been reported in several experimental models, such as in the prevention of methyl linoleate hydroperoxide formation (Hopia and Heinonen, 1999), the scavenging of superoxide radical generated either by an enzymatic system or non-enzymatically (Robak and Gryglewski, 1988). The inhibition of XO was also observed with rutin (Selloum et al., 2001).

**Total radical-trapping antioxidant parameter (TRAP)**

The TRAP assay has been used to determine the antioxidant activity of single compounds as well as different plant extracts. In the absence of antioxidants, the reaction of ABTS oxidation induced by free radical formed upon ABAP decomposition starts without any lag time, resulting in the appearance of a green colour in the sample due to the formation of the ABTS cation radical ($ABTS^+$) (Bartosz et al., 1998). In the sample containing only ABTS and ABAP in phosphate buffer, the rate of absorbance increased initially in approximately a linear manner then showed a slight decline. If the antioxidant was present in the sample, the reaction will be inhibited. The induction time (lag time) of the reaction is proposed as a parameter to enable the determination of the antioxidant content. In the presence of 0.1 μM quercetin in the reaction mixture, no lag time was observed while the same concentration of CHE or EAE extracts gave a lag time of about 5 min. Increasing the
Figure 3. Effects of TCRE on the activity of XO as measured by the production of uric acid: A = BXO, B = SXO, C = HXO, CE = crude extract, AE = aqueous extract, PE = protein extract, EAE = ethyl acetate extract, CHE = chloroform extract.

Figure 4. Effect of T. communis L. extracts, quercetin and BSA on the ABTS oxidation (A,B); chloroform and ethyl acetate extracts in comparison with quercetin (C,D); protein extract in comparison with BSA. (A) 1-DMSO, 2-quercetin (0.1 μM), 3-ethyl acetate extract (0.1 μM)/quercetin equivalent and 4-chloroform extract (0.1 μM)/quercetin equivalent (B) 1-DMSO, 2-quercetin (1 μM), 3-ethyl acetate extract (1 μM)/quercetin equivalent and 4-chloroform extract (1 μM)/quercetin equivalent (C) 1-H2O, protein: 2-(0.1 μg/mL), 3-(0.4 μg/mL) and 4-(0.81 μg/mL) (D) 1-H2O, BSA: 2-(0.1 μg/mL), 3-(0.4 μg/mL) and 4-(0.81 μg/mL).

PE gave a lag time of 5 and 9.5 min, respectively. The maximal rate of ABTS oxidation was not influenced significantly by BSA 0.8 μg/mL (3.63%), in contrast the PE decreased the ABTS oxidation rate by 50.9% (Fig. 4). The non-enzymatic methods showed that the T. communis L. extracts have a potential antioxidant activity in the same order as obtained by using the reduction of cytochrome c, an enzymatic method, in which the antioxidant activity followed a decreasing order ethyl acetate extract and chloroform extract > protein. The present study confirms that T. communis L. root extracts represent a significant source of antioxidant compounds which may cause a remarkable antioxidant activity. This could be compared with the antiinflammatory effect of T. communis ethanol extract, which show an inhibitory effect on granuloma formation in rats comparable to that of reference drugs, such as benzydamine HCl and hydrocortisone. The antiinflammatory activity of the plant did not affect the body weight (Mascolo et al., 1987). Capasso et al. (1983) showed that T. communis ethanol extract has antiinflammatory properties more potent than phenylbutazone in a short duration test and similar to phenylbutazone in chronic inflammation.

In conclusion, T. communis L. is an efficient inhibitor of xanthine oxidase and has a significant antioxidant and free radical scavenging properties, which could be attributed to phenolic compounds. These properties may explain the beneficial effects of this plant reported in traditional medicine. Therefore, it was suggested that this plant could be used as an additive in the food industry providing good protection against oxidative damage.

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