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Original Article

Cytoprotective Effects of Hydrophilic and Lipophilic Extracts of *Pistacia vera* against Oxidative Versus Carbonyl Stress in Rat Hepatocytes

Jafar Shahraki[a, b], Mona Zareh[a], Mohammad Kamalinejad[a] and Jalal Pourahmad[a*]

[a]*Faculty of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, P.O. Box 14155-6153, Iran.* [b]*Department of Pharmacology and Toxicology, Faculty of Pharmacy, Zabol University of Medical Sciences, Zabol, Iran.*

Abstract

This study was conducted to evaluate the cytoprotection of various extracts and bioactive compounds found in Pistacia vera againts cytotoxicity, ROS formation, lipid peroxidation, protein carbonylation, mitochondrial and lysosomal membrane damages in cell toxicity models of diabetes related carbonyl (glyoxal) and oxidative stress (hydroperoxide). Methanol, water and ethyl acetate were used to prepare crude pistachios extracts, which were then used to screen for *in-vitro* cytoprotection of freshly isolated rat hepatocytes against these toxins. The order of protection by Pistacia vera extracts against both hydroperoxide induced oxidative stress (ROS formation) and glyoxal induced protein carbonylation was: pistachio methanolic extract > pistachio water extract, gallic acid, catechin > α-tochoferol and pistachio ethyl acetate extract. Finally due to higher protection achieved by methanolic extract even compared to sole pretreatment of gallic acid, catechin or α-tochoferol, we suggest that cytoprotection depends on the variety of polar and non-polar compounds found in methanolic extract, it is likely that multiple cytoprotective mechanisms are acting against oxidative and carbonyl induced cytotoxicity. To our knowledge, we are the first to report the cytoprotective activity of Pistacia vera extracts against oxidative and carbonyl stress seen in type 2 diabetes hepatocytes model.

Keywords: *Pistacia vera*; Cytotoprotection; Protein carbonylation; Oxidative stress; Lysosomes; Mitochondria.

Introduction

Diabetes mellitus is a metabolic disorder characterized by elevated blood glucose (hyperglycemia)due to insufficiency in secretion or action of endogenous insulin. Elevated plasma glucose leads to the formation and accumulation of advanced glycation end products (AGEs) (advanced glycation end products) and advanced lipoxidation end products (ALEs) (advanced lipoxidation end products) believed to cause the long term micro-vascular and macrovascular complications of diabetes such as cardiovascular disease, retinopathy, neuropathy, and nephropathy (1). AGEs and ALEs are modifications of proteins or lipids that became non-enzymatically glycated and oxidized after contact with aldose sugars. AGEs formation are a two-staged reaction. Early glycation and oxidation processes that are reversible, result in the formation of Schiff bases and Amadori products. Further glycation through Maillard reaction of proteins and lipids causes molecular rearrangements that lead to the generation of stable AGEs(2).The Maillard reaction, begins

* Corresponding author:
E-mail: j.pourahmadjaktaji@utoronto.ca

from Schiff bases and the Amadori product, a 1-amino-1-deoxyketose, produced by the reaction of the carbonyl group of a reducing sugar, like glucose, with proteins, lipids, and nucleic acid amino groups. During Amadori reorganization, these highly reactive intermediate carbonyl groups, known as α-dicarbonyls or oxoaldehydes, products of which include 3-deoxyglucosone (3-DG), glyoxal (GO) and methylglyoxal (MGO), accumulate. Such buildup is referred to as "carbonyl stress"(2).

Diabetes has been associated with increased levels of ROS(reactive oxygen species) along with a decreased ability to prevent oxidative stress (3-5). Chronic hyperglycemia leads to reactive oxygen species (ROS) and reactive carbonyl species (RCS) formation, both of which have been implicated as possible causative factors for tissue damage, and the development of long-term complications in diabetes (1, 6, 7). Carbonyl stress is caused by a generalized increase in the concentration of reactive carbonyl precursors of AGEs, glycoxidation and lipoxidation products(2).

Adherence to a diabetic diet is an important aspect of controlling elevated blood sugar in patients with diabetes. The American Diabetes Association (ADA) has provided guidelines for a diabetic diet. The ADA diet is a balanced, nutritious diet that is low in fat, cholesterol, and simple sugars containing most notably fruits, vegetables, and nuts. Weight reduction and exercise are also important treatments for diabetes (8).

Control blood glucose levels for prevention of the complications of diabetes is one of the main goal of diabetes treatments. It›s also important to bear in mind that the cost of drug therapy is relatively small compared to the cost of managing the long-term complications associated with poorly controlled diabetes(8). Since the oral hypoglycemic agents may cause side effects (9, 10), non-pharmacological strategies to reduce or prevent the onset of diabetic complications are of interest. Therefore, studying dietary sources of antioxidants or natural preventing AGEs formation compound may be useful in the context of ameliorating oxidative and carbonyl stress seen in type 2 diabetes.There are some evidences that show nuts have good effects in diabetic patients. ADA has claimed an ounce of nuts can go a long way in providing key healthy fats along with hunger management (8).

Pistacia vera Linn., a member of the Anacardiaceae family, is a native of the arid zones of Central and West Asia and distributed throughout the Mediterranean basin. Pistachio is the nut of the tree, having an edible green kernel enclosed in a woody shell (11). It has been previously shown that pistachios possess multiple pharmacological effects such as antimicrobial (12, 13), anti-hyperlipidemia (14) anti-nociceptive, anti-inflammatory (11) anti-protozoal (15), hepato protective activity and anti-erectile dysfunction effect(16).

Antioxidant effect, reducing plasma lipid levels, and body weight-lowering effects of pistachios are well known. Pistachios with this characteristics, looks a food supplement for the prevention of diabetes complications progression. In this study, we investigated the protective effects of water, methanol and ethyl acetate extracts of pistachios against both oxidative stress and carbonyl stress in diabetic hepatocyte model.

Experimental

Chemicals

Collagenase, Glyoxal (GO), Cumene hydroperoxide (CHP) (cumene hydroperoxide), Trichloroacetic acid (TCA), 2,4-dinitrophenyl hydrazine, 2,7 dichlorofluorescin diacetate (DCFD), dimethyl sulfoxide (DMSO), Methanol , Ethanol, Trypan blue, Rhodamine 123, bovine serum albumin (BSA), N-(2-hydroxyethyl) piperazine-Ń-(2-ethanesulfonic acid) (HEPES), Acridine orange (AO), Thiobarbituric acid (TBA), Rhodamine, N-Ethylmaleimide (NEM) , Orthophthaldehyde (OPT), and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of crude pistachios extracts

Whole pistachios purchased from arboretum in Kashan, Isfahan Province, Iran. 6 g of pistachios were crushed into a fine powder with a glass mortar and pestle. The ground pistachios powders were added to 30 mL water, methanol, and ethyl acetate in 125 mL Erlenmeyer flasks. The flasks

were placed on stirrer and mixed for 3 days.

Each mixture was separated by vacuum filtration, and the liquid extract (filtrate) was collected. These processes repeated for three times, then filtrate placed in rotary and solvents: water, methanol, and ethyl acetate were evaporated from the filtrates. The extracts were weighed and frozen at -70 °C until further use. The extracts were used within 6 months, and were dissolved in water, methanol, and DMSO(17). In concentration that we used in these experiments water, methanol, and DMSO alone did not affect hydroperoxide or glyoxal cytotoxicity.

