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Inhibitory Effect of Xanthine Oxidase from *Tamus communis* Roots Extracts/Fraction

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ABSTRACT

In the course of our phytochemical studies of plant *Tamus communis L.,* methanol soluble extract (EMeOH) (138 g) was chromatographed on a silica gel column. The column was eluted with chloroform and then with chloroform /methanol mixtures of increasing polarity. A total of 52 fractions (400 ml each) were collected and grouped according to their TLC behaviour into 6 main fractions (I-VI). Total phenolic and flavonoid contents in these extracts were determined by a colorimetric method. Values varied between 73.143±0.009 and 29.214±0.003 equivalent Gallic acid/g lyophilisate. All the extracts showed inhibitory properties on xanthine oxidase, the IC_{50} ranges from 0.029±0.017 mg/ml to 0.237±0.026 mg/ml. The extracts exhibited an additional superoxide scavenging capacity by using both enzymatic methods and IC_{50} values range from 0.039±0.023 mg/ml to 0.141±0.086 mg/ml. These results show that *Tamus communis L.* extracts have strong anti-oxidant effects and may have some clinical benefits.

Keywords: Xanthine oxidase, Antioxidant, Superoxide scavenger, *Tamus communis L.*

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1. INTRODUCTION

Oxidative stress is defined in general as excess formation and/or incomplete removal of highly reactive molecules such as reactive oxygen species (ROS). In vivo, some of these ROS play a positive role such as energy production, phagocytosis, regulation of cell growth and intracellular signaling [1;2] however, excessive amounts can cause alteration in cellular reduction-oxidation (redox) balance, and disrupt normal biological functions. One of the very important enzyme that has been reported to increase during oxidative stress is xanthine oxidase (XO), which is conventionally seen as a late enzyme of purine catabolism, catalysing the oxidation of hypoxanthine to xanthine and of xanthine to uric acid [3]. Hyperuricemia leads to the accumulation of uric acid in joints and kidneys causing acute arthritis and uric acid nephrolithiasis. One therapeutic approach for gout is the use of XO inhibitors such as allopurinol [4]. However, the use of allopurinol may cause a number of undesirable side effects [5], ranging from mild skin allergy to a concerted allopurinol hypersensitivity syndrome [6;7]. Thus, there is a need to develop compounds with XO inhibitory activities but devoid of the undesirable effects of allopurinol.

There is a growing interest in natural phenolic antioxidants, present in medicinal and dietary plants that might help attenuate oxidative damage [8]. Epidemiological studies have shown that many of these antioxidant compounds possess anti-inflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial, or antiviral activities to a greater or lesser extent [9;10], and it was found that their antioxidant activity surpasses the effect of known antioxidants, such as the vitamins A and E [11]. *Tamus communis* L. plant is commonly used in folk medicine for various types of pathological cases. Previous studies have demonstrated certain antioxidant effects [12]. The purpose of this study was to investigate the antioxidant effect of root extracts of *Tamus communis L.* (TCE) and its active fractions using in vitro assays.

2. MATERIALS AND METHODS

2.1 Materials

Tamus communis was harvested from natural resources from Setif province in the northeast of Algeria, during the spring (May–June) mainly at flowering stage. Plant parts were dried for 7-10 days in shadow at room temperature then powdered and stocked in darkness until use. The

authenticated was confirmed by Prof. Oudjhih University of Batna, Algeria. A specimen was deposited at the Laboratory of Botany, Faculty of natural and life sciences, University Ferhat Abbas Setif, Algeria. Bovine milk obtained from a local farm. All the other reagents were purchased from Sigma chemicals (Germany) and Fluka.

2.2 Methods

2.2.1. Extraction

Roots parts of the plant were cut into pieces, air dried and ground to a fine powder. The dried-powdered roots of the plant (2 Kg) was extracted with 85 % methanol (10 L) incubated for 5 days at room temperature. The extract was filtered through Whatman No. 41 filter paper to obtain particle free extract. The combined methanolic extracts were pooled, concentrated and dried under vacuum. Finally extracts were stored at 4°C and were used to explore their antioxidant activity.

