Effect of Long-Term Dietary Administration of Oregano on the Alleviation of Carbon Tetrachloride-Induced Oxidative Stress in Rats

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This study aimed at evaluating the protective effect of long-term dietary oregano on the alleviation of carbon tetrachloride-induced oxidative stress in rats. Twenty-four female Wistar rats were allocated to four groups of six animals each. Groups 1 (control) and 2 (CCl_4) were fed a basal diet, while groups 3 (oregano) and 4 (oregano + CCl_4) were fed the basal diet supplemented further with ground oregano at 1% level. Following six-week feeding, the rats of groups 2 and 4 were given a single intraperitoneal injection of CCl_4 at a dose of 1 mL/kg bw. Six hours after the CCl_4 injection, all animals were sacrificed, and serum, liver, kidney, and heart tissue samples were collected. Analysis results showed that the addition of oregano significantly increased the total phenolic content and the Trolox equivalent antioxidant capacity of the basal diet but had no effect on its lipid peroxidation index. Treatment with CCl_4 of rats from the CCl_4 group caused a significant increase in aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP) in serum, whereas it decreased cholesterol and triglyceride content as compared to the control. It also increased the lipid peroxidation index and decreased the scavenging activities of the 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS) radical cation, the hydroxyl anion radical, the superoxide anion radical, and the hydrogen peroxide in all tested tissues, as compared to that of the control. Without CCl_4 treatment, diet supplementation with oregano had no effect on these biochemical parameters, excluding the hydroxyl radical scavenging activity, which was increased in all tested tissues as compared to that of the control. Feeding oregano before CCl_4 treatment resulted in a significant decline of the increase in AST, ALT, and ALP activities ($P < 0.05$ vs CCl_4 group), but the recorded values could not attain those of the control group ($P < 0.05$ vs control group). It significantly increased the reduced cholesterol and triglycerides ($P < 0.05$ vs CCl_4 group) to values not differing from those of the control. It also resulted in a significant reduction of the increased malondialdehyde ($P < 0.05$ vs CCl_4 group) to values that could not attain the levels of the control but had no significant effect ($P > 0.05$) on the reduced ABTS radical cation scavenging activity. It increased significantly the reduced hydroxyl anion radical scavenging activity ($P < 0.05$ vs CCl_4 group) to values that could not attain those of the control in all tested tissues except kidney. Additionally, it resulted in a significant elevation of the decreased superoxide anion radical scavenging activity in serum and liver but had no effect in kidney and heart, whereas it also resulted in a significant elevation of the decreased hydrogen peroxide scavenging activity in liver, kidney, and heart but had no effect in serum. These results suggest that dietary oregano may effectively improve the impaired antioxidant status in CCl_4-induced toxicity in rats.

KEYWORDS: Oxidative stress; oregano; rats; dietary; carbon tetrachloride

INTRODUCTION

Epidemiological studies consistently indicate a positive association between the consumption of food of plant origin and the prevention of diseases (*1*). Some of the beneficial effect is attributed to the presence of phenolic phytochemicals that are considered to play an important role as dietary antioxidants for the prevention of the oxidative damage caused by reactive oxygen species in living systems (*2*). Reactive oxygen species such as the superoxide radicals ($O_2^{\bullet-}$), hydroxyl radicals ($^{\bullet}OH$), and the nonfree radical species singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2)

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are byproducts of regular cell metabolism capable of oxidizing cellular lipids, proteins, and nucleic acids, causing progressive decline in cell function that can lead to various pathophysiological disorders (*3–5*). Since reactive oxygen species are destructive in nature, cells have several antioxidant defense mechanisms of enzymatic, nonenzymatic, or dietary origin to scavenge them. However, if these defense mechanisms are overwhelmed by an excessive generation of reactive oxygen species within the organism due to a variety of factors, a situation of oxidative stress occurs. Oxidative stress, defined as the imbalance between oxidants and antioxidants in favor of the oxidants within the organism, has been suggested to be a critical concept in maintaining a healthy biological system (*3*).

The protective effect of phenolic phytochemicals in biological systems is ascribed to their capacity to transfer hydrogen to free radicals, chelate metal catalysts, activate antioxidant enzymes, and inhibit oxidases (*6–8*). In the search for phytochemicals with potential antioxidant activity, much attention has been focused, in the last several years, on aromatic plants, especially those of the Labiatae family, traditionally used in folk medicine. Among them, oregano, a characteristic spice of Mediterranean cuisine obtained by grinding dried leaves and flowers of *Origanum vulgare* subsp. *hirtum* plants, is well-known for its antioxidative activity (*9*). Carvacrol and thymol, the two main phenols that constitute about 78−82% of the essential oil of oregano, are principally responsible for this activity (*10*). Additional minor constituents such as γ-terpinene and p-cymene, two monoterpene hydrocarbons that constitute about 5 and 7%, respectively, of the essential oil, contribute also to this activity but their contribution is uncertain, as it is the effect of all the constituents working together (*11*). Potential precursor substances with antioxidant activity are also a variety of nonvolatile glycosidically bound compounds that exhibit biological activity after enzymatic or acid hydrolysis (*12*).