Animal treatment and hepatocyte preparation

Male Sprague–Dawley rats (280–300 g) purchased from Pasteur Institute (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 Shahid 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 (18)). Hepatocytes were isolated by collagenase liver perfusion as described previously by Moldeus and Orrenius (19). Isolated hepatocytes (10^6 cells/mL) (10 mL) were suspended in Krebs–Henseleit buffer(pH 7.4) containing 12.5 mM HEPES in continually rotating 50ml round-bottom flasks, under an atmosphere of 95% O2 and 5% CO2 in a water bath of 37°C. Stock solutions of chemicals, were prepared immediately prior to use. Each flask contained 10 mL of hepatocyte suspension. Hepatocytes were pre incubated for 30 min prior to the addition of chemicals. Stock solutions of all chemicals (×100 concentrated) were prepared fresh prior to use(20). To avoid either non-toxic or severe toxic conditions in this study, EC50 concentrations were used for algae lysate. To incubate treatments with the required concentration, 100 µL sample of concentrated stock solution (×100 concentrated) was added to the rotating flask containing 10 mL of hepatocyte suspension. In the case of CHP toxicity assessments, all the extracts/inhibitors were added 30 min prior to addition of CHP into hepatocytes (21, 22). In the case of glyoxal toxicity assessments, all the extracts/inhibitors were pre-mixed with glyoxal 30 min prior to addition to hepatocytes. This treatment will increase the carbonyl scavenging activity of all the extracts/inhibitors (23).

Hepatocyte viability

Hepatocyte viability was tested microscopically by plasma membrane disruption, as determined by the trypan blue (0.1% w/v) exclusion test(24). Hepatocyte viability was assessed every 30 min during the 3 h incubation period, and the cells were at least 80–90% viable before use. To avoid either non-toxic or severe toxic conditions in this study, $EC50_{2h}$ concentrations were used for either glyoxal (GO) or Cumene hydroperoxide (CHP). The $EC50_{2h}$ of a chemical in hepatocyte cytotoxicity assessment technique is defined as the concentration, which decreased the hepatocyte viability to 50% following the 2 h incubation period.

Lipid peroxidation determination in hepatocyte

Lipid peroxidation was assayed by measuring the amount of thiobarbituric acid reactive substances (TBARS), formed during the lipid hydroperoxide decomposition, mostly formed from malondialdehyde (MDA) with the pink adduct being measured at 532 nm.

1ml aliquots of hepatocyte suspension (10^6 cells/mL) were treated with trichloroacetic acid (TCA) (250 µL, 70% w/v) to stop the reaction and lyse the cells. After this, 1 mL of thiobarbituric, acid (TBA) (0.8% w/v) was added to the cells to determine the formation of lipid peroxidation products. Suspensions were incubated in a boiling water bath for 20 min, after which they were cooled on ice for 5 min, and centrifuged at 4000 rpm for 5 min. The supernatant was

read at 532 nm using a Beckman DU®-7 spectrophotometer (25). The concentrations of TBARs were expressed as μM concentration of MDA and calculated by using a molar extinction coefficient of 1.56×10^5 mol^{-1} cm^{-1}.

Determination of reactive oxygen species

Hepatocyte reactive oxygen species (ROS) generation induced by the cumenehydroperoxide was determined by adding dichlorofluorescin diacetate (DCFD) to the hepatocyte incubate. DCFD penetrates hepatocytes and is hydrolyzed to form non-fluorescent dichlorofluorescin. Dichlorofluorescin is then oxidized by ROS to form the highly fluorescent dichlorofluorescein which effluxes the cell. After incubation with CHP, 1 mL samples of hepatocytes were withdrawn at 90 min and centrifuged at 50 rpm for 1 min. The cells were resuspended in Krebs–Henseleit buffer and 1.6 μL DCFD was added(26). Cells were allowed to incubateat37 °C for 10 min. Excitation and emission wavelengths were 490 and 520 nm, respectively.

Measurement of reduced and oxidized glutathione

We measured the concentration of the reduced (GSH) and oxidized (GSSG) glutathione using a spectro fluorometric method (27). In order to confirm the linearity of the reaction rate in the adopted method, we used commercially purified GSH and GSSG to calibrate the standard curve. For GSH measurement, the final reaction mixture volume was 200 μL, which contained 180 μL of phosphate–EDTA buffer (0.1 M sodium phosphate–0.005 M EDTA, pH=8.0), 10 μL of o-Phthalaldehyde(OPT, 100 μg per 100 μL methanol) and 10 μL of diluted sample (1:10 in phosphate–EDTA buffer). The reaction mixture was incubated for 15 min at room temperature, and the fluorescence was measured at excitation and emission wavelength of 350 nm and 450 nm, respectively using Hitachi F-2500 fluorescence spectrophotometer. The GSH content was expressed as μM per 10^6 cells/mL. GSSG was then measured using the same method outlined above, except that the sample was diluted with 10 volumes of 0.1 N NaOH containing 0.04 M of N-ethylmaleimide, instead of phosphate–EDTA buffer, in order to prevent the further oxidation of GSH to GSSG the pH of solution was adjusted to 12. The GSSG content was also expressed as μM per 10^6 cells/mL.

Mitochondrial membrane potential assay

The uptake and retention of the cationic fluorescent dye, rhodamine 123, has been used for the estimation of mitochondrial membrane potential. This assay is based on the fact that rhodamine 123 accumulates selectively in the mitochondria by facilitated diffusion. However, when the mitochondrial potential is decreased, the amount of rhodamine 123 that enters the mitochondria is also decreased as there is no facilitated diffusion. Thus, the amount of rhodamine 123 in the supernatant is increased and the amount in the pellet is decreased. Samples (500 μL) were taken from the cell suspension incubated at 37 °C at different time points, and centrifuged at 50×g for 1 min. The cell pellet was then resuspended in 2 mL of fresh incubation medium containing 1.5 μM rhodamine 123 and incubated at 37 °C in a thermostatic bath for 10 mins with gentle shaking. Hepatocytes were separated by centrifugation and the amount of rhodamine 123 appearing in the incubation medium was measured fluorimetrically, using Hitachi F-2500 fluorescence spectrophotometer set at 490 nm excitation and 520 nm emission wavelengths. The capacity of mitochondria to take up the rhodamine 123 was calculated as the difference in fluorescence intensity between control and treated cells (28).

Lysosomal membrane integrity assay

Hepatocyte lysosomal membrane stability was determined from the redistribution of the fluorescent dye, acridine orange(29). 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 fluorimetrically Hitachi F-2500 fluorescence spectrophotometer set at 495 nm excitation and 530 nm emission wavelengths.

Table 1. Protective effects of different pistachios extracts against cumene hydroperoxide and glyoxal induced cytotoxicity.

Addition	Percent Cytotoxicity (%)		
	1 h	2 h	3 h
control	10 ± 2	17 ± 2	24 ± 2
+cumene hydroperoxide(120 µM)	31 ± 3[a]	52 ± 3[a]	87 ± 3[a]
+butylatedhydroxytoluene (50 µM)	11 ± 2[b]	19 ± 2[b]	28 ± 3[b]
+penicillamine (5 mM)	18 ± 2[b]	38 ± 3[b]	54 ± 4[b]
+pistachios ethyl acetate extract (150 µg/mL)	22 ± 2[b]	41 ± 3[b]	45 ± 3[b]
+pistachios methanolic extract (150 µg/mL)	13 ± 2[b]	23 ± 2[b]	32 ± 3[b]
+pistachios water extract (150 µg/mL)	17 ± 3[b]	28 ± 2[b]	43 ± 4[b]
+α-tochoferol(100 µM)	23 ± 2[b]	44 ± 3[b]	49 ± 4[b]
+gallic acid (100 µM)	21 ± 3[b]	32 ± 3[b]	46 ± 3[b]
+catechin (5 mM)	19 ± 2[b]	30 ± 3[b]	44 ± 4[b]
+glyoxal (5 mM)	36 ± 3[a]	48 ± 4[a]	90 ± 5[a]
+butylatedhydroxytoluene (50 µM)	21 ± 3[b]	37 ± 2[b]	48 ± 5[b]
+penicillamine (5 mM)	15 ± 2[b]	35 ± 3[b]	46 ± 4[b]
+pistachios ethyl acetate extract (150 µg/mL)	18 ± 2[b]	35 ± 3[b]	67 ± 4[b]
+pistachios methanolic extract (150 µg/mL)	13 ± 2[b]	24 ± 2[b]	38 ± 3[b]
+pistachios water extract (150 µg/mL)	14 ± 2[b]	29 ± 3[b]	49 ± 3[b]
+α-tochoferol(100 µM)	19 ± 3[b]	38 ± 3[b]	69 ± 3[b]
+gallic acid (100 µM)	17 ± 2[b]	32 ± 2[b]	53 ± 3[b]
+catechin (5 mM)	16 ± 2[b]	30 ± 2[b]	50 ± 4[b]

Hepatocytes (10^6cells/mL) were incubated in Krebs–Henseleit buffer pH 7.4 at 37 °C for 3.0 h following the addition of $EC50_{2h}$ of cumene hydroperoxide and glyoxal. Cytotoxicity was determined as the percentage of cells that take up trypan blue (Pourahmad and O'Brien, 2000).

