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Evaluation of Extracts and Isolated Fraction from *Capparis spinosa* L. Buds as an Antioxidant Source

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The antioxidant activity of extracts from *Capparis spinosa* L. buds was evaluated using different in vitro tests: ascorbate/Fe^{2+}-mediated lipid peroxidation of microsomes from rat liver; bleaching of 1,1-diphenyl-2-picryl-hydrazyl radical; and autoxidation of Fe^{2+} ion in the presence of bathophenanthroline disulfonate. The methanolic extract showed strong activities in all of these in vitro tests. The amount of total phenols was determined in the methanolic extract. In addition, the level of rutin was calculated as 0.39% (w/w) by HPLC analysis. Our findings indicate the following: (a) the antioxidant efficiency of the methanolic extract may be attributed to its phenolic content; and (b) the antioxidant activity of the methanolic extract was maintained after removal of glucosinolates, confirming that these compounds do not interfere with the antioxidant properties of the extract. The results obtained from this study exalt the nutritional value of the flowering buds (capers) which are widely used as a source of flavor.

KEYWORDS: ***Capparis spinosa*****; capers; natural antioxidants; glucosinolates; rutin**

INTRODUCTION

The generation of reactive oxygen species (ROS) beyond the antioxidant capacity of a biological system gives rise to oxidative stress (*1*). It is well-known that free radical oxidative stress is implicated in the pathogenesis of a variety of human diseases. Cells and tissue normally possess antioxidant defense mechanisms to ensure the removal of reactive oxygen species; some are controlled endogenously (superoxide dismutase) and others are provided by dietary and other means (ascorbic acid, α-tocopherol, and β-carotene) (*2*). In the context of cellular prooxidant states and lipid peroxidation, simple plant components as part of the regular diet, other than traditional nutrients, may have influence in the treatment/amelioration/prevention of many chronic diseases, such as cancer, and cardiovascular and inflammatory damages including cellular degeneration related to aging (*3*).

Capparis spinosa L. (Capparidaceae) is one of the most commonly found aromatics in the Mediterranean kitchen, and it is also important in the commercial preparation of frozen food.

The aromatic part of the caper is the floral bud, which is gathered just before it blossoms. The plant is typically not cultivated, but rather the wild buds are harvested by seasonal pickers. Before commercial packaging, the buds are stored under salt.

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Previous chemical studies on *Capparis spinosa* have reported the presence of alkaloids, lipids, flavonoids, and glucosinolates (*4*), which are naturally occurring products belonging to the order Capparales, known as flavor compounds, cancer preventing agents, and biopesticides (*5*).

The objectives of the present work are the following: (a) to evaluate the antioxidant activity of the methanolic extract from *Capparis spinosa* buds; (b) to compare the antioxidant potential of the extract with that of rutin, which is one of the most commonly found flavonoid glycosides from aerial parts of *Capparis spinosa*; (c) to determine the differences in the antioxidant activity of the methanolic extract after removal of the glucosinolates.

The antioxidant properties were estimated by measuring the inhibition of ascorbate/Fe^{2+}-induced lipid peroxidation on microsomes from rat liver, the direct scavenging effect of the stable free radical on 1,1-diphenyl-2-picryl-hydrazyl (DPPH), and the autoxidation of Fe^{2+} ion in the presence of bathophenanthroline disulfonate (*6*).

MATERIALS AND METHODS

Materials. Plants of *Capparis spinosa* L. were collected during Spring 1999 from Lipari, in the Aeolian Archipelago (Italy), and they were authenticated by the botanists of the Pharmaco-Biological Department of the University of Messina (Italy).

Chemicals. Ascorbic acid, boric acid, 1,1-diphenyl-2-picryl-hydrazyl (DPPH), (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]) (HEPES), iron sulfate heptahydrate, ossalic acid, potassium chloride, rutin, 1,1,3,3-tetraethoxypropane, thiobarbituric acid (TBA), and ba-

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thophenanthroline disulfonate were purchased from Sigma (Sigma Chemical Co., St. Louis, MO). *p*-Dimethylaminocinnamaldehyde was from Merck (E. Merck, Darmstadt, Germany); butylated hydroxytoluene (BHT) was from Fluka (Fluka Chemie AG, Buchs, Switzerland); and Folin-Ciocalteu reagent was from Carlo Erba Reagenti (Milano, Italy). All solvents were HPLC grade, and were purchased from Carlo Erba.