Several studies have shown that phenolic phytochemicals are extensively metabolized in vivo, mainly during transfer across the small intestine, by colonic microflora, and in the liver, resulting in significant alteration in their redox potentials (*13*). Thus, to delineate the protective potential of these compounds, it is essential to screen their efficacy as bioavailable in vivo using cell systems and animal models (*14*). The antioxidant potential of oregano and its constituents has predominantly been derived from in vitro investigations. There have been several reports on the in vitro potential of either the essential oil or an aqueous extract of oregano to inhibit lipid oxidation when added in various food systems (*9, 12, 15*) or in fresh porcine muscle tissue (*8*), respectively. In vivo investigations have only occasionally been carried out and concern primarily the potential of ground oregano or its essential oil to inhibit lipid oxidation in chicken, turkey, and rabbit meat (*16–26*) and eggs (*27, 28*), when supplemented into the animal feeds. Nevertheless, the potential of dietary-supplemented ground oregano to act as an effective antioxidant in different tissues and organs of a living organism has not yet been adequately investigated.

A common denominator in pathogenesis of most chronic diseases in living organisms is the involvement of oxidative stress. Although it has often been assumed that antioxidants in dietary plants protect against diseases related to oxidative stress, results from relevant clinical studies with single compounds such as β-carotene or vitamins C or E have not supported any protective effect; indeed, supplementation with β-carotene has resulted in adverse disease outcomes in clinical studies (*29–31*). One reason for the ineffective clinical trials could be that the protective effects of dietary plants result from the action of lesser-known antioxidant constituents or from a concerted action of the cocktail of antioxidants present in plants. These raise the possibility that a variety of antioxidants are necessary to maintain the proper redox status in a biological system. Although it would be much simpler to test the protective effect of a single or a limited number of antioxidants, such an opportunity might never be found if it actually is the case that a number, maybe hundreds, of dietary antioxidants such as carotenoids, polyphenols, or flavonoids are bioactive and work synergistically. Thus, combinations of electron-donating antioxidants in the diet consisting of individual antioxidants that occur naturally in plants may be a better concept than individual dietary antioxidants. Considering the above along with recent reports on the antioxidative potential of an oregano diet administered to poultry in the form of ground plant (*25*) rather than the essential oil (*17–22*), we decided to carry out the present study to investigate whether the long-term dietary administration of ground oregano could ameliorate the adverse effects of oxidative stress in different tissues and organs in an animal model system.

Since CCl_4 is a classic model compound for inducing free radical damage in liver, CCl_4 poisoning was chosen as a primary rodent model for this study. CCl_4 is reductively bioactivated by cytochrome P450 to the trichloromethyl free radical ($^{\bullet}CCl_3$), which, in the presence of oxygen, is subsequently converted into a peroxy radical ($^{\bullet}OOCCl_3$). These free radicals trigger cell damage through covalent binding to cellular macromolecules and lipid peroxidation, which affects the permeability of the membrane, inducing the disintegration and solubilization of membrane structure.

MATERIALS AND METHODS

Chemicals. Analytical-grade homovanillic acid (HVA), nicotinamide adenine dinucleotide (NADH), horseradish peroxidase type VI, nitroblue tetrazolium (NBT), L-ascorbic acid, benzoic acid, phenazine methosulfate, gallic acid, diformazan, Tris-HCl buffer, phosphate-buffered saline (PBS), ethylenediaminotetraacetic acid sodium salt (EDTA), hydrogen peroxide 30% solution, butylated hydroxytoluene, 2-thiobarbituric acid (TBA), trichloroacetic acid, α-tocopherol reference standard, 1,1,3,3-tetraethoxypropane, and Folin-Ciocalteau phenol reagent were obtained from Sigma-Aldrich GmbH. 6-Hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS), and potassium persulfate were obtained from Aldrich Chemical Co. Methanol, dimethylformamide (DMF), ferric sulfate, sodium carbonate, and sodium hydroxide were from Merck.

Animals and Diets. Twenty-four adult female Wistar rats from the stock colony of our laboratory, four-months old, weighing about 155 $\pm$ 10 g, were used in this study. Animals were housed individually in polypropylene cages in a room maintained at 22−24 °C with a controlled 12-h light−dark cycle and had free access to feed and tap water. **Table 1** presents the ingredients and the composition of the basal diet, as it was formulated to meet the nutrient requirements of rats.

Following 1 week acclimatization, the rats were allocated randomly to four groups of six animals each, ensuring the groups were balanced for body weight. Groups 1 (control) and 2 (CCl_4) were fed a basal diet, while groups 3 (oregano) and 4 (oregano + CCl_4) were fed the basal diet supplemented further with ground oregano at 1% level. Feeding of the experimental diets to rats lasted six weeks. Body weight and food intake were recorded at the end of the experimental period. All procedures were in accordance with the guiding principles of the European Community Council Directive (89/609/EEC) for the care and use of laboratory animals (*32*).