2.2.3. Fractionation

The residual solid material (169 g) was extracted thoroughly with methanol. The methanol soluble fraction (138 g) was chromatographied on a silica gel column. The column was eluted with chloroform and then with chloroform /methanol mixtures of increasing polarity. A total of 52 fractions (400 ml each) were collected and grouped according to their TLC behavior into 6 main fractions (I-VI) (Fig.1).

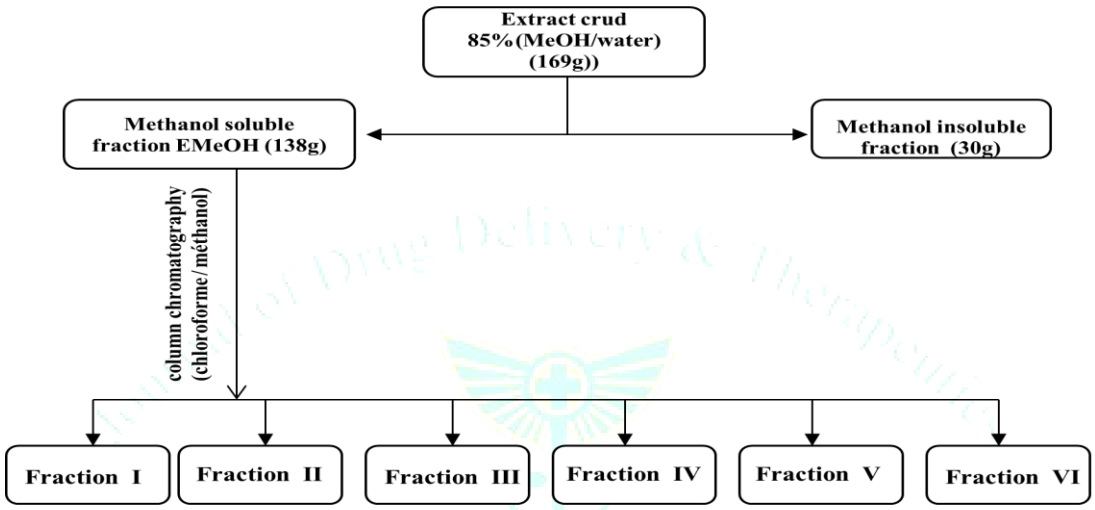

Figure 1: Different steps of extraction

2.2.3. Determination of total phenolics

Total phenolic content of *Tamus communis.L* extract was determined with the Folin–Ciocalteu's reagent (FCR) according to the published method [13]. Each sample (0.5 ml) was mixed with 2.5 ml FCR (diluted 1:10, v/v) followed by 2 ml of Na_2CO_3 (7.5%, v/v) solution. The absorbance was then measured at 765 nm after incubation at 30°C for 90 min. Results were expressed as Gallic acid equivalent (mg Gallic acid/g dried extract).

2.2.4. Determination of flavonoid

Flavonoids were quantified using aluminium chloride reagent $AlCl_3$ [14]. Flavonoids were measured as quercetin equivalents (Quer-Eq). 1mL of *T. communis* samples are dissolved in methanol, then 1 ml of $AlCl_3$ (2 % in MeOH) was added, after incubation for 10 min, the absorbance was measured at 430 nm.

2.2.5. Purification of milk xanthine oxidoreductase (XOR)

XOR was routinely purified in our laboratory from mammalian milk (bovine), in the presence of 10mM of dithiothreitol, by ammonium sulphate fractionation, followed by affinity chromatography on heparin-agarose [15]. XOR concentration was determined from the UV-visible spectrum by using an absorption coefficient of 36000 $M^{-1}cm^{-1}$ at 450 nm. The purity of enzyme was assessed on protein/flavin ratio (PFR = A_{280}/A_{450}) (Bray, 1975) and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (10%) [16]. The XOR activity was 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} [17]. Assays were performed at room temperature in air-saturated 50 mM phosphate buffer, pH 7.4, supplemented with 0.1 mM EDTA.

