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Effect of *Rhus coriaria* L. (Anacardiaceae) on Superoxide Radical Scavenging and Xanthine Oxidase Activity

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***Rhus coriaria* L. (Anacardiaceae) is a well-known spice in the Middle-East and grown in the Central and East Anatolia region of Turkey. A methanolic extract (water-soluble part constituents) of *R. coriaria*, was found to be an uncompetitive inhibitor of xanthine oxidase and scavenger of superoxide radical in vitro with IC_{50} values of 172.5 μg/mL and 232 μg/mL respectively. Superoxide radicals were generated either by an enzymatic or a non-enzymatic system, and scavenging ability was evaluated by the inhibition of nitroblue tetrazolium reduction. This study provides evidence that a crude extract of *R. coriaria* exhibits interesting antioxidant properties, expressed either by the capacity to scavenge superoxide radical or to uncompetitively inhibit xanthine oxidase.**

Keywords: *Rhus coriaria* L; Superoxide radical scavenging activity; Xanthine oxidase inhibition

INTRODUCTION

Hydrogen peroxide, hydroxyl radicals, peroxide anions and superoxide anion are collectively known as reactive oxygen species (ROS). It is accepted that ROS play different roles in vivo. Some are beneficial and are related to their involvement in energy production, phagocytosis, regulation of cell growth and intercellular signaling, and synthesis of biologically important compounds.[1] However, ROS may be very damaging to the body since they can attack lipids in cell membranes, proteins in tissues or enzymes, carbohydrates and DNA, to induce oxidations which cause membrane damage, protein modification (including enzymes), and DNA damage. This oxidative damage is considered to play a causative role in aging and several degenerative diseases associated with it, such as heart disease, cataracts, cognitive dysfunction and cancer.[2–4]

Xanthine oxidase (XOD, EC1.2.3.2) is a key enzyme that catalyzes the oxidation of hypoxanthine to xanthine and then xanthine, in the presence of molecular oxygen as electron acceptor, to yield uric acid, superoxide anions and hydrogen peroxide.[5] These reactions are as follows:[6]

$$\text{Hypoxanthine} + O_2 + H_2O \rightarrow \text{Xanthine} + H_2O_2 \quad (1)$$

$$\text{Xanthine} + 2O_2 + H_2O \rightarrow \text{Uric acid} + 2O_2^{\bullet -} + 2H^+ \quad (2)$$

$$\text{Xanthine} + O_2 + H_2O \rightarrow \text{Uric acid} + H_2O_2 \quad (3)$$

Consequently, xanthine oxidase is considered to be an important biological source of superoxide radicals. XOD-derived superoxide anions have been linked to postischemic tissue injury and edema[7] as well as to changes in vascular permeability.[8] XOD can also oxidize synthetic purine drugs, thus neutralizing their biological activity.[9] The inhibition of this enzyme is therefore useful in the treatment of several diseases, such as gout and kidney stone production where uric acid levels are decreased and in ischemia-reperfusion processes[10] where it prevents damage caused by the free radicals produced.

Several compounds present in plants are reported to be inhibitors of xanthine oxidase[11–13] and to possess antioxidant properties.

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In this study, the scavenging effect has been investigated of the effects of *R. coriaria* fruits on superoxide radical employing either a xanthine/xanthine oxidase system or an NADH/phenazine methosulfate system, as well as their effect on the activity of a ROS-producing enzyme, xanthine oxidase itself.

MATERIALS AND METHODS

Chemicals

Xanthine, xanthine oxidase from buttermilk, β-nikotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), nitroblue tetrazolium chloride (NBT), were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade and were used without further purification.

Collection of Plant Material

R. coriaria fruits were collected from Gurun-Sivas, Turkey during September 1999 and identified by Dr. Erol Dönmez at the Department of Biology, Cumhuriyet University, Sivas, Turkey.

