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A search for hepatoprotective activity of aqueous extract of *Rhus coriaria* L. against oxidative stress cytotoxicity

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ABSTRACT

The protective effects of different concentrations of aqueous extract of *Rhus coriaria* L. fruit (75 and 100 μg/ml) and also gallic acid (100 μM) as one of its main components were examined against oxidative stress toxicity induced by cumene hydroperoxide (CHP) in isolated rat hepatocytes. Both extract concentrations and gallic acid (100 μM) significantly ($P < 0.05$) protected the hepatocyte against all oxidative stress markers including cell lysis, ROS generation, lipid peroxidation, glutathione depletion, mitochondrial membrane potential decrease, lysosomal membrane oxidative damage and cellular proteolysis. Aqueous extracts of *Rhus coriaria* L. (75 and 100 μg/ml) were more effective than gallic acid (100 μM) in protecting hepatocytes against CHP induced lipid peroxidation ($P < 0.05$). On the other hand gallic acid (100 μM) acted more effective than aqueous extracts of *Rhus coriaria* L. (75 and 100 μg/ml) at preventing hepatocyte membrane lysis ($P < 0.05$). In addition H_2O_2 scavenging effect of both extract concentrations (75 and 100 μg/ml) were determined in hepatocytes and compared with gallic acid (100 μM). Gallic acid (100 μM) was more effective than aqueous extracts of *Rhus coriaria* L. (75 and 100 μg/ml) at H_2O_2 scavenging activity ($P < 0.05$).

1. Introduction

Sumac is the common name for a genus (Rhus) that contains over 250 individual species of flowering plants in the family Anacardiaceae (USDA, 2007). This genus is found in temperate and tropical regions worldwide, with representative members by geographic location. In general, sumac can grow in non-agriculturally viable regions, and various species have been used by indigenous cultures for medicinal and other purposes. *Rhus coriaria* (tanner's sumac), which grows wild in the region from the Canary Islands through the Mediterranean region to Iran and Afghanistan, is commonly used as a spice by grinding the dried fruits with salt, and is also widely used as a medicinal herb in the Mediterranean and Middle East, particularly for wound healing (Sezik et al., 1991). In Persian traditional medicine, sumac (*Rhus coriaria*) is believed to have atheroprotective effects and is consumed in some Persian dishes. This spice comes from the berries of a wild bush that grows in all Mediterranean areas.

The research efforts on sumac extracts to date indicate a promising potential for this plant family to provide renewable bioproducts with the following reported desirable bioactivities: antifibrogenic (Lee et al., 2003), antifungal (McCutcheon et al., 1994), anti-inflammatory (Fourie and Snyckers, 1984), antimalarial (Ahmed et al., 2001), antimicrobial (McCutcheon et al., 1992), antitumorigenic activities (Lee et al.,2004; Park et al., 2004), antioxidant (Lee et al., 2002), antithrombin (Kuo et al., 1991), antiviral (Lin et al., 1999), hypoglycaemic (Giancarlo et al., 2006), and leukopenic (Du et al., 1999; Yang and Du, 2003). Sumac may also have potential for the prevention or treatment of atherosclerosis and its clinical manifestations (Zargham and Zargham, 2008).

Previous studies have suggested that extract of *Rhus coriaria* L. fruits may be a source of natural antioxidants (Ozcan, 2003; Candan and Sokmen, 2004). Sumac is a rich source of hydrolysable tannins (Kosar et al., 2007). Tannin and its derivatives are strong antioxidants. Tannins have also been shown in vitro and in vivo to exhibit anticarcinogenic properties, such as the induction of cell cycle arrest and apoptosis as well as the inhibition of tumor formation and growth in animals (Perchellet et al., 1992).

Oxidative stress plays an important role in the initiation and progression of many liver diseases. Due to antioxidant and free radical scavenging activities of sumac, we planned to study liver protective effects of aqueous extract of *Rhus coriaria* L. fruit against

Abbreviations: ACMS, accelerated cytotoxicity mechanisms screening; ANOVA, analysis of variance; BSA, bovine serum albumin; CHP, cumene hydroperoxide; DCF, dichlorofluorescein; DCFH-DA, dichlorofluorescin diacetate; GLU/GO, glucose/glucose oxidase; GSH, glutathione (reduced form); GSSG, glutathione (oxidized form); HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; ROS, reactive oxygen species; rpm, rotations per minute; SD, standard deviation; TBARS, 2-thiobarbituric acid-reactive substances; $\%\Delta\Psi m$, percentage of mitochondrial membrane potential decline.