2.2.6. Effects of *Tamus communis* extracts on the generation of $O_2^{\bullet-}$ radicals

Anti-radical activity was determined according to Robak and Gryglewski [18], by monitoring the effect of TCE on superoxide anion radicals ($O_2^{\bullet-}$) produced by xanthine/xanthine oxidase 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 (50mM, pH 7.4), supplemented with 0.1mM EDTA and various concentrations of TCE. The reactions were started by addition of XO. Within 2 min, reduced cytochrome c was determined at 550 nm against enzyme-free mixture using. The cytochrome c activity was calculated using an absorption coefficient of 21.100 M^{-1} cm^{-1}, and the sensibility of the reaction was determined by using bovine erythrocytes superoxide.

2.2.7. Effects of *Tamus communis* extracts on Xanthin Oxidase activity

The effect of TPE on the xanthine oxidation was examined at 295 nm following the production of uric acid using an absorption coefficient of 9600 M^{-1} cm^{-1} by

spectrophotometer [17]. Assays were performed at room temperature, in presence of final concentration of 100 µM of xanthine, in air saturated sodium phosphate buffer (50 mM, pH 7.4), with various amounts of TCE dissolved in MeOH. Control experiments revealed that solvent didn't influence the activity of XO at this concentration. The reaction was started by the addition of XO (1176 nmol of urate /min/mg protein) for Enzyme activity of the control sample was set to 100 % activity. The percent inhibition was calculated by using the following formula.

$$\text{The inhibition } (\%) = \frac{\text{Control OD - Sample OD}}{\text{Control OD}} \text{ X } 100$$

Statically analysis

All determinations were conducted in triplicate or more and all results were calculated as mean ± standard deviation (SD).

3. RESULTS AND DISCUSSION

3.1. Determination of total polyphenol and flavonoids contents

Total phenolic and flavonoids contents were expressed as mg gallic acid equivalents per gram dry weight (mg GA-Eq/g) and mg quercetin and rutin equivalents per gram dry weight (mg Q-Eq/g) (Table 1) respectively.

There was a wide range of phenol concentration in different extracts. The highest level of polyphenols was recorded in FII followed by EMeOH (Table 1). Flavonoids were quantified using $AlCl_3$ method described by Bahorun *et al.* [14]. Quercetin (Quer) and Rutin (Rut) are used as standards. Results showed that EMeOH gave the greatest level (Table 1). In this study, an attempt was made to quantify and identify polyphenols in *Tamus communis.*

Table 1: Total phenolic and flavonoid content in extracts/ fractions of *T. communis*

Extracts	ɔtal phenolic content ıg GA-Eq/g extract)	Total flavonoid content	
		mg Q-Eq/g extract	mg R-Eq/g extract
CE	29.571±0. 11 ^{a}	2.091±0.05 $^{a'}$	3.404±0.030 $^{a''}$
MeOHE	69.786±0.10 ^{b}	8.080±0.07 ^{c}	19.387±0.11 $^{b''}$
FI	34.500±0.03 ^{a}	0.321±0.07 $^{b'}$	0.962±0.02 $^{a''}$
FII	73.143±0.09 ^{b}	0.755±0.04 $^{b'}$	2.092±0.007 $^{a''}$
FIII	37.500±0.06 ^{a}	0.621±0.04 $^{b'}$	1.479±0.03 $^{a''}$
FIV	30.5±0.06 ^{a}	0.141±0.09 $^{b'}$	0.487±0.07 $^{c'}$
FV	32.071±0.03 ^{a}	0.394±0.07 $^{b'}$	1.070±0.26 $^{a''}$
FVI	29.214±0.03 ^{a}	0.359±0.02 $^{b'}$	0.954±0.06 $^{a''}$

Each value represents the mean ± SD (n = 3). Total phenolic content was expressed as mg Gallic acid equivalent/g dried extract. Total flavonoid content was expressed as mg Quercetin and Rutin equivalent/g dried extract. Lines with different letters indicate activities significantly different ($p \leq 0.05$).