The ground oregano consisted of flowered tops, leaves, stems, and stalks of *Origanum vulgare* subsp. *hirtum* plants that had been dried and ground to pass a 2-mm screen. According to the supplier (Ecopharm

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Table 1. Composition of Basal Diet

components	g/kg feed	chemical analysis	%
wheat	230.0	moisture	11.4
wheat bran	150.0	crude protein	22.9
corn	222.5	fat	4.1
lucerne	40.0	crude fiber	4.5
corn gluten	25.0	ash	5.3
soybean meal	120.0		
herring meal	85.0		
dried skim milk	50.0		
yeast	25.0		
beetroot molasses	15.0	calculated analysis	
soybean oil	10.0	calcium	0.93
limestone	5.0	phosphorus	0.77
dicalcium phosphate	12.5	lysine	1.25
sodium chloride	4.0	methionine + cystine	0.80
vitamin premix[a]	0.10	sodium chloride	0.86
trace-mineral premix[b]	0.50	metabolizable energy, cal/kg	2870.0

[a] Supply per kilogram of feed: 7500 IU of vitamin A, 1200 IU of vitamin D$_3$, 7.77 mg of vitamin E, 1.12 mg of vitamin K$_3$, 0.71 mg of vitamin B$_1$, 2.58 mg of vitamin B$_2$, 0.35 mg of vitamin B$_6$, 0.04 mg of vitamin B$_{12}$, 12.57 mg of niacin, 0.24 mg of pantothenic acid, 0.01 mg of ascorbic acid, and 150 mg of choline hydrochloride. [b] Supply per kilogram of feed: 501.5 mg of Zn, 500 mg of Mn, 400 mg of Fe, 75.24 mg of Cu, 2.58 mg of Co, 15.13 mg of I, and 1.57 mg of Se.

Hellas), the ground oregano contained 1.22% carvacrol and 0.07% thymol, whereas its proximate analysis (*33*) showed 8.9% moisture, 13.3% crude protein, 4.1% fat, 19.0% crude fiber, and 9.1% ash.

Experimental Design. At the end of the six-week period, all rats of groups 2 (CCl$_4$) and 4 (oregano + CCl$_4$) were administered a single intraperitoneal injection of CCl$_4$ at a dose of 1 mL/kg bw as a 50% paraffin oil solution to induce liver injury. The rats of the groups 1 (control) and 3 (oregano) received paraffin oil at a dose of 1 mL/kg bw in the same manner. Six hours after the CCl$_4$ or vehicle injection, all animals were sacrificed by decapitation under diethyl ether anesthesia.

Blood samples were drawn by cardiac puncture into tubes, and serum was separated by centrifugation at 2000*g* for 10 min at 4 °C. Liver, kidney, and heart tissue samples were excised immediately, blotted off blood, rinsed in pH 7.4 PBS, vacuum packaged, and stored at −80 °C until analyzed. Before their analysis, tissues were thawed for 30 min, and 0.5- or 1-g samples were accurately weighed into 15-mL centrifugal tubes, homogenized (Ultraturrax T25, Janke & Kunkel GmbH, IKA-Labortechnik) at medium speed for 10 s with 4.6 or 9.2 mL, respectively, of PBS, pH 7.4, and centrifuged at 3000*g* for 15 min.

Determination of Total Phenolic Content in Diets and Oregano. The amount of total phenolics in oregano and diets was determined by the Folin-Ciocalteau procedure (*34*). In this analysis, the sample (0.5 g) was transferred to a 50-mL centrifugal tube where 25 mL of 80% aqueous methanol was also added. The mixture was homogenized at high speed for 30 s, and the homogenate was filtered. Filtrate aliquots of 0.2 mL in case of diets or 0.05 mL in case of oregano were transferred to 15-mL tubes, where 6.3 or 6.45 mL of distilled water was also added, respectively. Following the addition of water, 0.5 mL of Folin-Ciocalteau reagent was also added, and the content of each tube, after standing for 3 min, was mixed (Vortex apparatus, Scientific Industries, Inc., model K-550-GE) with 3 mL of 7.5% Na$_2$CO$_3$ and allowed to incubate for 60 min at room temperature. Following incubation, the mixture was centrifuged at 2000*g* for 5 min, and its absorbance was measured at 760 nm (Spectrophotometer Shimadzu, model UV-160A).

Since the concentration of total phenolics was calculated in gallic acid equivalents per gram of sample, the calibration curve was constructed using gallic acid as a reference standard. This standard solution was prepared by weighing 20 mg of gallic acid into a 50-mL volumetric flask and diluting to volume with 80% aqueous methanol. Volumes of 0.05−2.5 mL from this solution that corresponded to 20−100 μg of gallic acid were placed in 15-mL tubes, diluted to 6.5 mL with water, mixed with 0.5 mL of Folin-Ciocalteau reagent and 3 mL of 7.5% Na$_2$CO$_3$, and their absorbance was measured at 760 nm.

Total phenolics were calculated in milligrams of gallic acid equivalents per gram by referring to slope and intercept data of the computed least-squares fit of this standard calibration curve, using the formula, gallic acid (μg) = absorbance/0.008.