Preparation of Extracts. According to the method of Elliot and Stowe (*7*), the fresh buds were separated from the aerial parts, weighed (500 g), then immersed in boiling methanol for 2 min, and finally left in cooled methanol overnight before filtration.

The residue was ground and extracted with warm (35 °C) 80% methanol (500 mL × 2). The solvent was evaporated from the combined filtrates under reduced pressure to obtain the extract (yield 1.68%).

A part of the methanolic extract of *Capparis spinosa* buds obtained as described above was preliminarily passed through an alumina acid column. A glucosinolate fraction was eluted by sodium sulfate (1%). The presence of glucosinolates in the eluted fraction was confirmed by TLC, using as solvent system butanol/ethanol/water (4:1:2, v/v upper phase), and for detection the *p*-dimethylaminocinnamaldehyde reagent (Ehrlich's reagent) (*8*).

The methanolic extract residue after removal of the glucosinolate fraction was recovered on the alumina acid column.

Determination of Total Phenolic Compounds. The levels of total phenolic compounds were determined in the methanolic extract. An aliquot of extract (0.1 mL of 10 mg/mL) was mixed with 0.2 mL of Folin-Ciocalteu reagent, 2 mL of H_2O, and 1 mL of 15% Na_2CO_3, and the absorbance was measured at 765 nm after 2 h incubation at room temperature. Rutin was used as the standard for the calibration curve, and the total phenolics were expressed as milligrams of rutin equivalents per gram of methanolic extract.

TLC and HPLC−DAD Analysis. The presence of rutin was preliminarily assayed in the methanolic extract by TLC, using precoated silica gel 60 F_{254} plates (Merck), eluted with ethyl acetate/formic acid/water (8:1:1). For detection, the plates were sprayed with boric acid−oxalic acid reagent (*9*), heated, and examined in long-wave UV light. For quantification of rutin, an HPLC−DAD analysis was performed: three replicates from each methanolic extract were filtered through a 0.45-μm filter. Samples of 20 μL of extract were analyzed using an HPLC system (Hewlett Packard, 1100 pump) with an autosampler, coupled with a photodiode array detector (DAD) operated by HP ChemStation software. A reversed-phase C_{18} Nucleosil column was used (250 × 4 mm, 5 μm) (Hewlett Packard), and maintained at room temperature.

The mobile phase was methanol/acetic acid 1% aqueous solution, 43:57 (v/v) at a flow rate of 1 mL/min. Rutin was identified by its retention time and its UV spectrum, which were compared to those of the authentic standard. Quantitation of rutin was performed by using a six points regression curve (r^2= 0.999) obtained by employing the authentic standard.

Isolation of Liver Microsomes. Male Sprague Dawley rats (180−200 g) (Morini s.a.s. Reggio Emilia, Italy) were acclimatized for two weeks at 23 ± 2 °C with free access to standard rodent chow and water before treatment.

Healthy rats were selected and sacrificed by decapitation under ether anesthesia after overnight starvation. Liver microsomes were isolated as described by Fowler et al. (*10*). The livers were quickly removed and homogenized with a glass potter in ice-cold KCl (1.15%) buffered with 0.02 M HEPES (pH 7.5) at 4 °C, to make a 20% (w/v) mixture. The homogenate was centrifuged at 670*g* for 10 min to remove nuclei and cell debris, then at 10000*g* for 15 min to remove mitochondria, and again at 105000*g* for 60 min. The microsomal pellet was resuspended in HEPES−KCl buffer (pH 7.5) and the last step was repeated. Finally, aliquots of microsomal pellets were stored at −80 °C until use. For the experiment, the microsomes were thawed, diluted in ice-cold HEPES−KCl buffer, and washed with centrifugation at 105000*g* for 45 min.

The pellet was resuspended in HEPES−KCl buffer, and microsomal protein concentrations were determined according to the method of Bradford (*11*).

Assay of Nonenzymatic Lipid Peroxidation. The liver microsomal pellet was heat-inactivated at 90 °C for 1.5 min to remove all enzymatic factors, and then it was resuspended in KCl/HEPES. For ascorbate/Fe^{2+}-induced lipid peroxidation, the incubation mixture (1 mL) consisted of microsomal suspension (0.5 mg of proteins), various concentrations of the tested compounds in DMSO, and ascorbic acid (0.1 mM). The reaction was started by the addition of a freshly prepared $FeSO_4$ solution (10 μM). The mixture was incubated for 1 h at 37 °C (*12*). As a marker of lipid peroxidation, the formation of thiobarbituric acid reactive substances (TBARS) was determined (*13*). TCA/TBA/HCl solution (2 mL, 15% w/v trichloracetic acid, 0.375% w/v thiobarbituric acid, 0.25 N hydrochloric acid) was added to the sample mixture which was then heated for 15 min in a boiling-water bath. After the mixture cooled the flocculent precipitates were removed by centrifugation (1000*g* for 10 min).