3. 2. Xanthine oxidase purification

Isolation of XO, as the widespread enzymes among different species, involves the extraction of the enzyme from a wide range of materials (bacteria, milk, organs of different animals, etc.) and its purification from crude extract. XO is concentrated in the milk fat/lipid globule membrane (MFGM), in which it is the second most abundant protein, after butyrophilin.

Freshly purified bovine milk XOR showed an ultraviolet / visible spectrum with three major peaks at 280, 325, 450 nm, with A280/A450 (protein to flavin ratio, PFR) of 5.078 indicating a high degree of purity [19]. Run on SDS-PAGE, purified enzyme showed quite similar patterns with one major band of approximately 150 KDa (Fig. 2)

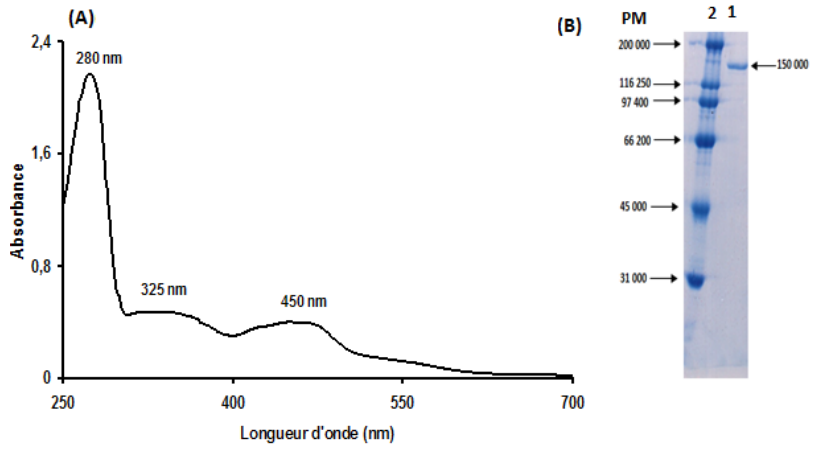

Figure 2: Spectre d'absorption UV-Vis (**A**) SDS-PAGE of XOR (**B**) **B1:** XO purified (150 000 Da), **B2:** markers (Myosin 200 000, β-galactosidase 116 250, Phosphorylase b 97 400, Serum albumin 66 200, Ovalbumin 45 000, Carbonic anhydrase 31 000 Da).

3. 3. Effects of TCE on the generation of $O_2^{\bullet-}$ by the xanthine/xanthine oxidase system

Cytochrome c^{3+} has been extensively used for the $O_2^{\bullet-}$ detection produced in biological systems due to its fast superoxide-mediated reduction to cytochrome c^{2+} [20]. The effect of TCE at different concentrations were studied for their ability to scavenge $O_2^{\bullet-}$ generated by the xanthine/xanthine oxidase system. The amount of $O_2^{\bullet-}$ generated was determined by measuring the reduction of cytochrome c. Under our experimental conditions, the activity of cytochrome c in the absence of extracts was (2135.91 nmols/min/mg protein) reduced by $O_2^{\bullet-}$ generated from XO. The reduction of cytochrome c^{3+} was almost totally inhibited by SOD (330 U/mL). Results showed that all the extracts were able to inhibit cytochrome c^{3+} (Fig. 3). The superoxide scavenging effect was found to increase with increasing concentration of TCE. The FV and EMeOH were the most potent scavenger of $O_2^{\cdot-}$ (IC_{50} of 0,039±0,023 mg/ml) followed by FIII (0,040±0,023 mg/ml) (Figure 1).