Extract Preparation

Pericarps of respective plant fruits were liberated from the kernels, air-dried (100 g) and successively extracted in a Soxhlet apparatus with methanol (1 L), at $\leq$60°C for 4 h. The extract was then filtered and concentrated in vacuo at 45°C. The resulting extract was resuspended in water and partitioned with chloroform to remove less polar principles. The water-soluble part was then lyophilised and kept in the dark at 4°C until used.

Enzymatic Assay of Superoxide Radicals

Superoxide radical generated by the xanthine–xanthine oxidase system was determined spectrophotometrically by monitoring its ability to reduce nitroblue tetrazolium (NBT).[14] Various concentrations of the extract were added to a reaction mixture containing 100 μM xanthine, 600 μM NBT, 0.05 U/mL xanthine oxidase and 0.1 M phosphate buffer (pH 7.4) and then made up to a final volume of 2.0 mL with buffer. After incubation at 25°C for 10 min, the absorbance was measured at 560 nm, compared with the control samples where the xanthine oxidase was absent. Percent scavenging of superoxide was calculated from the optical density of the treated and control samples in which there was no inhibitor.

Nonenzymatic Assay of Superoxide Radicals

The superoxide-producing system was set up by mixing phenazine methosulfate (PMS), NADH, and oxygen (air), and the production of superoxide was estimated by the nitroblue tetrazolium method. Measurement of superoxide radical scavenging activity was carried out on the basis of the method described by Fernandes *et al.*[15] with slight modifications. The reaction mixture (300 μL) containing 3 μM PMS, 78 μM NADH, and 25 μM NBT in 19 μM phosphate buffer pH 7.4 was incubated for 2 min at room temperature and the absorption measured at 560 nm against a blank containing PMS. Different concentrations of compounds were preincubated for 2 min before adding NADH.

Effect on Xanthine Oxidase Activity

The enzyme activity was measured spectrophotometrically by determining uric acid formation at 295 nm with xanthine as substrate.[11] Samples were dissolved in 0.1 M potassium phosphate buffer solution (PBS) pH 7.4 for each concentration. Then 50 μL of sample solution and 400 μL xanthine/PBS was added to 530 μL H_2O and mixed, and then 20 μL of 1 unit/mL xanthine oxidase/PBS was added, mixed vigorously, and the absorption measured for 2 min at 295 nm.

Protein Determination

Protein was determined by the procedure with comassie Brilliant Blue G-250 using bovine serum albumin as a standard.[16]

RESULTS AND DISCUSSION

Diets rich in fruits and vegetables significantly reduce the incidence and mortality rates of cancer, cardiovascular disorders, and other degenerative diseases caused by oxidative stress.[17,18] The protection of the organism against oxidative stress relies not only on endogenous antioxidants but also on exogenous compounds taken in food and beverages.[19]

Rhus coriaria L. (Anacardiaceae), a well-known spice, is familiar to the people in the Middle-East and grown in the Central and the East Anatolia region of Turkey. It has been used as a folk medicine for the treatment of diarrhoea and bleeding, and also an anticeptic.[20] Previous phytochemical investigations of this species have demonstrated that it contains gallotannins and polyphenolic components.[21,22]

In the present work, an effective antioxidant activity was found in the methanolic extract of *Rhus coriaria* L. fruits which exhibited superoxide radical scavenging activity using the xanthine–xanthine oxidase system (Figure 1A), with an IC_{50}

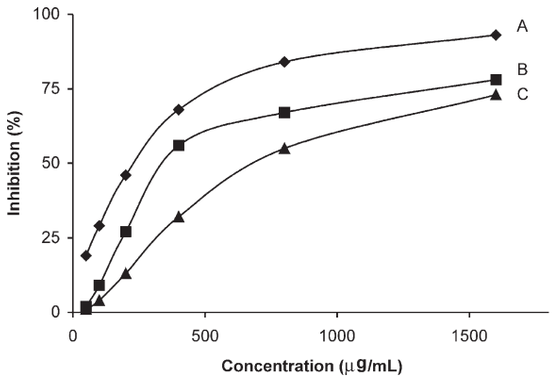

FIGURE 1 Effect of *R. coriaria* on; (A) NBT reduction induced by superoxide radical generated in an X/XOD system; (B) XOD activity, and (C) NBT reduction induced by superoxide radical generated in an NADH/PMS system. Values are the mean from four experiments performed in triplicate.

of 232 µg/mL. When this method is used, the effect of the extract on the XOD activity must be checked, because an inhibitory effect on the enzyme itself would also lead to a decrease in NBT reduction.[19] In this regard, the effect of the extract on the XOD activity was evaluated by the metabolic conversion of xanthine to uric acid.