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various cellular and sub-cellular features of oxidative stress, using accelerated cytotoxicity mechanisms screening (ACMS) techniques and isolated Sprague–Dawley rat hepatocytes as cellular model. These cells are the most similar mammalian cells to human liver hepatocytes (Pourahmad et al., 2008).

2. Materials and methods

2.1. Preparation of fruit extract

Rhus coriaria L. fruits were collected from Alborz Mountains, Aderan region, North West of the capital Tehran, Iran. The collected fruits were scientifically approved in the Department of Botany, Shaheed Beheshti University (Voucher number: 917A, deposited in: Shaheed Beheshti University Herbarium (SBU)). Fresh fruits were cleaned, and then dried in shade at room temperature. Fruits were decocted in water for 30 min. Then the extract was filtered and concentrated to the desired level with honey-like viscosity, and stored in the freezer. Moisture of extract was determined as follows: 2 g of final extract was placed in an oven in 60–65 °C for 72 h, and then weighed, and weight loss used as a moisture indicator. The final extract contained 24% water. This extract was dissolved in distilled water (DW) at the desired concentrations just before use (Ahmadiani et al., 2001).

We chose a wide concentration range for aqueous extract of *Rhus coriaria* L. fruit in our pilot study and their inhibitory effects against cumene hydroperoxide (CHP) induced hepatocyte toxicity were evaluated (data not shown). By omitting non effective, poor effective or toxic concentrations, the 75 and 100 μg/ml concentrations were selected.

2.2. Chemicals

Rhodamine 123, collagenase, bovine serum albumin (BSA), *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), reduced and oxidized glutathione (GSH and GSSG), Acridine orange, 2′,7′-dichlorofluorescin diacetate (DCFH-DA), Trichloroacetic acid, Trypan blue and heparin were purchased from Sigma–Aldrich Co. (Taufkrichen, Germany). All other chemicals were of the highest commercial grade available.

2.3. Animals

Male Sprague–Dawley rats (280–300 g) purchased from Institute Pasteur (Tehran, Iran), fed with a standard chow diet and water ad libitum, used for hepatocyte preparation. All experiments were conducted according to ethical standards and protocols approved by the Committee of Animal Experimentation of Shaheed Beheshti University of Medical Sciences, Tehran, Iran. The ethical standards were based on "European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes" Acts of 1986, and the "Guiding Principles in the Use of Animals in Toxicology", adopted by the Society of Toxicology in 1989, for the acceptable use of experimental animals. All animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86–23, revised 1985).

2.4. Isolation and incubation of hepatocytes

Hepatocytes were obtained by collagenase perfusion of the liver and viability was assessed by plasma membrane disruption determined by trypan blue (0.2 w/v) exclusion test (Pourahmad and O'Brien, 2000). Cells were suspended at a density of 10^6 cells/ml in round-bottomed flasks rotating in a water bath maintained at 37 °C in Krebs–Henseleit buffer (pH = 7.4), supplemented with 12.5 mM HEPES under an atmosphere of 10% O_2, 85% N_2, and 5% CO_2.

2.5. Cell viability

The viability of isolated hepatocytes was assessed from the intactness of the plasma membrane as determined by the trypan blue (0.2% w/v) exclusion test (Pourahmad and O'Brien, 2000). Aliquots of the hepatocyte incubate were taken at different time points during the 3 h incubation period. At least 80–90% of the control cells were still viable after 3 h.

2.6. Determination of reactive oxygen species

To determine the rate of hepatocyte reactive oxygen species (ROS) generation induced by cumene hydroperoxide, dichlorofluorescin diacetate (DCFH-DA) was added to the hepatocytes. It penetrates hepatocyte cells and becomes hydrolyzed to non-fluorescent dichlorofluorescin. The latter then reacts with ROS to form the highly fluorescent dichlorofluorescein (DCF), which effluxes the cell. The fluorescence intensity of DCF was measured using a Shimadzu RF5000U fluorescence spectrophotometer. Excitation and emission wavelengths were 500 and 520 nm, respectively. The results were expressed as fluorescent intensity per 10^6 cells (Shen et al., 1996).