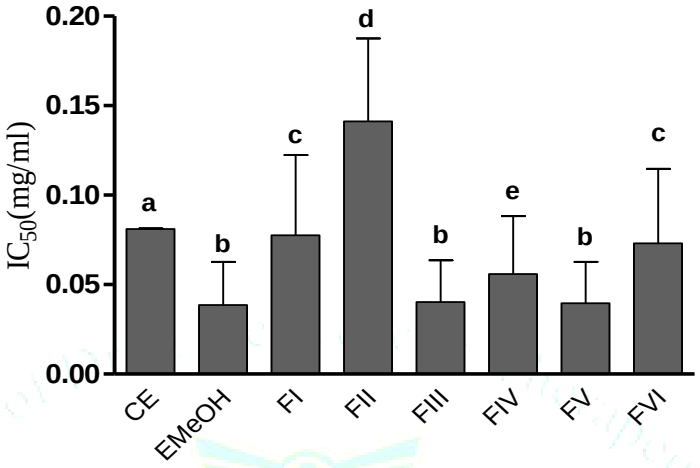

Figure 3: Comparison of inhibitory concentration of extracts/fractions of *T.communis* for 50% of Cyt C activity which reduced by O2•- generated from XO. Amount of superoxide anion radicals in the control sample without extract was set to 100%. Each value is represented as mean ± S.D (n = 3). Different letters indicate activities significantly different ($p \leq 0.05$).

Xanthine oxidase activity, has been reported to increase during oxidative stress [21], this enzyme is considered to be an important biological source of $O_2^{\cdot-}$ which has been linked to post-ischemic tissue injury and odema [22;21].

3.4. Effects of TCE on XO activity

At the identical concentrations of TCE, we observed significant inhibition of XO activity in dose dependent manner. The inhibitory effects of TCE were compared with allopurinol, clinically used as a drug for the XO inhibitor (Fig. 4).

The results demonstrated that FIII possessed high XO inhibitory activity (IC_{50} 0.029±0.017 mg/ml) followed by EMeOH and FII (0.030 ±0.017 mg/ml) (Fig. 4).

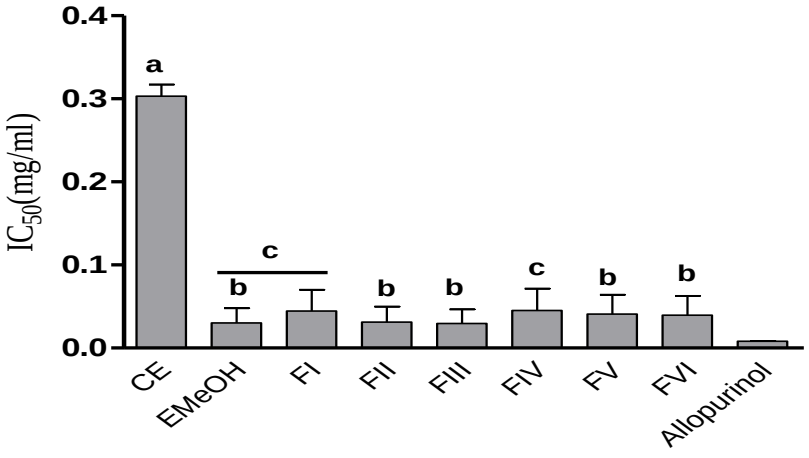

Figure 4. Inhibitory actions of extracts/fractions of *T. communis* on xanthine oxidase activity. Results are expressed as percentage of control where no inhibitor was added. Each value is represented as mean ± S.D (n = 3). Different letters indicate activities significantly different ($p \leq 0.05$).

3.5. Evaluation of inhibition and scavenging effects of TCE

The antioxidative properties of many compounds are brought by several different mechanisms, such as scavenging of free radicals, chelating of metal ions, such as iron and copper, and inhibition of enzymes responsible for free radical generation [23]. The half-maximal inhibitory concentrations of tested compounds for xanthine oxidase inhibition and reduction of the superoxide level are shown in (Fig. 5). Several data has been reported in many experimental models indicating that phenolic compounds are considered as antioxidants not only because they act as free radical scavengers, but also because of their ability to inhibit XO [24;25]. This data showed that IC_{50} to scavenge superoxide radical of CE is low compared of the inhibition of the IC_{50} of XO, this behaviour may be explicated by the fact that CE had a scavenging effect rather than an inhibitory action. The inhibition of Cyt c reduction is due to the inhibitory effect of XO and / or to the scavenging effect on the $O_2^{\bullet-}$ produced by this enzyme [26].