Values are expressed as mean±SD of three separate experiments (n=5).

[a] Significant difference in comparison with control hepatocytes ($P < 0.05$).

[b] Significant difference in comparison with cumene hydroperoxideor glyoxal treated hepatocytes ($P < 0.05$).

Determination of protein carbonyl content

The total protein bound carbonyl content was determined by derivatizing the protein carbonyl adducts with 2,4dinitrophenylhydrazine (DNPH). Briefly, 0.5 mL of cells (0.5×10^6 cells) was incubated for a 1 h at room temperature with 0.5 mL of DNPH (0.1% w/v) in 2N HCl. 1 mL of TCA (20% w/v) was added to the suspension to stop the reaction. The sample was centrifuged at 500 rpm to obtain the cellular pellet, and the supernatant was removed. DNPH was removed by extracting the pellet three times using 0.5 mL of ethyl acetate: ethanol (1:1) solution. After the extraction, the pellet (cellular protein) was dried under a gentle stream of nitrogen and dissolved in 1 mL of Tris-buffered 8.0 M guanidine–HCl (pH 7.2). The solubilized hydrazones were measured at 370 nm. The concentration of DNPH derivatized proteins was determined by the molar extinction coefficient of $22.000M^{-1}\,cm^{-1}$(30).

Statistical analysis

Levene's test was used to check the homogeneity of variances. Data was analyzed using one-way analysis of variance 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 $P < 0.05$.Dataare presented as mean ±SD (n=5).

Results

The $EC_{50, 2h}$ concentration (the concentration which decreases the hepatocyte viability by 50% following the 2 h of exposure) found for CHP and GO were equivalent to 120 µM and 5mM, respectively. As shown in Table 1 CHP and GO

Table 2. Protective effects of different pistachios extracts against cumene hydroperoxide and glyoxal induced ROS formation**.**

Addition	ROS formation		
	15 min	30 min	60 min
control	114 ± 9	158 ± 7	193 ± 9
+cumene hydroperoxide(120 μM)	225 ± 8[a]	291 ± 9[a]	531 ± 12[a]
+butylatedhydroxytoluene (50 μM)	127 ± 5[b]	182 ± 8[b]	241 ± 12[b]
+pistachios ethyl acetate extract (150 μg/mL)	174 ± 5[b]	246 ± 8[b]	417 ± 14[b]
+pistachios methanolic extract (150 μg/mL)	146 ± 4[b]	183 ± 6[b]	305 ± 11[b]
+pistachios water extract (150 μg/mL)	167 ± 5[b]	213 ± 8[b]	361 ± 10[b]
+α-tochoferol(100 μM)	181 ± 9[b]	250 ± 8[b]	422 ± 11[b]
+gallic acid (100 μM)	172 ± 7[b]	225 ± 8[b]	385 ± 9[b]
+catechin (5 mM)	169 ± 5[b]	217 ± 8[b]	368 ± 9[b]
+glyoxal (5 mM)	194 ± 8[a]	256 ± 9[a]	449 ± 9[a]
+butylatedhydroxytoluene (50 μM)	117 ± 8[b]	163 ± 5[b]	228 ± 7[b]
+pistachios ethyl acetate extract (150 μg/mL)	152 ± 4[b]	204 ± 8[b]	339 ± 7[b]
+pistachios methanolic extract (150 μg/mL)	126 ± 5[b]	177 ± 6[b]	270 ± 7[b]
+pistachios water extract (150 μg/mL)	154 ± 9[b]	193 ± 10[b]	293 ± 8[b]
+α-tochoferol(100 μM)	160 ± 5[b]	210 ± 8[b]	342 ± 8[b]
+gallic acid (100 μM)	153 ± 5[b]	199 ± 7[b]	299 ± 8[b]
+catechin (5 mM)	158 ± 5[b]	197 ± 7[b]	295 ± 8[b]

Hepatocytes (10^6 cells/mL) were incubated in Krebs–Henseleit buffer pH 7.4 at 37°C for 1.0 h following the addition of $EC50_{2h}$ of cumene hydroperoxide and glyoxal. DCF formation was expressed as fluorescent intensity units (Shen *et al*., 1996).
Values are expressed as mean±SD of three separate experiments (n=5).
[a] Significant difference in comparison with control hepatocytes ($P < 0.05$).
[b] Significant difference in comparison with cumene hydroperoxide or glyoxal treated hepatocytes ($P < 0.05$).

significantly increased hepatocyte cytotoxicity (hepatocyte membrane lysis) compared to control hepatocytes (P < 0.05).CHP and GO induced cytotoxicity were significantly (P < 0.05) prevented by methanolic, water and ethyl acetate extracts of *Pistacia vera* at concentration of 150 μg/mL. The order of protection against cell death was: pistachio methanolic extract > pistachio aqueous extract (water extract) > non-polar pistachio extract (ethyl acetate extract). Pistachios extract (150 μg/mL) as well as BHT(butylated hydroxytoluene), penecilamin, α-tochoferol, gallic acid and catechin were not cytotoxic toward intact rat hepatocytes and there was no loss of cell viability over a 3 h incubation period.CHP and GO induced cytotoxicity were prevented by antioxidants (BHT, penecilamin, α-tochoferol, gallic acid and catechin).Gallic acid and catechinare generally used as reference standards in antioxidant and radical scavenging studies and also are major polyphenol components in the pistachio(31).

When hepatocytes were incubated with CHP and GO at this $EC_{50, 2h}$ concentration, ROS formation determined by the oxidation of DCFH to DCF significantly increased (Table 2). ROS formation was significantly (P < 0.05) prevented by methanolic, water and ethyl acetate extracts of *Pistacia vera* at concentration of 150 μg/mL.

When examined against CHP and GO induced ROS formation in hepatocytes, all three extracts prevented ROS formation, in the following order, from most protectiveto least protective: pistachio methanolic extract >pistachio aqueous extract >pistachio ethyl acetate extract.

CHP and GO induced ROS generation were prevented by antioxidants (BHT, α-tochoferol, gallic acid, catechin)(Table2). Pistachios extracts (150 μg/mL) as well as BHT, penecilamin, α-tochoferol, gallic acid and catechin did not induce any ROS formation (oxidative stress marker) over a 3 h incubation period in rat hepatocytes(data not shown).

As shown in Table 3, CHP and GO induced

Table 3. Protective effects of different pistachios extracts against cumene hydroperoxide and glyoxal induced lipid peroxidation.