Estimation of the Trolox Equivalent Antioxidant Capacity in Diets and Oregano. For estimating the antioxidant capacity of oregano and diets, an improved version of the Trolox equivalent antioxidant capacity (TEAC) assay applicable to both lipophilic and hydrophilic antioxidants was applied (*35*). This improved version involves direct production of the blue/green ABTS radical cation chromophore through reaction between a 7 mM aqueous solution of ABTS with 2.45 mM (final concentration) aqueous solution of potassium persulfate in the dark for 12−16 h, at ambient temperature. The radical cation was stable in this form for more than 2 days when stored in the dark at room temperature. Addition of antioxidants to the preformed radical cation causes decolorization to an extent that depends on the antioxidant activity and calculated relative to the reactivity of Trolox as a standard.

For the determination of the antioxidant capacity of oregano and diets, the ABTS radical cation solution was further diluted with methanol to an absorbance of 0.7 at 734 nm. Samples (0.5 g) of oregano and diets were placed into 50-mL centrifugal tubes, and 25 mL of 80% aqueous methanol was also added. The mixtures were homogenized at high speed for 1 min, and the homogenates were filtered. The aqueous methanol extracts of oregano and diets were further diluted with methanol such that, after addition of a 50-μL aliquot to 2.45 mL of the diluted ABTS radical cation solution, they resulted in inhibition of the blank absorbance between 20−80%. Absorbance readings were taken at 734 nm exactly 1 min after initial mixing, whereas appropriate solvent blanks were run in each assay. In addition, a calibration curve was generated by running a series of Trolox standards (final concentration 0−15 μM) in methanol, plotting the recorded percentage inhibition values of absorbance at 734 nm versus the corresponding mass of the analyte, and computing slope, intercept, and least-squared fit of standard curve. TEAC values of the samples (micromoles of Trolox equivalents per gram) were realized by reference to standard curve and multiplying with appropriate dilution factor. All determinations were carried out in triplicate.

Determination of Lipid Peroxidation in Diets and Oregano. Lipid peroxidation in oregano and diets was determined on the basis of their malondialdehyde (MDA) content. MDA, the compound used as an index of lipid peroxidation, was determined by a selective third-order derivative spectrophotometric method (*36*). In this analysis, samples (1 g) were homogenized in the presence of 8 mL of 5% aqueous trichloroacetic acid and 5 mL of 0.8% butylated hydroxytoluene in hexane, and the mixture was centrifuged. The top layer was discarded, and a 2.5-mL aliquot from the bottom layer was mixed with 1.5 mL of 0.8% aqueous 2-thiobarbituric acid to be further incubated at 70 °C for 30 min. Following incubation, the mixture was cooled under tap water and submitted to conventional spectrophotometry in the range of 400−650 nm. Third-order derivative spectra were produced by digital differentiation of the normal spectra using a derivative wavelength difference setting of 21 nm. The concentration of MDA in analyzed samples (nanograms per gram) was calculated on the basis of the height of the third-order derivative peak at 521.5 nm by referring to slope and intercept data of the computed least-squares fit of standard calibration curve prepared using 1,1,3,3-tetraethoxypropane.

Determination of Some Biochemical Parameters in Serum. The liver function markers aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP), cholesterol, and triglycerides were measured in the serum of rats with a Clinical Chemistry Analyzer, model Flexor e (Vital Scientific NV).

Determination of the Lipid Peroxidation Index in Rat Tissues. Lipid peroxidation in rat serum, liver, kidney, and heart tissue samples was estimated on the basis of their MDA content using a slightly modified third-order derivative spectrophotometric method (*36*). Depending on the type of sample, 0.5−2-mL aliquots from serum or tissue homogenate were transferred into 15-mL tubes, and distilled water was added so that the final volume within each tube became 3 mL. A 0.5-mL volume of 35% aqueous trichloroacetic acid was then added, and the tubes were centrifuged at 2000*g* for 3 min. A 2.5-mL aliquot of the supernatant was transferred into another tube and mixed with 1.5

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mL of 0.8% aqueous 2-thiobarbituric acid to be further incubated at 70 °C for 30 min. Following incubation, the mixture was analyzed as above.

Determination of the ABTS Radical Cation Scavenging Activity in Rat Tissues. To determine the ABTS radical cation scavenging activity in serum, liver, kidney, and heart tissue samples, the ABTS radical cation solution prepared as above was diluted with 5 mM PBS, pH 7.4, to an absorbance of 0.7 at 734 nm (*35*). Samples of serum or tissue homogenates were diluted with water so that, after addition of a 50-μL aliquot of each sample solution to 2.45 mL of the diluted ABTS radical cation solution, they produced inhibition of the blank absorbance between 20 and 80%. Absorbance readings and calculations were carried out as previously described.