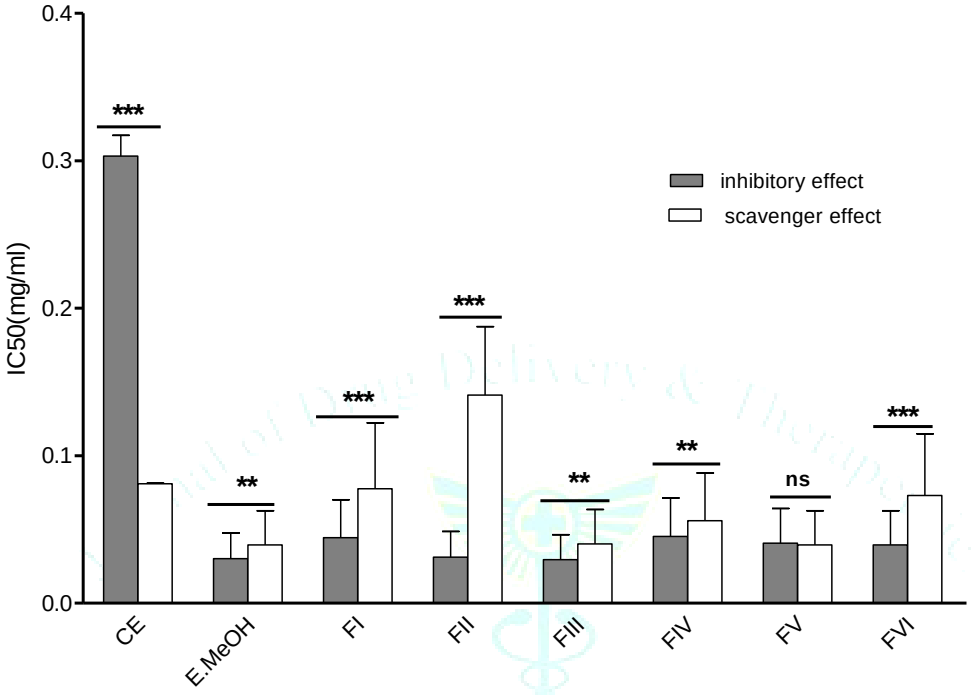

Figure 5. Evaluation of extracts/fractions as inhibitors of xanthine oxidase and as scavengers of superoxide produced by the action of XO enzyme. Each value is represented as mean ± S.D (n = 3). *** $p < 0.001$, **$p<0.01$, ns : non-significant.

CONCLUSION

It has already been shown that the increase in xanthine oxidase activity is an important cause of the oxidative stress that occurs in the disease [27;28]. Xanthine oxidase inhibitors (XOI) are typically used in the treatment of diseases related to hyperuricemia such as gout. Allopurinol is an allosteric xanthine oxidase inhibitor, which can decrease the damaging effect of xanthine oxidase in radical- mediated diseases, but the use of allopurinol is restricted by formation of oxypurinol, which is known to cause side effects [29]. Thus, a prompted search for new XO inhibitors that are structurally distinct from pureness is a necessity [30]. Hence, phytochemicals or extracts, which inhibit XO and the $O_2^{\bullet-}$ regeneration by the enzymatic pathway, would be beneficial in preventing these side effects. The potent capacity in inhibition of XO and $O_2^{\bullet-}$ scavenging activity exhibited by TCE and the different fractions can be explained by their phenolic compounds levels and/or the other type of phenolic compounds, to be discovered antioxidant compounds [31]. Enroth *et al.,* [32] confirm existing structure-activity relationship. In phenolic compound, especially flavonoid, the hydroxyl groups at C-5 and C-7 and the double bond between C-2 and C-3 were essential for a high inhibitory activity on xanthine oxidase. Furthermore, they suggested that phe A planar flavone skeleton alone is insufficient to induce xanthine oxidase inhibition. It may be speculated that at least one hydroxyl group is necessary, favorable at position 7, to achieve xanthine oxidase inhibition by flavones [32].

The results clearly indicated that TCE are potent scavengers of $O_2^{\bullet-}$This can prevent the formation of ROS, which can react with biological macromolecules leading to tissue damages [33].

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