Addition	Lipid Peroxidation (µmol/10^6 cells)		
	15 min	30 min	60 min
control	0.34 ± 0.02	0.38 ± 0.01	0.43 ± 0.03
+cumene hydroperoxide (120 µM)	1.32 ± 0.04[a]	1.47 ± 0.06[a]	1.78 ± 0.04[a]
+butylatedhydroxytoluene (50 µM)	0.46 ± 0.01[b]	0.52 ± 0.04[b]	0.60 ± 0.05[b]
+pistachios ethyl acetate extract (150 µg/mL)	0.51 ± 0.03[b]	0.62 ± 0.05[b]	0.70 ± 0.06[b]
+pistachios methanolic extract (150 µg/mL)	0.33 ± 0.02[b]	0.40 ± 0.04[b]	0.41 ± 0.05[b]
+pistachios water extract (150 µg/mL)	0.37 ± 0.01[b]	0.42 ± 0.03[b]	0.44 ± 0.04[b]
+α-tochoferol(100 µM)	0.55 ± 0.03[b]	0.67 ± 0.04[b]	0.72 ± 0.04[b]
+gallic acid (100 µM)	0.40 ± 0.02[b]	0.47 ± 0.03[b]	0.50 ± 0.01[b]
+catechin (5 mM)	0.38 ± 0.03[b]	0.44±0.02[b]	0.48 ± 0.05[b]
+glyoxal (5 mM)	1.04 ± 0.06[a]	1.31 ± 0.04[a]	1.52 ± 0.03[a]
+butylatedhydroxytoluene (50 µM)	0.49 ± 0.03[b]	0.63±0.05[b]	0.70 ± 0.06[b]
+pistachios ethyl acetate extract (150 µg/mL)	0.52 ± 0.02[b]	0.57 ± 0.04[b]	0.67 ± 0.05[b]
+pistachios methanolic extract (150 µg/mL)	0.38 ± 0.04[b]	0.42 ± 0.03[b]	0.53 ± 0.04[b]
+pistachios water extract (150 µg/mL)	0.42 ± 0.02[b]	0.48 ± 0.03[b]	0.61 ± 0.03[b]
+α-tochoferol(100 µM)	0.55 ± 0.03[b]	0.60 ± 0.01[b]	0.71 ± 0.05[b]
+gallic acid (100 µM)	0.47 ± 0.04[b]	0.50 ± 0.02[b]	0.65 ± 0.03[b]
+catechin (5 mM)	0.44 ± 0.01[b]	0.49 ± 0.04[b]	0.63 ± 0.06[b]

Hepatocytes (10^6cells/mL) were incubated in Krebs–Henseleit buffer pH 7.4 at 37°C for 1.0 h following the addition of $EC50_{2h}$ of cumene hydroperoxide and glyoxal. Lipid peroxidation was determined by measuring thiobarbituric acid reactive substances as µM concentration of malondialdehyde (Smith *et al*., 1982).
Values are expressed as mean±SD of three separate experiments (n=5).
[a] Significant difference in comparison with control hepatocytes ($P < 0.05$).
[b] Significant difference in comparison with cumene hydroperoxideorglyoxal treated hepatocytes ($P < 0.05$).

lipid peroxidation were also significantly (P < 0.05)prevented by methanolic, water and ethyl acetate extracts of *Pistacia vera* at concentration of 150 µg/mL. The order of protection by nut extracts against oxidative stress induced lipid peroxidation in hepatocytes was same as the order of protection elicited against cell death. Pistachios extracts (150 µg/mL) as well as BHT, penecilamin, α-tochoferol, gallic acid and catechin did not induce any thiobarbitoric acid reactive substances formation(lipid peroxidation marker) over a 3 h incubation period in rat hepatocytes(data not shown).

As shown in Table 4 incubation of hepatocytes with CHP and GO, also caused rapid hepatocyte glutathione (GSH) depletion another marker of cellular oxidative stress, demonstrated as intracellular GSH decrease and extracellular GSSG increase. Most of the CHP and GO induced GSH depletion could be attributed to the expulsion of GSSG (Table 4). Methanolic, water and ethyl acetate extracts of *Pistacia vera* at concentrations of 150 µg/mL significantly (P < 0.05) prevented both CHP and GO induced GSH depletion. Methanolic extract of *Pistacia vera* was the most protective among its extracts. Gallic acid, *α*-tochoferol and catechin significantly ($P < 0.05$) prevented both CHP and GO induced intracellular GSH decrease and extracellular GSSG increase (Table 4). Pistachios extracts (150 µg/mL) as well as BHT, penecilamin, α-tochoferol, gallic acid and catechin did not induce any significant alteration in hepatocytes GSH and GSSG contents over a 3 h incubation period (data not shown).

As a consequence of hepatocyte ROS formation, CHP and GO induced a rapid decline of mitochondrial membrane potential, an apparent marker of mitochondrial dysfunction. Mitochondrial membrane potential decrease was prevented by methanolic, water and ethyl acetate extracts of *Pistacia vera* at concentration

Table 4. Protective effects of different pistachios extracts against cumene hydroperoxide and glyoxal induced GSH depletion.

Addition	IntracellularGSH (μM/10^6 cells)90 min	Extra cellularGSSG (μM/10^6 cells) 90 min
control	42 ± 4	4.8 ± 0.7
+cumene hydroperoxide (120 μM)	11 ± 2[a]	14 ± 1.9[a]
+GSH (2 mM)	53 ± 4[b]	5.3 ± 0.8[b]
+pistachios ethyl acetate extract (150 μg/mL)	22 ± 2[b]	8.9 ± 0.5[b]
+pistachios methanolic extract (150 μg/mL)	37 ± 3[b]	7.7 ± 0.5[b]
+pistachios water extract (150 μg/mL)	32 ± 3[b]	8.4 ± 0.4[b]
+α-tochoferol(100 μM)	20 ± 3[b]	9.1 ± 0.3[b]
+gallic acid (100 μM)	28 ± 2[b]	9 ± 0.2[b]
+catechin (5 mM)	30 ± 2[b]	8.7 ± 0.7[b]
+glyoxal (5 mM)	19 ± 3[a]	13 ± 2.2[a]
+GSH (2 mM)	56 ± 4[b]	4.8 ± 0.5[b]
+pistachios ethyl acetate extract (150 μg/mL)	27 ± 3[b]	8.3 ± 0.9[b]
+pistachios methanolic extract (150 μg/mL)	43 ± 3[b]	6.6 ± 0.6[b]
+pistachios water extract (150 μg/mL)	40 ± 4[b]	7.4 ± 0.8[b]
+α-tochoferol(100 μM)	25 ± 2[b]	8.5 ± 0.2[b]
+gallic acid (100 μM)	34 ± 3[b]	8.2 ± 0.6[b]
+catechin (5 mM)	39 ± 3[b]	7.6 ± 0.4[b]

Hepatocytes (10^6 cells/mL) were incubated in Krebs–Henseleit buffer pH 7.4 at 37 °C for 90 min following the addition of $EC50_{2h}$ of cumene hydroperoxide and glyoxal. Intracellular GSH and extra cellular GSSG were determined fluorimetrically as described byHissin and Hilf, 1978.

Values are expressed as mean±SD of three separate experiments (n=5).

[a] Significant difference in comparison with control hepatocytes ($P < 0.05$).

[b] Significant difference in comparison with cumene hydroperoxide glyoxal treated hepatocytes ($P < 0.05$).

(150 μg/mL) as well as by cyclosporine, α-tochoferol, catechin and gallic acid (Table 5). Methanolic extract of *Pistacia vera* was the most protective among other treatments. Pistachios extracts (150 μg/mL) as well as BHT, penecilamin, α-tochoferol, gallic acid and catechin did not induce any damage to rat hepatocytes mitochondria and there was no mitochondrial membrane potential decrease over a 3 h incubation period of above mentioned treatments in rat hepatocytes (data not shown).

When hepatocyte lysosomes were loaded with acridine orange (a lysosomotropic agent), a significant redistribution of acridine orange into the cytosolic fraction ensued within 120 min of incubation with CHP and GO, indicating sever oxidative damage to lysosomal membrane (Table 6). CHP and GO induced acridine orange release were again prevented by methanolic, water and ethyl acetate extracts of *Pistacia vera* at concentration of 150 μg/mL as well as bychloroqunie, α-tochoferol, catechin and gallic acid (Table 6). Pistachios extracts (150 μg/mL) as well as BHT, chloroqunie, penecilamin, α-tochoferol, gallic acid and catechin did not induce any damage to rat hepatocytes lysosomes and there was no acridine orange release into cytosol over a 3 h incubation period of above mentioned treatments in acridine orange loaded hepatocytes (data not shown).

Catechin, gallic acid, α-tochoferol, penicillamine and *Pistacia vera* extracts at concentration of 150 μg/mL were also protective against CHP and GO induced protein carbonylation in rat hepatocytes (Table 7).