The absorbance of the resulting solution was measured at 535 nm, using a spectrophotometer (Shimadzu UV-1601). The percentage of inhibition is expressed as the inhibition of lipid peroxidation of sample compared to that of a blank using the following formula:

$$\%I = \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \times 100$$

where % I = percentage of inhibition, A_{blank} = absorbance of the blank, and A_{sample} = absorbance of the sample. Butylated hydroxytoluene (BHT) was tested and used as reference standard. All tests were performed in triplicate, and the results were averaged.

Measurement of the DPPH Radical-Scavenging Activity. The method of Ohinishi et al. (*14*), with minor modification, was used for assessment of the free radical-scavenging activity of *Capparis spinosa* extracts and rutin.

After dissolving the test sample in methanol, aliquots (0.5 mL) were mixed with 3 mL of a 0.1 mM methanolic solution of DPPH. The mixture was shaken vigorously and then kept in the dark for 30 min at room temperature. The decrease in absorbance of the resulting solution was measured at 517 nm with a spectrophotometer (Shimadzu UV-1601). DPPH solution was freshly prepared prior to use, and BHT was tested and used as reference standard. All tests were performed in triplicate, and the results were averaged.

The inhibition percentage (% I) of the DPPH$^\bullet$ radical was calculated according to the following formula:

$$\%I = \frac{A_{C(0)} - A_{A\,(t)}}{A_{C(0)}} \times 100$$

where $A_{C(0)}$ is the absorbance of the control DPPH solution at t = 0 min and $A_{A\,(t)}$ is the absorbance after addition of test samples at t = 30 min.

The extract concentration required to reduce the absorbance of DPPH control solution by 50% (EC_{50}) was obtained by interpolation of concentration−inhibition curves.

Autoxidation of Fe^{2+} Ion in the Presence of Bathophenanthroline Disulfonate. Fe^{2+} autoxidation was evaluated by determining the Fe^{2+} concentration with bathophenanthroline disulfonate, a reagent that avidly binds Fe^{2+} forming a complex with a maximum absorption at 540 nm (*6*). A stock solution of $FeSO_4$ was prepared daily. The samples (1 mL) contained 10 mM Tris−HCl (pH 7.1), 0.05 mM $FeSO_4$, and various concentrations of the tested compounds. All the incubations were carried out at 37 °C. The reaction was started by addition of $FeSO_4$. Aliquots (0.5 mL) of a 1 mM bathophenanthroline disulfonate solution were added to the samples at appropriate intervals of time, and the decrease in absorbance of the resulting solutions was measured at 540 nm with a spectrophotometer (Shimadzu UV-1601).

Rutin was tested and used as reference standard. Bathophenanthroline disulfonate solution was freshly prepared before use. All tests were performed in triplicate, and the results were averaged.

RESULTS

The level of total phenolic compounds was 100.51 mg of rutin equivalents per gram of methanolic extract.

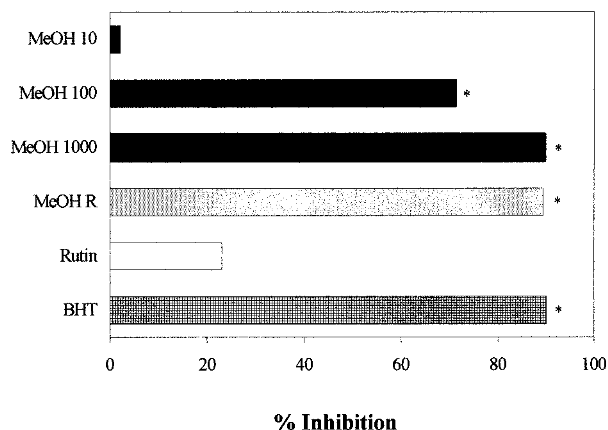

Figure 1. Effect of *Capparis spinosa* L. extracts on ascorbate/Fe^{2+}-induced lipid peroxidation from rat liver microsomes. Notes: % Inhibition = (mean absorbance of the blank − mean absorbance of sample)/mean absorbance of blank. * = Values significantly different at $p < 0.01$ (ANOVA followed by Student's *t*-test as post-test). MeOH = methanolic extract at 10, 100, and 1000 μg/mL concentrations. MeOH R = methanolic extract residue at 1000 μg/mL concentration, after removal of glucosinolates. Rutin tested at 3.90 μg/mL corresponding to 1000 μg/mL methanolic extract. BHT at 2.20 μg/mL concentration.