The activity of XOD was measured as 935.1 ± 5.2 nmol of uric acid produced min^{-1} (mg of protein)$^{-1}$. *R. coriaria* L. at concentrations between 50 and 1500 µg/mL inhibited the activity of this enzyme (Figure 1B).

To characterize the mechanism of *R. coriaria* L. action, XOD was chosen as an enzyme whose action was affected by *R. coriaria* L. These selections were made on the basis of the high stability of XOD and the ease with which its activity can be determined.

To evaluate XOD inhibition by *R. coriaria* L. a steady-state analysis of XOD activities was performed in which the concentrations of xanthine and *R. coriaria* were varied systematically. It was found that *R. coriaria* L. is an uncompetitive inhibitor of XOD with respect to xanthine as substrate; the lines at different concentrations of *R. coriaria* L. in the Lineweaver–Burk plot are parallel (Figure 2). Therefore, the binding site of xanthine oxidase with *R. coriaria* L. is not the molybdenum site (substrate binding site) but the iron–sulfur group (Fe_2–S_2) in the enzyme molecule.[11] The type of inhibition observed in the Lineweaver–Burk was also confirmed by using Cornish–Bowden and Dixon plots (not shown). The plot of the y-axis of the Lineweaver–Burk intercept versus *R. coriaria* L. concentration (Figure 2, inset) gave an apparent K_i value of 172.5 µg/mL for XOD. In the absence of *R. coriaria* L. an apparent K_m value of 1.85 µM was observed for XOD, which approximates the literature K_m value with xanthine as substrate (1.7 µM).[23] For further clarification, the effect of the extract was also determined on superoxide generated by the NADH/PMS system (Figure 1C), which indicated an I_{50} of 662 µg/mL. Considering the results obtained, it may be anticipated that the extract of *R. coriaria* L. has antioxidant activity, shown here by the scavenging of superoxide radical and xanthine oxidase inhibition.

Although the extract is not very potent, purified constituents would necessarily show a higher potency. Their isolation, characterisation and biological in vitro testing may provide a lead compound for the development of more potent agents as potential drugs for the treatments of diseases related to xanthine oxidase.

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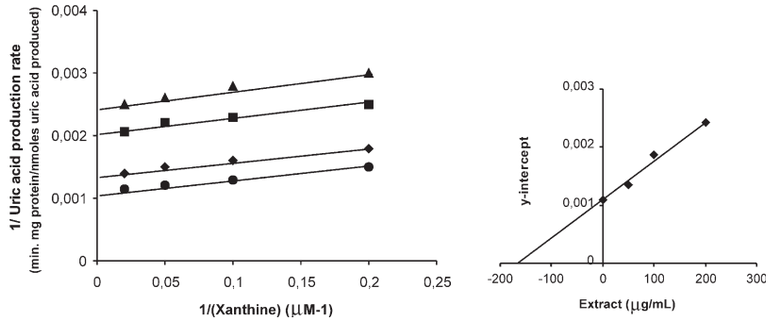

FIGURE 2 Lineweaver–Burk plot of steady-state analysis of XOD inhibition by *R. coriaria*. The reaction mixture contained 6 milliunits/ml XOD. Xanthine concentration varied at a series of fixed concentrations of *R. coriaria*; Results show an uncompetitive type of inhibition. *R. coriaria* concentrations used were (from bottom to top) 0, 50, 100, 200 µg/mL. The inset is a secondary plot of the y-intercept versus *R. coriaria* concentration. A K_i value of 173 µg/mL was estimated for the inhibition of XOD by *R. coriaria*. Values are the mean from three experiments performed in triplicate.

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