When catechin, *α*-tochoferol and gallic acid were compared for protectiveness against CHP and GO induced carbonyl stress in hepatocytes, catechin and gallic acid, were more protective than *α*-tochoferol against protein carbonylation at 3 h (Table 7). There was no significant difference between catechin and methanolic extract of

Table 5. Protective effects of different pistachios extracts against cumene hydroperoxideand glyoxal induced mitochondrial membrane potential decline.

Addition	Percent of Mitochondrial membrane potential decline (%ΔΨm)		
	15 min	30 min	60 min
control	2 ± 1	4 ± 1	6 ± 1
+cumene hydroperoxide (120 µM)	19 ± 2 [a]	24 ± 3 [a]	36 ± 2 [a]
+cyclosporine (2 mM)	2 ± 1[b]	6 ± 1[b]	10 ± 1[b]
+pistachios ethyl acetate extract (150 µg/mL)	9 ± 1[b]	14 ± 1[b]	23 ± 2[b]
+pistachios methanolic extract (150 µg/mL)	6 ± 1[b]	9 ± 1[b]	16 ± 2[b]
+pistachios water extract (150 µg/mL)	6 ± 1[b]	12 ± 2[b]	21 ± 2[b]
+α-tochoferol(100 µM)	10 ± 2[b]	16 ± 2[b]	25 ± 2[b]
+gallic acid (100 µM)	8 ± 1[b]	14 ± 2[b]	24 ± 2[b]
+catechin (5 mM)	7 ± 1[b]	13 ± 1[b]	22 ± 2[b]
+glyoxal (5 mM)	16 ± 2 [a]	20 ± 2 [a]	26 ± 2 [a]
+cyclosporine (2 mM)	3 ± 1[b]	7 ± 1[b]	10 ± 2[b]
+pistachios ethyl acetate extract (150 µg/mL)	7 ± 1[b]	15 ± 2[b]	19 ± 2[b]
+pistachios methanolic extract (150 µg/mL)	4 ± 1[b]	10 ± 2[b]	13 ± 2[b]
+pistachios water extract (150 µg/mL)	5 ± 1[b]	11 ± 2[b]	16 ± 2[b]
+α-tochoferol(100 µM)	8 ± 1[b]	16 ± 1[b]	20 ± 2[b]
+gallic acid(100 µM)	7 ± 1[b]	14 ± 2[b]	18 ± 2[b]
+catechin (5 mM)	6 ± 1[b]	13 ± 2[b]	17 ± 2[b]

Hepatocytes (10^6 cells/mL) were incubated in Krebs–Henseleit buffer pH 7.4 at 37°C for 1.0 h following the addition of $EC50_{2h}$ of cumene hydroperoxide and glyoxal. Mitochondrial membrane potential was determined as the difference in mitochondrial uptake of the rhodamine 123 between control and treated cells and expressed as fluorescence intensity unit. Our data were shown as the percentage of mitochondrial membrane potential collapse (%ΔΨm) in all treated (test) hepatocyte groups (Andersson *et al*., 1987).
Values are expressed as mean±SD of three separate experiments (n=5).
[a] Significant difference in comparison with control hepatocytes ($P < 0.05$).
[b] Significant difference in comparison with cumene hydroperoxide or glyoxal treated hepatocytes ($P < 0.05$).

Pistacia vera at protecting hepatocytes against CHP and GO induced protein carbonylation. Pistachios extracts (150 µg/mL) as well as BHT, Penecilamin, *α*-tochoferol, gallic acid and catechin did not induce any protein carbonylation in rat hepatocytes over a 3 h incubation period (data not shown).

Discussion

Epidemiologic and clinical studies have demonstrated that nut consumption decreases the risk of different diseases. Pistachios is a nut with an edible green kernel, good taste and high nutritional value. Pistachios component such as polyphenols, tocopherols, and lutein are bioaccessible during human gastric digestion and therefore available for absorption in the upper GI tract(32). Recent publications have shown beneficial effects of pistachios on cardiovascular disease risk factors, lipid parameters, endothelial function, inflammation, and oxidative status(33, 34). In a randomized cross-over controlled feeding study, the inclusion of pistachios decreased total cholesterol, LDL cholesterol, non–high-density lipoprotein cholesterol, and plasma stearoyl-coenzyme A desaturase activity in a dose dependent manner (33). When pistachios were given to 32 normo-lipidemic healthy young men for 4 weeks, significant decreases in blood glucose, total cholesterol, and serum interleukin-6 were observed, with improved endothelium vasodilation and total antioxidant status (34). The consumption of pistachio nuts has been shown to significantly decrease oxidative stress, improving total cholesterol and its LDL levels in healthy volunteers (35). Li *et al.* showed pistachio consumption decreased plasma triacylglycerols and body weight when compared with a carbohydrate snack in obese subjects(36).

Table 6. Protective effects of different pistachios extracts against cumene hydroperoxide and glyoxal induced Lysosomal membrane leakiness in rat hepatocytes.

Addition	Percent of acridine orange redistribution		
	15 min	30 min	60 min
control	2 ± 1[b]	3 ± 1[b]	5 ± 1[b]
+cumene hydroperoxide (120 µM)	11 ± 2[b]	17 ± 2[b]	28 ± 2[b]
+chloroquine (100 µM)	3 ± 1[b]	3 ± 1[b]	6 ± 1[b]
+pistachios ethyl acetate extract (150 µg/mL)	5 ± 1[b]	12 ± 2[b]	20 ± 2[b]
+pistachios methanolic extract (150 µg/mL)	3 ± 1[b]	9 ± 1[b]	14 ± 2[b]
+pistachios water extract (150 µg/mL)	5 ± 1[b]	11 ± 2[b]	17 ± 2[b]
+α-tochoferol(100 µM)	6 ± 1[b]	13 ± 2[b]	19 ± 3[b]
+gallic acid (100 µM)	3 ± 1[b]	9 ± 1[b]	16 ± 1[b]
+catechin (5 mM)	4 ± 1[b]	10 ± 2 [b]	17 ± 1[b]
+glyoxal (5 mM)	8 ± 1[b]	13 ± 2[b]	21 ± 2[b]
+chloroquine (100 µM)	2 ± 1[b]	3 ± 1[b]	8 ± 1[b]
+pistachios ethyl acetate extract (150 µg/mL)	6 ± 1 [b]	8 ± 1[b]	15 ± 2[b]
+pistachios methanolic extract (150 µg/mL)	3 ± 1[b]	6 ±1[b]	9 ± 1[b]
+pistachios water extract (150 µg/mL)	7 ± 1 [b]	8 ± 1[b]	12 ± 2[b]
+α-tochoferol(100 µM)	8 ± 1 [b]	9 ± 1[b]	15 ± 2[b]
+gallic acid (100 µM)	3 ± 1[b]	6 ± 1[b]	9 ± 1[b]
+catechin (5 mM)	7 ± 1[b]	9 ± 1[b]	13 ± 2[b]

Hepatocytes (10^6cells/mL) were incubated in Krebs–Henseleit buffer pH 7.4 at 37°C for 1.0 h following the addition of $EC50_{2h}$ of cumene hydroperoxide and glyoxal. Lysosomal membrane damage was determined as intensity unit of diffuse cytosolic green fluorescence induced by acridine orange following the release from lysosomes. Our data were shown as the percentage of lysosomal membrane leakiness in all treated (test) hepatocyte groups (Pourahmad *et al*., 2001)

Values are expressed as mean±SD of three separate experiments (n=5).

[a] Significant difference in comparison with control hepatocytes ($P < 0.05$).

[b] Significant difference in comparison with cumene hydroperoxide or glyoxal treated hepatocytes ($P < 0.05$).

Compared with other tree nuts, pistachios are very rich in phytosterols, potassium, vitamin B6, carotenoids, and tocopherols (37) and have been ranked among the 50 foods highest in antioxidants (38). Extensive in vivo and in vitro experiments on the effect of have shown beneficial health activities as protective agents against cancer and cardiovascular, inflammatory, and aging disorders, and human pathogens (39, 40).