Assessment of the Hydroxyl Anion Radical Scavenging Activity in Rat Tissues. The hydroxyl radical scavenging activity in rat serum, liver, kidney, and heart tissue samples was assessed according to a slightly modified method based on benzoic acid hydroxylation (*37*). In this method, the hydroxyl radicals generated by the Fenton reaction were trapped by benzoate to give the fluorescent hydroxybenzoate, and the antioxidant status against hydroxyl radicals was estimated by competition between benzoate and the antioxidant present in tissues. Highly reactive hydroxyl radicals were generated by the addition of 200 μL of 10 mM $FeSO_4$, 200 μL of 10 mM EDTA, and 200 μL of 10 mM H_2O_2 to 1.4 mL of 50 mM PBS, pH 7, in which 200 μL of 10 mM benzoic acid and 400 μL of serum or tissue homogenate were first added. The mixture was incubated for 2 h at 37 °C and centrifuged at 2000*g* for 5 min, and the fluorescence of the formed hydroxybenzoate was measured using an excitation of 305 nm and an emission of 407 nm. In this test, the $^{\bullet}OH$ generated by the Fenton reaction was trapped by benzoate to give the fluorescent hydroxybenzoate. All substances able to decrease $^{\bullet}OH$ formation can reduce fluorescence. The scavenging activity against $^{\bullet}OH$ was expressed as the percentage of fluorescence inhibition of samples compared to a reference of distilled water.

Determination of the Superoxide Anion Radical Scavenging Activity in Rat Tissues. Determination of the superoxide anion radical scavenging activity in serum, liver, kidney, and heart tissue samples was carried out according to a slightly modified literature method (*38*). In this assay, 100 μL of an NBT solution (12.255 mg/10 mL), 100 μL of a phenazine methosulfate solution (4.59 mg/50 mL), and 30 μL of the sample homogenate or 100 μL of an ascorbic acid solution that served as control were transferred into a 15-mL centrifuge tube in which 670 or 600 μL, respectively, of a 16 mM, pH 8.0, Tris-HCl buffer solution was also added so the final volume within the tube became 900 μL. Generation of superoxide anions started by the addition, under vortexing, of 100 μL of an NADH solution (16.59 mg/10 mL), and the reaction mixture was incubated at room temperature for 5 min. A 2.5-mL volume of DMF was then added to extract the chromogen formed by the reduction of NBT, and the mixture was stirred vigorously for 1 min, allowed to stand for 10 min, and centrifuged for 5 min at 1500*g*. The absorbance of the chromogen extracted in the DMF layer was measured at 560 nm against a blank sample. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity of the sample. The percentage inhibition of superoxide anion generation was calculated using the formula, inhibition % = [(A_o − A_1)/A_o] × 100, where A_o is the absorbance of the control, and A_1 is the absorbance of the sample.

Determination of the Hydrogen Peroxide Scavenging Activity in Rat Tissues. Determination of hydrogen peroxide scavenging activity in serum, liver, kidney, and heart tissue samples was carried out by a method based on the oxidation of HVA by H_2O_2 in the presence of horseradish peroxidase, to a fluorescent biphenyl dimmer (*39*). Samples with H_2O_2 scavenging activity could prevent the oxidation of HVA by removing H_2O_2 and causing a decrease in fluorescence that is proportional to their antioxidant activity. According to a slight modification of this assay, aliquots of 50, 100, or 200 μL of the sample homogenate were mixed with 100 μL of 0.15 mM H_2O_2 solution and 1950, 1900, or 1800 μL, respectively, of 0.01 M phosphate buffer, pH 7.4, so the final volume within the tube became 2100 μL. The mixture was vortex-mixed, and 200 μL of 1.25 mM HVA solution followed by 200 μL of a horseradish peroxidase (2 U) solution were added, mixed, and incubated for 5 min at 20 °C. The fluorescence intensity

Table 2. Total Phenolic Content, Antioxidant Capacity, and Lipid Peroxidation Status of Diets and Oregano Herb

	total phenolic content, gallic acid equivalents, mg/g	antioxidant capacity, Trolox equivalents, μmol/g	lipid peroxidation, MDA concn, ng/g
control diet	1.8 ± 0.2[a]	52 ± 2[a]	269 ± 19[a]
oregano diet	3.1 ± 0.2[b]	63 ± 2[b]	231 ± 22[a]
oregano herb	123.2 ± 3.5[c]	1162 ± 40[c]	

[a−c] Values in the same column with a superscript in common do not differ significantly at $P > 0.05$. Data are means ± SD of triplicate analysis.

was measured using an excitation of 315 nm and an emission of 425 nm. Appropriate solvent blanks were run in each assay. All determinations were carried out in triplicate. The obtained results were presented as the percentage fluorescence inhibition using the formula, inhibition ratio (%) = [($A − A_b$) − ($A_1 − A_b$)] × 100/($A − A_b$), where A is the fluorescence of the HVA solution and A_1 is the fluorescence of the reaction mixture containing the test sample. The fluorescence of the blank (A_b) was obtained without adding HVA to the reaction mixture and was subtracted from both A and A_1, allowing, thus, the influence of factors interfering with H_2O_2 and HVA−peroxidase system to be avoided.

Statistical Analysis. Data were subjected to analysis of variance in the general linear model using the SPSS 10.05 statistical package (SPSS Ltd.). The homogeneity of the variances was tested by Levene's test. When significant treatment effects were disclosed at the probability level of $P < 0.05$, the Tukey's test was applied to determine statistical differences between means.