The effect of *Capparis spinosa* extract on ascorbate-induced lipid peroxidation of microsomes from rat liver was investigated. As shown in **Figure 1**, the methanolic extract exhibited a dose-dependent inhibitory activity on ascorbate-induced lipid peroxidation. It is evident that this extract at 100 and 1000 μg/mL concentrations inhibits significantly ($p < 0.01$) lipid peroxidation by 71.50% and 90%, respectively. The effect of the highest dose is comparable to that of BHT, which produced an inhibition of 90.13% ($p < 0.01$). The antioxidant activity of the methanolic extract was maintained even after removal of the glucosinolate fraction.

The methanolic extract of *Capparis spinosa* buds contains 0.39% w/w of rutin as revealed by HPLC analysis. In the lipid peroxidation assay, rutin, tested at the concentration found in the methanolic extract, produced an inhibition of 23%.

Free radical scavenging effect of *Capparis spinosa* L. extracts was determined using the DPPH test. The methanolic extract showed antioxidant properties for the scavenger activity against the stable radical DPPH, expressed as percent inhibition (**Figure 2**). The strong activity of the methanolic extract is clearly evident even after removal of glucosinolates. Rutin was tested at concentrations corresponding to its content in the methanolic extract, reaching 47.86% of inhibition at the highest dose. Results of EC_{50} values are shown in **Table 1**.

Autoxidation of Fe^{2+} was assayed in the presence of bathophenanthroline disulfonate. Decrease in absorbance at 540 nm was monitored as a color change of the test solution from pink to yellow at different intervals during a time of 20 min as a result of autoxidation of Fe^{2+} ion. The methanolic extract of *Capparis spinosa* increased the rate of Fe^{2+} autoxidation markedly, both at 100 and 1000 μg/mL. Autoxidation of iron increased considerably, even after removal of glucosinolates from the methanolic extract. Rutin, tested at 3.90 μg/mL concentration (corresponding to 0.39% w/w of extract), enhanced iron autoxidation to a lesser extent as compared to that of the methanolic extract at 1000 μg/mL concentration (**Figure 3**).

The glucosinolate fraction did not show evidence of any antioxidant activity in all of these in vitro tests (data not shown).

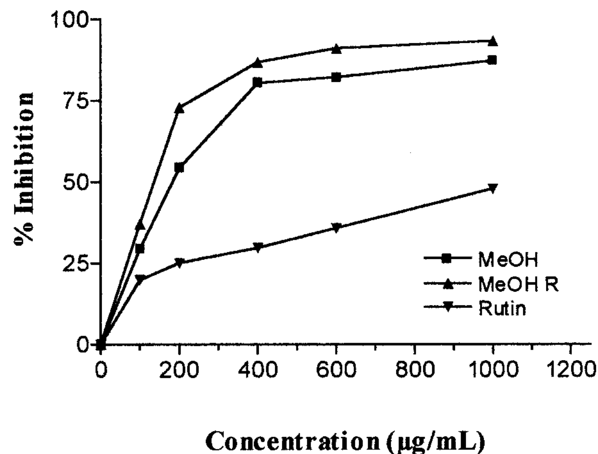

Figure 2. Effect of *Capparis spinosa* L. extracts on DPPH radical scavenging activity. Notes: % Inhibition = (absorbance of DPPH solution − (absorbance of DPPH + test sample))/absorbance of DPPH solution. MeOH = methanolic extract. MeOH R = methanolic extract residue after removal of glucosinolates. Rutin tested at 0.39, 0.78, 1.56, 2.34, and 3.90 μg/mL corresponding to 100, 200, 400, 600, and 1000 μg/mL methanolic extract, respectively.

Table 1. Effect of *Capparis spinosa* Extracts on DPPH Test: EC_{50}[a]

sample	EC_{50} (μg/mL)
MeOH	177.451 ± 6.132
MeOH R	117.036 ± 5.640
rutin	6.130 ± 2.170
BHT	119.188 ± 3.451

[a] All values are shown as the mean ± standard deviation of at least three experiments, each in triplicate. MeOH = methanolic extract. MeOH R = methanolic extract residue after removal of glucosinolates.