Polyphenols have been shown to have higher antioxidant capacity in vitro compared with vitamins and carotenoids and they represent the main dietary antioxidant (41). Catechins, a polar phenolic compound have been shown to be particularly effective in cardiovascular disease prevention and in decreasing the oxidation of LDL (42). Eleven flavonoids (flavanols, flavonols, and flavanones) and phenolic acids were identified in natural, raw, shelled *Pistacia vera.* Gallic and chlorogenic acids are dominant polar anti-oxidant components of pistachios. On the other hand tocopherols, and lutein are dominant lipid soluble anti-oxidant component of pistachios (32).

There are some studies on nuts that show the hydrophilic extract had a much higher antioxidant activity and activity against lipid peroxidation when compared to the lipophilic extract(43).These studies is consistent with our observation of protection by pistachios extracts against rat hepatocytes ROS formation and lipid peroxidation (Tables 2, 3).

In the current study, the ROS and carbonyl scavenging activity of different extracts of *Pistacia vera* were evaluated in an specific *in-vitro* cellular model of diabetic complications in liver and we tried to figure out whether different aqueous, methanolic and ethyl acetate extracts of *Pistacia vera*. kernel could protect hepatocytes against CHP and GO induced oxidative stress and

Table 7- Protective effects of different pistachios extracts against cumene hydroperoxide and glyoxal induced protein carbonylation.

Addition	Protein Carbonylation (nmol/10^6 cells)		
	1 h	2 h	3 h
control	10 ± 1	13 ± 2	12 ± 2
+cumene hydroperoxide (120 µM)	195 ± 11[a]	204 ± 13[a]	189 ± 7[a]
+penicillamine (5 mM)	24 ± 2[b]	22 ± 2[b]	26 ± 2[b]
+pistachios ethyl acetate extract (150 µg/mL)	132 ± 4[b]	151 ± 5[b]	149 ± 4[b]
+pistachios methanolic extract (150 µg/mL)	117 ± 7[b]	110 ± 4[b]	128 ± 6[b]
+pistachios water extract (150 µg/mL)	126 ± 4[b]	122 ± 8[b]	136 ± 4[b]
+α-tochoferol (100 µM)	133 ± 4[b]	159 ± 3[b]	151 ± 4[b]
+gallic acid (100µM)	129 ± 3[b]	127 ± 4[b]	139 ± 5[b]
+catechin(5 mM)	119 ± 4[b]	115±5[b]	130 ± 6[b]
+glyoxal (5 mM)	323 ± 8[a]	348 ± 9[a]	336 ± 11[a]
+penicillamine (5 mM)	18 ± 1[b]	19 ± 2[b]	15 ± 2[b]
+pistachios ethyl acetate extract (150 µg/mL)	142 ± 4[b]	148 ± 5[b]	146 ± 9[b]
+pistachios methanolic extract (150 µg/mL)	124 ± 7[b]	138 ± 4[b]	133 ± 4[b]
+pistachios water extract (150 µg/mL)	132 ± 4[b]	145 ± 6[b]	142 ± 5[b]
+α-tochoferol (100 µM)	144 ± 4[b]	150 ± 5[b]	148 ± 4[b]
+gallic acid (100 µM)	137 ± 7[b]	148 ± 5[b]	145± 8[b]
+catechin (5 mM)	125 ± 3[b]	139 ± 4[b]	135 ± 4[b]

Hepatocytes (10^6cells/mL) were incubated in Krebs–Henseleit buffer pH 7.4 at 37 °C for 3.0 h following the addition of $EC50_{2h}$ of cumene hydroperoxide and glyoxal. Protein carbonylation was measured as DNPH-derivatized samples as nM concentration/10^6cells (Hartley *et al*., 1997).

Values are expressed as mean±SD of three separate experiments (n=5).

[a] Significant difference in comparison with control hepatocytes ($P < 0.05$).

[b] Significant difference in comparison with cumene hydroperoxideor glyoxal treated hepatocytes ($P < 0.05$).

carbonyl stress cytotoxicity. We also compared the inhibitory effects of different extracts of *Pistacia vera*. with gallic acid (100 µM), catechin(5 mM) and α-tochoferol (100 µM). Our results showed that when isolated hepatocytes were incubated with CHP or gloxal there was an initial rapid increase in ROS formation, which was prevented by all different extracts of *Pistacia vera*. kernel and also *α*-tochoferol, catechin and gallic acid. Lipid peroxidation was also markedly increased following ROS formation and different extracts of *Pistacia vera*. kernel prevented both malondialdehyde formation and cytotoxicity as well as subsequent lipid peroxidative sub-cellular organelle damage (mitochondria/lysosomes). Our data showed that different extracts of *Pistacia vera*. kernel also prevented hepatocyte ROS formation induced by the intracellular H_2O_2 generation system (glucose/glucose oxidase) (Table 2), suggesting that antioxidants and radical scavenging components of *Pistacia vera*. kernel extract including gallic acid, catechin or epi-catechin can easily cross the cell membrane and cope with the intracellular ROS formation. Previously it was shown that hepatocyte ROS formation induced by glucose/glucose oxidase, could be prevented by hydroxyl radical scavengers such as dimethyl sulfoxide and mannitol(44). Lipid peroxidation is probably the most extensively investigated process induced by free radicals. This process and subsequent sub-organelle (mitochondria/ lysosomes) membrane damages are regarded as an important mechanism underlying the toxicity of several oxidative xenobiotics (45). CHP induced cellular lipid peroxidation is a common model for evaluating the effectiveness of antioxidants (46). In this study, different extracts of *Pistacia vera*. were able to inhibit the lipid peroxidation and all the sub-cellular subsequent events (*i.e.* mitochondrial/lysosomal membrane damege) induced by CHP or GO. Glutathione (GSH) is an intracellular antioxidant that prevents intracellular ROS formation and

lipid peroxidation. As an antioxidant, it has been involved in cell protection from the noxious effect of oxidative stress, both directly and as a cofactor of glutathione peroxidases and these reactions generate oxidized glutathione (GSSG) (47). So glutathione depletion is a marker of cellular oxidative stress and could be attributed to the expulsion of GSSG. Our results showed that when isolated hepatocytes were incubated with CHP or GO, glutathione depletion was occurred as a consequence of ROS formation. Glutathione depletion and lysosomal membrane leakage which observed in our study could also accelerate and exacerbate the oxidative stress cytotoxicity(48). ROS can target intracellular organelles such as mitochondria and lysosomes. Our results showed (Tables 5 and 6) that the decline in mitochondrial membrane potential and lysosomal membrane damage was occurred in isolated hepatocytes incubated with CHP or GO. So it seems that damage to mitochondrial and lysosomal membranes could be a lipid peroxidative consequence of ROS formation. On the other hand, in tralysosomal Haber–Weiss reaction and hydroxyl radical formation could destabilize the lysosomal membrane integrity. These events could finally result in lipid membranes lysis and cellular proteolysis, a process that ends in cellular death(48). Considering the fact that gallic acid, a polyphenolic compound, is a ferric (Fe^{3+}) chellator (49, 50)and our results showed that there was no significant difference between 150 µg/mL of the methanolic extract and gallic acid (100 µM) at preventing CHP or GO induced lysosomal membrane damage, we can therefore suggest that gallic acid, a major polyphenol component in the *Pistacia vera*(31), probably might be a lysosomotrophic agent and by penetrating inside the lysosomes it could chelate Fe ions and block the Haber–Weiss reaction and subsequent lysosomal derived oxidative stress and lysosomal/mitochondrial toxic interaction inside the hepatocytes.