RESULTS AND DISCUSSION

So far as plant phenolics constitute one of the major groups of compounds acting as primary antioxidants or free radical scavengers, it was reasonable to determine at the start of this study their total amount and their scavenging activity in both the ground oregano and the diets given to rats. Analysis results (**Table 2**) showed that oregano contained total phenolics equivalent to 123.2 mg of gallic acid/g, a value slightly higher than that reported (101.7 mg of gallic acid equivalents/g) for oregano by other workers (*40*). Additionally, the antioxidant capacity of oregano was found to be 1162 μmol of Trolox equivalents/g (**Table 2**), a value that was slightly higher than that reported (1007 μmol of Trolox equivalents/g) for oregano by other workers (*40*). The addition of oregano to the basal diet at the level of 10 g/kg significantly increased ($P < 0.05$) the total phenolic content from 1.8 to 3.1 mg of gallic acid equivalents/g and the antioxidant capacity from 52 to 63 μmol of Trolox equivalents/g (**Table 2**). Nevertheless, this addition had no effect on the lipid peroxidation index of the diets (**Table 2**).

To assess reliably the oxidative status in animal models, a variety of methods have been proposed. However, there has been little direct comparison between the different methods using identical samples. Thus, currently there is no consensus on which methods are the most useful, reliable, accurate, or specific for different types of oxidative insults, and it is extremely difficult to provide absolute reference values for specific markers in different living systems (*41*). The only definitive way to demonstrate excessive free radical activity in in vivo animal studies is by means of electron spin resonance, but the expense of such instruments hinders their use in clinical practice. Therefore, investigators must still rely upon measurement of the oxidation products of free radical activity in biological fluids and tissues.

Elevated levels of serum liver markers are a direct reflection of oxidative injury in liver. Serum transaminases are sensitive

Table 3. Determination of Some Liver Function Markers in Serum[d]

groups	AST, IU/L	ALT, IU/L	ALP, IU/L	cholesterol, mg/100 mL	triglycerides, mg/100 mL
control	126 ± 8^a	55 ± 3^a	102 ± 9^a	67 ± 1^a	53 ± 1^a
CCl$_4$	567 ± 36^b	351 ± 24^b	329 ± 22^b	60 ± 2^b	48 ± 2^b
oregano	112 ± 11^a	66 ± 4^a	93 ± 8^a	67 ± 2^a	53 ± 1^a
CCl$_4$ + oregano	338 ± 22^c	211 ± 12^c	262 ± 123^c	66 ± 1^a	51 ± 1^a

[a–c] Values in the same column with a superscript in common do not differ significantly at $P > 0.05$. [d] Values are means $\pm$ SD of six samples analyzed.

Table 4. Changes in the Levels of MDA in Rat Tissues

groups	MDA concentrations[d] (ng/g) serum	liver	kidney	heart
control	16 ± 1a	363 ± 18a	437 ± 16a	146 ± 6a
CCl$_4$	29 ± 2^b	725 ± 23^b	846 ± 34^b	334 ± 12^b
oregano	18 ± 1a	396 ± 15a	424 ± 18a	154 ± 9a
CCl$_4$ + oregano	22 ± 1^c	576 ± 18^c	637 ± 16^c	246 ± 14^c

[a–c] Values in the same column with a superscript in common do not differ significantly at $P > 0.05$. [d] Values are means $\pm$ SD of six samples analyzed.

Table 5. ABTS Radical Cation Scavenging Activity in Rat Tissues

groups	Trolox equivalent antioxidant capacity[c] (μmol/g) serum	liver	kidney	heart
control	1.6 ± 0.12^a	3.6 ± 0.07^a	1.3 ± 0.08^a	1.0 ± 0.04^a
CCl$_4$	1.1 ± 0.08^b	2.7 ± 0.08^b	0.8 ± 0.06^b	0.6 ± 0.08^b
oregano	1.6 ± 0.10^a	3.6 ± 0.12^a	1.3 ± 0.11^a	1.0 ± 0.05^a
CCl$_4$ + oregano	1.3 ± 0.06^b	2.9 ± 0.07^b	0.9 ± 0.03^b	0.8 ± 0.06^b

[a,b] Values in the same column with a superscript in common do not differ significantly at $P > 0.05$. [c] Values are means $\pm$ SD of six samples analyzed.