2.7. Lipid peroxidation assay

Hepatocyte lipid peroxidation was determined by measuring the amount of thiobarbituric acid-reactive substances (TBARS) formed during the decomposition of lipid hydroperoxides by following the absorbance at 532 nm in a Beckman DU®-7 spectrophotometer (Smith et al., 1982).

2.8. Intracellular GSH and extra cellular GSSG assessment

GSH and GSSG were determined according to the spectrofluorometric method (Hissin and Hilf, 1976). Each sample was measured in quarts cuvettes using a fluorimeter set for 350 nm excitation and 420 nm emission wavelengths.

2.9. Mitochondrial membrane potential assay

Mitochondrial uptake of the cationic fluorescent dye, rhodamine123, has been used for estimation of mitochondrial membrane potential (Andersson et al., 1987). The amount of rhodamine123 remaining in the incubation medium was measured fluorometrically using a Shimadzu RF5000U fluorescence spectrophotometer set at 490 nm excitation and 520 nm emission wavelengths. The capacity of mitochondria to uptake the rhodamine123 was calculated as the difference (between control and treated cells) in rhodamine123 fluorescence. Our data were shown as the percentage of mitochondrial membrane potential collapse ($\%\Delta\Psi m$) in all treated (test) hepatocyte groups (Andersson et al., 1987).

2.10. Lysosomal membrane integrity assay

Hepatocyte lysosomal membrane stability was determined from the redistribution of the fluorescent dye, acridine orange (Pourahmad et al., 2001). Aliquots of the cell suspension (0.5 ml) that were previously stained with acridine orange (5 μM) were separated from the incubation medium by 1 min centrifugation at 1000 rpm. The cell pellet was then resuspended in 2 ml of fresh incubation medium. This washing process was carried out for two times to remove the fluorescent dye from the media. Acridine orange redistribution in the cell suspension was then measured fluorometrically using a Shimadzu RF5000U fluorescence spectrophotometer set at 495 nm excitation and 530 nm emission wavelengths. Lysosomal membrane damage was determined as difference in redistribution of acridine orange from lysosomes into cytosol between treated cells and control cells. Our data were shown as the percentage of lysosomal membrane leakiness in all treated (test) hepatocyte groups (Pourahmad et al., 2001).

2.11. Determination of proteolysis

Proteolysis was monitored using a fluorescence assay for tyrosine release (adapted from Novak et al., 1988). An aliquot of the hepatocyte suspension was precipitated with an equal volume of 20% trichloroacetic acid and allowed to stand overnight at 4 °C. The sample was vortexed and centrifuged in a bench top clinical centrifuge (at 13250 rpm for 15 min). One ml of the supernatant was removed and placed in a test tube to which was added one ml of 0.2% solution of 1-nitroso-2-naphthol and one ml acid nitrite reagent (10 mg/ml $NaNO_2$ in 20% HNO_3). The solution was vortexed, covered with parafilm, and incubated at 37 °C for 30 min. Five ml of ethylene dichloride was added to the test tube. The mixture was vortexed vigorously and the sample was centrifuged for 10 min at high speed. The fluorescence of the aqueous phase was read in a Shimadzu RF5000U spectrophotometer (excitation at 460 nm and emission at 570 nm). The tyrosine content of the sample was determined from a standard curve constructed from known concentrations of tyrosine (0–100 μM).

2.12. Statistical analysis

Levene's test was used to check the homogeneity of variances. Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD as the *post hoc* test. Results were presented as mean ± SD of triplicate samples. The minimal level of significance chosen was $P < 0.05$.

3. Results

As shown in Table 1 cumene hydroperoxide (CHP) produced a marked increase in cytotoxicity, TBARS, and reactive oxygen species (ROS) generation. Aqueous extract of *Rhus coriaria* L. fruit at concentrations of 75 and 100 μg/ml and gallic acid (100 μM) significantly ($P < 0.05$) prevented CHP induced hepatocyte membrane lysis as well as ROS formation and lipid peroxidation. Gallic acid