Our *in-vitro* experiments have shown the best cytoprotective effect for the methanolic extract against hydroperoxide induced lipid peroxidation, cytotoxicity and ROS formation. This could be due to inactivation of cytoprotective components in the methanolic extract to hepatocyte metabolising enzymes, such as glucuronidation or methylation enzymes (51). Another study also confirmed the radical scavenging ability of methanolic extracts of peanut hulls (52). They found that the extract when added to 1,1-diphenyl-2-picrylhydrazyl radicals, H_2O_2 or a source of superoxide or hydroxyl radicals caused their disappearance suggesting that the extract had *in-vitro* radical scavenging ability(52). Their study concluded that this peanut hull methanolic extract acted as both an oxygen scavenger and as an antioxidant. It has been reported that walnuts (with skin) contain~40×more total antioxidant capacity compared to hazelnuts (with skin) as well most other nuts (53). The FRAP values for walnuts were also found to be ~32× more effective reducing ability compared to hazelnuts. It was already proposed that a direct reaction of epicatechin with methylglyoxal(MGO) could induce trapping ability of methylglyoxal in aqueous Maillard systems (54). The chemical structure of two adducts were identified in which MGO covalently bound to the A ring of epicatechin. However the Maillard reaction reported involved incubating 10 mM epicatechin with 10 mM MGO for 30minat 125 °C so it seemed unlikely that the reaction would occur under physiological conditions(23). However, in the present study, catechin or galic acid was found to prevent GO cytotoxicity towards hepatocytes under physiological conditions (37 °C, pH 7.4), at lower dicarbonyl concentrations. Furthermore hepatocyte protein carbonylation induced by GO was also prevented by catechin and galic acid. The phenolic compounds were also effective when added after GO or when premixed with the GO, prior to addition to hepatocytes, and suggests that catechin and gallic acid trap dicarbonyls, thereby reducing toxicity and reversing early stage carbonyl formation(23).

We have also shown that phenolic compounds protect cells from the cytotoxic effects of dicarbonyls. This likely occurs by trapping dicarbonyls or by reversing dicarbonyl induced Schiff base formation when added after the dicarbonyl. D-penicilamine was also shown to prevent GO cytotoxicity towards hepatocytes by trapping gloxal(23). An HPLC analysis of the pistachio methanolic extract revealed the presence

of catechin, gallic acid, ellagic acid, coumaric acid, caffeine and also α-tochoferol(55). Due to the variety of compounds found in both polar and non-polar extracts, it is likely that multiple cytoprotective mechanisms are acting against oxidative and carbonyl induced cytotoxicity. For example, synergistic effects between both hydrophilic and lipophilic antioxidants could exist in which antioxidants recharge neighbouring antioxidants (53). Another study found that quercetin and epicatechin regenerated α-tocopherol thereby eliciting a co-antioxidant effect (56). Almond extracts also demonstrated excellent metal chelating abilities (57). In summary our study has shown that pistachio extracts, particularly the methanolic extract prevented *in-vitro* hepatocyte oxidative stress (lipid peroxidation) induced by hydroperoxide. They also prevented carbonyl stress (protein carbonylation) and cytotoxicity induced by the dicarbonyl glyoxal that has been implicated in diabetic complications. Additionally the flavonoids catechin, galic acid found in nuts and *α*-tochoferol effectively prevented early stage protein carbonylation (Schiff base formation) induced by the reactive dicarbonyl glyoxal under physiological conditions.

Conclusion

To our knowledge, we are the first to report the cytoprotective activity of *Pistachio vera* extracts in this particular *in-vitro* cellular model. Overall, *Pistacia vera* may provide a useful approach to counter elevated oxidative and carbonyl stress seen in type 2 diabetes, likely by increasing plasma antioxidants and bioactive compounds which could prevent protein modification and ameliorate oxidative stress in the early stages.

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References

(1) Brownlee M. Biochemistry and molecular cell biology of diabetic complications. *Nature* (2001) 414: 813-820.
(2) Goldin A, Beckman JA, Schmidt AM and Creager MA. Advanced glycation end products: sparking the development of diabetic vascular injury. *Circulation* (2006) 114: 597-605.
(3) Rosca MG, Monnier VM, Szweda LI and Weiss MF. Alterations in renal mitochondrial respiration in response to the reactive oxoaldehyde methylglyoxal. *Am. J. Physiol. Renal. Physiol*. (2002) 283: 52-59.
(4) Baynes JW. Role of oxidative stress in development of complications in diabetes. *Diabetes* (1991) 40: 405-412.
(5) Baynes JW and Thorpe SR. Role of Oxidative Stress in Diabetic Complications. *Diabetes* (1999) 48: 1-9.
(6) Sheetz MJ and King GL. Molecular understanding of hyperglycemia's adverse effects for diabetic complications. *JAMA* (2002) 288: 2579-2588.
(7) Beisswenger PJ, Howell SK, Nelson RG, Mauer M and Szwergold BS. Alpha-oxoaldehyde metabolism and diabetic complications. *Biochem. Soc. Trans*. (2003) 31: 1358-1363.
(8) Franz M, Bantle J, Beebe C, Brunzell J, Chiasson J, Garg A, Holazmeister L, Hoogwere B, Mauer-Davis E, Mooradian A, Purnell J and Wheeler M. Evidence-based nutrition principles and recommendations for the treatment and prevention of diabetes and related complications. *Diabetes Care* (2002) 25: 148-198.
(9) Krentz AJ, Ferner RE and Bailey CJ. Comparative tolerability profiles of oral antidiabetic agents. *Drug Saf*. (1994) 4: 223-241.
(10) Lebovitz HE. Differentiating members of the thiazolidinedione class: a focus on safety. *Diabetes Metab. Rev*. (2002) 18: 23-29.
(11) Giner-Larza E, Manez S, Giner-Pons R and Carmen-Recio M. On the anti-inflammatory and anti-phospholipase A (2) activity of extracts from lanostane-rich species. *Ethnopharmacol.* (2000) 73: 61-69.
(12) Shtayeh A and Abu-Ghdeib SIM. Antifungal activity of plant extracts against dermatophytes. *Mycoses* (1999) 42: 665-672.
(13) Magiatis P, Melliou E, Skaltsounis A, Chinou I and Mitaku S. Chemical composition and antimicrobial activity of the essential oils of Pistacia lentiscus var. *Planta Med*. (1999) 65: 749-752.
(14) Bombi G, Pinna W and Sau F. Total blood lipids and lipoproteins in sheep fed Pistacia lentiscus drupe. *Boll. Soc. Ital. Biol.Sper*. (1988) 64: 93-99.
(15) Orhan I, Aslan M, Sener B, Kaiser M and Tasdemir D.

In-vitro antiprotozoal activity of the lipophilic extracts of different parts of Turkish Pistacia vera L. *Phytomed.* (2006) 13: 735-739.

(16) Parvardeh S, Niapour M and Hosseinzadeh H. Hepatoprotective activity of Pistacia vera L. gum extract in mice. *J. Med. Plants* (2002) 1: 27-34.

(17) Ahmadiani A, Javan M, Semnanian S, Barat E and Kamalinejad M. Anti-inflammatory and antipyretic effects of Trigonella foenum-graecum leaves extract in the rat. *J. Ethnopharmacol*. (2001) 75: 283-286.

(18) Publication NIoHN. *Guide for the Care and Use of Laboratory Animals,* USDoHaHuman and NIoH Services (ed.) (1985).

(19) Moldeus P, Hogberg J and Orrenius S. Isolation and use of livercells. *J. Methods Enzymol*. (1978) 52: 60-71.

(20) Pourahmad J, Amirmostofian M, Kobarfard F and Shahraki J. Biological reactive intermediates that mediate dacarbazine cytotoxicity. *Cancer Chemother. Pharmacol*. (2009) 65: 89-96.

(21) Shahraki J, Motallebi A and Pourahmad J. Oxidative mechanisms of fish hepatocyte toxicity by the harmful dinoflagellate Cochlodinium polykrikoides. *Mar. Environ. Res*. (2013) 88: 52-60.

(22) Pourahmad J, Eskandari MR, Kaghazi A, Shaki F, Shahraki J and Fard JK. A new approach on valproic acid induced hepatotoxicity: involvement of lysosomal membrane leakiness and cellular proteolysis. *Toxicol. In-vitro* (2012) 26: 545-51.

(23) Banach MS, Dong Q and O›Brien PJ. Hepatocyte cytotoxicity induced by hydroperoxide (oxidative stress model) or glyoxal (carbonylation model): prevention by bioactive nut extracts or catechins. *Chem. Biol. Interact*. (2009) 178: 324-331.

(24) Pourahmad J and O'Brien PJ. A comparison of hepatocyte cytotoxic mechanisms for Cu2+ and Cd2. *Toxicol.* (2000) 143: 263-273.