Table 6. Hydroxyl Anion Radical Scavenging Activity in Rat Tissues

groups	fluorescence inhibition[e] (%) serum	liver	kidney	heart
control	63.3 ± 2.2^a	63.8 ± 1.6^a	68.4 ± 0.8^a	57.8 ± 0.7^a
CCl$_4$	29.4 ± 1.4^b	33.4 ± 0.6^b	35.8 ± 1.7^b	34.1 ± 0.6^b
oregano	69.2 ± 1.0^c	67.3 ± 0.4^c	72.6 ± 1.1^c	62.2 ± 1.0^c
CCl$_4$ + oregano	36.8 ± 2.3^d	37.8 ± 1.1^d	39.1 ± 2.2^b	38.0 ± 0.8^d

[a–d] Values in the same column with a superscript in common do not differ significantly at $P > 0.05$. [e] Values are means $\pm$ SD of six samples analyzed.

indicators of liver cell injury and are most helpful in recognizing hepatic disease. **Table 3** presents the changes in the activities of AST, ALT, and ALP in serum. Without CCl$_4$ treatment, diet supplementation with oregano (group 3) had no effect on the activities of these liver function marker enzymes as compared to the control. Treatment with CCl$_4$ (group 2) caused a significant increase ($P < 0.05$) of these marker enzymes, whereas feeding oregano before CCl$_4$ treatment (group 4) resulted in a significant decline ($P < 0.05$) of this increase, but the recorded values could not attain those of the control group.

Considering that lipid levels could change acutely with liver damage, cholesterol and triglyceride content in serum was also measured. **Table 3** shows that, without CCl$_4$ treatment, diet supplementation with oregano (group 3) had no effect on these biochemical parameters as compared to the control. Treatment with CCl$_4$ (group 2) caused a significant decrease ($P < 0.05$) in both cholesterol and triglyceride content, whereas feeding oregano before CCl$_4$ treatment (group 4) resulted in restoration of these parameters to values that did not significantly differ ($P > 0.05$) from those of the control group. This is in agreement with recent studies on CCl$_4$ poisoning (42). This is in line with other studies reporting that hypolipidemic effect of fruits and vegetables was evident only in rats fed cholesterol-containing diets (43).

Traditionally, most markers of oxidative injury reflect the free radical attack on polyunsaturated fatty acids and the resulting lipid peroxidation. MDA is one of the most frequently used indicators of lipid peroxidation. Increased MDA can be interpreted as resulting from cellular membrane damage initially caused by increased formation of radicals. The changes in the levels of MDA in serum, liver, kidney, and heart tissues in the present study are shown in **Table 4**. Without CCl$_4$ treatment, diet supplementation with oregano (group 3) had no effect on the recorded MDA levels as compared to the control. Treatment with CCl$_4$ (group 2) caused a significant increase ($P > 0.05$) in MDA levels, whereas feeding oregano before CCl$_4$ treatment (group 4) resulted in a significant reduction ($P > 0.05$) of this increase although the recorded MDA values could not attain the levels of the control group. The lipid oxidation process seemed to be a very instant event because the products of peroxidation appeared in the circulation within 6 h after the intraperitoneal injection of CCl$_4$. It was also surprising that the

most significant rise in tissue MDA levels was seen in kidney rather than in liver. Two possible explanations might be considered. First, MDA production in the kidney may be endogenous, arising from oxidative damage produced by CCl$_4$ escaping first pass metabolism in the liver. Alternatively, the high levels of MDA may be a result of trapping of MDA−lipoprotein complexes within the kidney. The increase in hepatic MDA seen in our experiments was significant but modest compared to that seen in relevant studies. This may be due to the high discriminative power of the third-order derivative technique applied in this study for MDA determination, which eliminated interferences from other thiobarbituric acid reactive substances (TBA-RS) (36).

Comparing different analytical methods to measure total antioxidant status in serum, Prior and Cao (44) concluded that no single measurement of antioxidant status is going to be sufficient and that a "battery" of methods will be necessary to adequately assess oxidative stress in biological systems. Thus, the improved ABTS radical cation decolorization assay (35) was used to measure total antioxidant status in rat tissues. **Table 5** presents the ABTS radical cation scavenging activity recorded in serum, liver, kidney, and heart tissues of rats. Without CCl$_4$ treatment, diet supplementation with oregano (group 3) had no effect on the Trolox equivalent antioxidant capacity of the tested tissues. This is not in line with other studies showing that dietary supplementation of grapefruit juice resulted in significant increase of the antioxidant capacity in rat plasma (43). Treatment with CCl$_4$ (group 2) caused a significant decrease ($P < 0.05$) of these values in tested tissues, whereas feeding oregano before CCl$_4$ treatment (group 4) had no significant effect ($P > 0.05$) on this decline.

The deoxyribose method most often used for estimation of the hydroxyl radical scavenging activity is based on TBA-RS substances formation and, therefore, is not suitable for tissues where lipid oxidation also leads to formation of TBA-RS substances (45). Thus, in the present study a hydroxyl radical scavenging method that is based on benzoate hydroxylation and not on TBA-RS formation was applied (37). The results on the assessment of the hydroxyl anion radical scavenging activity in rat serum, liver, kidney, and heart tissues are presented in **Table 6**. Without CCl$_4$ treatment, diet supplementation with oregano (group 3) increased significantly ($P < 0.05$) the levels of the hydroxyl radical scavenging activity in all tested tissues

Table 7. Superoxide Anion Radical Scavenging Activity in Rat Tissues

groups	superoxide anion scavenging activity[d] (%)			
	serum	liver	kidney	heart
control	7.4 ± 1.2^a	54.9 ± 1.6^a	27.7 ± 0.8^a	43.5 ± 0.7^a
CCl$_4$	4.2 ± 0.3^b	41.4 ± 1.3^b	19.4 ± 1.7^b	37.1 ± 1.1^b
oregano	8.2 ± 0.8^a	57.3 ± 1.9^a	28.4 ± 1.1^a	43.4 ± 1.0^a
CCl$_4$ + oregano	5.6 ± 0.4^c	46.2 ± 0.7^c	23.2 ± 2.2^b	38.1 ± 0.9^b

$^{a-c}$ Values in the same column with a superscript in common do not differ significantly at $P > 0.05$. ^{d} Values are means $\pm$ SD of six samples analyzed.

Table 8. Hydrogen Peroxide Scavenging Activity in Rat Tissues

groups	hydrogen peroxide scavenging activity[d] (%)			
	serum	liver	kidney	heart
control	15.1 ± 1.4^a	71.1 ± 2.5^a	64.3 ± 1.9^a	52.5 ± 2.1^a
CCl$_4$	6.3 ± 0.7^b	57.1 ± 1.3^b	43.3 ± 1.1^b	38.5 ± 1.0^b
oregano	14.9 ± 1.7^a	70.9 ± 1.6^a	64.1 ± 2.3^a	51.9 ± 1.2^a
CCl$_4$ + oregano	8.0 ± 1.2^b	64.4 ± 0.7^c	54.5 ± 1.6^c	44.2 ± 0.8^c

$^{a-c}$ Values in the same column with a superscript in common do not differ significantly at $P > 0.05$. ^{d} Values are means $\pm$ SD of six samples analyzed.

as compared to the control. Treatment with CCl$_4$ (group 2) caused a significant decrease ($P < 0.05$) in the hydroxyl radical scavenging activity of all tested tissues as compared to the control, whereas feeding oregano before CCl$_4$ treatment (group 4) resulted in a significant decline ($P < 0.05$) of this decrease. However, the recorded values could not attain those of the control group in all tested tissues except kidney.

Even though superoxide radicals are not particularly reactive, they may play an important role during the peroxidation of unsaturated fatty acids and possibly other susceptible substances (*46*). By scavenging them, dietary oregano could reduce or eliminate the formation of hydrogen peroxide and the toxic hydroperoxyl radicals derived from superoxide anions. Reducing the formation of hydrogen peroxide would also reduce the formation of hydroxyl radicals generated by Fenton or Haber−Weiss reactions that could participate in chain reactions. The scavenging process can generally be followed by means of electron spin resonance, but the expense of such instruments hinders their use by the average laboratory. Thus, estimation of the superoxide anion scavenging activity in the present study was carried out by a commonly used method slightly modified (*38*). **Table 7** shows the superoxide anion radical scavenging activity in rat serum, liver, kidney, and heart tissues. Without CCl$_4$ treatment, diet supplementation with oregano (group 3) had no effect on the superoxide anion radical scavenging activity of all tested tissues as compared to the control. Treatment with CCl$_4$ (group 2) caused a significant decrease ($P < 0.05$) of the superoxide anion radical scavenging activity in all tested tissues. Feeding oregano before CCl$_4$ treatment (group 4) resulted in significant decline ($P < 0.05$) of this decrease in serum and liver tissues but had no effect on the superoxide anion radical scavenging activity in kidney and heart tissues.

Table 8 shows the hydrogen peroxide scavenging activity of rat serum, liver, kidney, and heart tissues. This activity was estimated by measuring hydrogen peroxide as the limiting factor of the peroxidase-mediated oxidation of HVA into a fluorescent dimmer (*39*). Because the assay detects small changes in fluorescence, it is highly sensitive. The results showed that, without CCl$_4$ treatment, diet supplementation with oregano (group 3) had no effect on the hydrogen peroxide scavenging activity of all tested tissues as compared to the control. Treatment with CCl$_4$ (group 2) caused a significant decrease

($P < 0.05$) of the hydrogen peroxide scavenging activity in all tested tissues as compared to the control. Feeding oregano before CCl$_4$ treatment (group 4) resulted in significant decline ($P < 0.05$) of this decrease in liver, kidney, and heart tissues, indicating the potential role of oregano constituents present in tissues in reducing the H$_2$O$_2$ content of the cells, but had no effect on the hydrogen peroxide scavenging activity in serum tissues.

In conclusion, the results of the various assays applied in this study indicate that the degree of the antioxidant activity exhibited by dietary oregano depends not only on the specific tissue but also on the particular assay applied. The present study gives evidence that dietary oregano has the potential to quench free radicals, inhibit lipid peroxidation, and improve the antioxidant status in rat tissues. It inhibits the leakage of liver marker enzymes into circulation and, therefore, limits the membrane damage caused by CCl$_4$ toxicity. It may be worth stating that, at the completion of the study, average body weight gain and feed efficiency values did not differ significantly among groups (data not shown), indicating that the addition of oregano to the diet had no adverse influence on growth rate. Therefore, if shown clinically to be safe and having beneficial effects in vivo, oregano might be utilized in novel applications as a nutritional supplement or a functional food component.

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