(25) Smith MT, Thor H, Hartzell P and Orrenius S. The measurement of lipid peroxidationin isolated hepatocytes. *Biochem*. (1982) 31: 19-26.

(26) Shahraki J, Motallebi AA, Aghvami M and Pourahmad J. Ichthyotoxic cochlodinium polykrikoides induces mitochondrial mediated oxidative stress and apoptosis in rat liver hepatocytes. *Iran. J. Pharm. Res*. (2013) 12: 829-844

(27) Hissin PJ and Hilf R. A fluorometric method for determination of oxidized and reduced glutathione in tissues. *Anal. Biochem*. (1976) 74: 214-226.

(28) Andersson BS, Aw TY and Jones DP. Mitochondrial transmembrane potential and pH gradient during anoxia. *Am. J. Physiol*. (1987) 252: 349-355.

(29) Pourahmad J, Mortada Y, Eskandari MR and Sharaki J. Involvement of lysosomal labilisation and lysosomal/ mitochondrial cross-talk in diclofenac induced hepatotoxicity**.** *Iran. J. Pharm. Res.* (2011) 10: 877-887.

(30) Hartley DP, Kroll DJ and Petersen DR. Prooxidant-initiated lipid peroxidation in isolated rat hepatocytes: detection of 4-hydroxynonenal- and malondialdehyde-protein adducts. *Chem. Res. Toxicol*. (1997) 10: 895-905.

(31) Kosar M and Bozan B. Antioxidant activity and phenolic composition of *Sumac(Rhus coriaria L.*) extracts. *Food Chem*. (2007) 103: 952-959.

(32) Mandalari G, Bisignano C, Filocamo A, Chessa S, Saro M, Torre G, Faulks RM and Dugo P. Bioaccessibility of pistachio polyphenols, xanthophylls, and tocopherols during simulated human digestion. *Nutrition* (2013) 29: 338-344.

(33) Gebauer SK, West SG, Kay CD, Alaupovic P, Bagshaw D and Kris-Etherton PM. Effects of pistachios on cardiovascular disease risk factors and potential mechanisms of action: a dose-response study. *Am. J. Clin. Nutr*. (2008) 88: 651-659.

(34) Sari I, Baltaci Y, Bagci C, Davutoglu V, Erel O, Celik H, Ozer O, Aksoy N and Aksoy M. Effect of pistachio diet on lipid parameters, endothelial function, inflammation, and oxidative status: a prospective study. *Nutrition* (2010) 26: 399-404.

(35) Kocyigit A, Koylu A and Keles H. Effects of pistachio nuts consumption on plasma lipid profile and oxidative status in healthy volunteers. *Nutr. Metab. Cardiovasc Dis*. (2006) 16: 202-209.

(36) Li Z, Song R, Nguyen C, Zerlin A, Karp H and Naowamondhol K. Pistachio nuts reduce triglycerides and body weight by comparison to refined carbohydrate snack in obese subjects on a 12-week weight loss program. *Am. Coll. Nutr*. (2010) 29: 198-203.

(37) Philips K, Ruggio D and Ashraf-Khorassani M. Phytosterol composition of nuts and seeds commonly consumed in the United States. *J. Agric. Food Chem*. (2005) 53: 9436-9445.

(38) Halvorsen B, Carlsen K, Philips K, Bohn S, Holte K and Jacobs DJ. Content of redox-active compounds (ie antioxidants) in food consumed in the United States. *Am. J. Clin. Nutr*. (2006) 84: 95-135.

(39) Lau F and Shukitt-Hale B. The beneficial effects of fruit pop-lyphenols on juice on cognitive and motor deficits in aging. *Nutrition* (2006) 22: 295-302.

(40) Zafra-Stone S, Yasmin T, Bagchi M, Chatterjee A, Vinson J and Bagchi D. Berry anthocyanins as novel antioxidants in human health and disease prevention. *Mol. Nutr. Food Res*. (2007) 51: 675-683.

(41) Richelle M, Tavazzi I and Offord E. Comparison of the antioxidant activity of commonly consumed polyphenolic beverages (coffee, cocoa and tea) prepared per cup serving. *J. Agric. Food Chem*. (2001) 49: 3438-3442.

(42) Velayutham P, Babu A and Liu D. Green tea catechins and cardiovascular health: an update. *Curr. Med. Chem*. (2008) 15: 1840-1850.

(43) Gentile C, Tesoriere L, Butera D, Fazzari M, Monastero M and Allegra M. Antioxidant activity of *Sicilian pistachio (Pistacia vera L. var. Bronte)* nut extract and its bioactive components. *J. Agric. Food Chem*. (2007) 55: 643-648.

(44) Siraki AG, Pourahmad J, Chan TS, Khan S and O'Brien PJ. Endogenous and endobiotic induced reactive

oxygen species formation by isolated hepatocytes. *Free Radic. Biol*. (2002) 32: 2-10.

(45) Nakagawa Y, Moldeus P and Moore GA. Cytotoxicity of ortho phenylphenol in isolated rat hepatocytes. *Biochem. Pharmacol*. (1992) 43: 159-165.

(46) Rigobello MP, Stevanato R, Momo F, Fabris S, Scutari G, Boscolo R and Folda A. Evaluation of the antioxidantproperties of propofol and its nitrosoderivative Comparison with homologue substituted phenols. *Free Radic. Res*. (2004) 38: 315-321.

(47) Pompella A, Visvikis A, Paolicchi A, De Tata V and Casini AF. The changing faces of glutathione, a cellular protagonist. *Biochem. Pharmacol*. (2003) 66: 1499-1503.

(48) Pourahmad J, Rezaei M, Rasekh HR and Ahmadiani A. Involvement of subcellular organelles in inflammatory pain-induced oxidative stress and apoptosis in the rat hepatocytes. *Arch. Iran. Med*. (2008) 11: 407-417.

(49) Perron NR and Brumaghim JL. A review of the antioxidant mechanisms of polyphenol compounds related to iron binding. *Cell Biochem. Biophys*. (2009) 53: 75-100.

(49) Dong Q, Banaich MS and O›Brien PJ. Cytoprotection by almond skin extracts or catechins of hepatocyte cytotoxicity induced by hydroperoxide (oxidative stress model) versus glyoxal or methylglyoxal (carbonylation model). *Chem. Biol. Interact*. (2010) 185: 101-109.

(51) Donovan JL, Crespy V, Manach C, Morand C, Besson C, Scalbert A and Remesy C. Catechin ismetabolized by both the small intestine and liver of rats. *J. Nutr*. (2001) 6: 1753-1757.

(52) Yen GC. Scavenging effect of methanolic extracts ofpeanuthulls onfree-radical and active-oxygen species. *J. Agric. Food Chem*. (2008) 42: 629-632.

(53) Blomhoff R, Carlsen MH, Andersen LF and R. JD. Health benefits of nuts: potential role of antioxidants. *Br. J. Nutr*. (2006) 96: 52-60.

(54) Totlani VM and Peterson DG. Epicatechin carbonyl-trapping reactions in aqueous Maillard systems: identification and structural elucidation. *J. Agric. Food Chem*. (2006) 54: 7311-7318.

(55) Papoutsi A, Kassi E, Chinou I, Halabalaki M, Skaltsounis LA and Moutsatsou P. Walnut extract (Juglans regia L.) and its component ellagic acid exhibit antiinflammatory activity in human aorta endothelial cells and osteoblastic activity in the cell line KS483. *Br. J. Nutr*. (2008) 99: 715-722.

(56) Pedrielli P and Skibsted LH. Antioxidant synergy and regeneration effect of quercetin, (−)-epicatechin, and (+)-catechin on alpha-tocopherol in homogeneous solutions of peroxidating methyl linoleate. *J. Agric. Food Chem*. (2002) 50: 7138-7144.

(57) Wijeratne SS, Abou-Zaid MM and Shahidi F. Antioxidant polyphenols in almond and its coproducts. *J. Agric. Food Chem*. (2006) 54: 312-318.

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