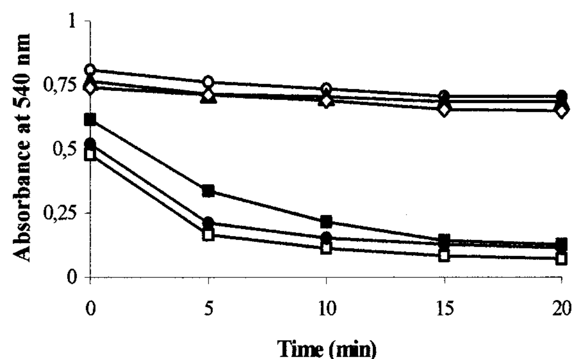

Figure 3. Effect of extracts from *Capparis spinosa* L. on the autoxidation of ferrous ion. Iron autoxidation was followed by determining the ferrous ion concentration according to the bathophenanthroline disulfonate method. -○- None; -▲- methanolic extract, 10 μg/mL; -■- methanolic extract, 100 μg/mL; -●- methanolic extract, 1000 μg/mL; -□- methanolic extract after removal of glucosinolates; -◇- rutin tested at 3.90 μg/mL concentration.

DISCUSSION

In this study, results clearly indicate that the methanolic extract from *Capparis spinosa* buds exhibited good antioxidant activities: it effectively preserved microsomes from iron-induced lipid peroxidation, reaching a 90% inhibition when tested at 1000 μg/mL concentration. Despite this result, the activity of the methanolic extract was lower than that of BHT. In fact, in the lipid peroxidation test when BHT was tested as reference pure compound, it required 2.20 μg/mL to produce a percent of inhibition of the same extent.

Moreover, the methanolic extract possessed hydroxyl radical scavenging properties acting as donor for hydrogen atoms or electrons in the DPPH test; it also increased iron autoxidation, markedly, suppressing the accessibility of iron to oxygen molecules by oxidizing ferrous ion to ferric state, which resulted in the inhibition of the hydroxyl radical production (*6*). These results suggest that the antioxidant activities of the methanolic extract are related to the high level of phenolic compounds.

Formation of reactive oxygen species is closely related to the redox state of transition metals; superoxide anion is readily produced through the one-electron reduction of oxygen by ferrous ion and it is largely dismutated into hydrogen peroxide by enzymatic and nonenzymatic mechanisms (*1*). This study showed the presence of rutin in the methanolic extract of *Capparis spinosa* buds. Yoshino and Murakami (*6*) reported that both rutin and its aglycon quercetin inhibited microsome peroxidation induced by Fe^{2+}. Formation of inactive iron−rutin complex, which is unable to generate the reactive hydroxyl radical, plays a principal role in the antioxidant action of flavonoids (*15*). Besides, concerning the role of rutin in the different assays, it is evident that other phenolic compounds may be involved in the antioxidant properties of the methanolic extract. Previous studies have reported the presence of other flavonoid compounds such as kaempferol and quercetin glycosides in addition to rutin (*4, 16*).

The present study also suggests that glucosinolates, a group of naturally occurring thioglucosides typically found in the order of Capparales, do not seem to interfere with the antioxidant properties of the extract. In fact, the antioxidant activity of the methanolic extract was maintained after removal of a glucosinolate fraction. In plants, glucosinolates co-occur with the endogenous thioglucosidase−myrosinase system which is responsible for the production of hydrolysis products including isothiocyanates (*17*). Traces of isothiocyanates were revealed by TLC (*9*) in the methanolic extract even after removal of glucosinolates, due to uncontrolled enzymatic reactions of the crushed capers during the extraction process.

Isothiocyanates are well-known for the important role they play in plant defense, and also in human affairs as flavor compounds, cancer-preventive agents, and biopesticides (*5, 18*). Antioxidant properties can be also assigned to the isothiocyanates, although few related data are reported (*19*) suggesting that these latter compounds may have a possible role in the antioxidant activity of *Capparis spinosa* extract.

The results obtained from this study bring attention to the antioxidant potential of and exalt the nutritional value of *Capparis* flowering buds, which are widely used as a source of flavor. There is currently a strong interest in the study of daily foods which are rich in antioxidant compounds such as flavonoids, anthocyanins, carotenoids, and vitamins. Epidemiological studies have shown that, in addition to the essential nutrients, there are many food products that contain a variety of biologically active compounds providing protection against many chronic diseases (*20*). On the effectiveness of naturally occurring compounds with antioxidative properties, an increased intake of caper buds with the diet, and their cultivation for such uses, should be encouraged.

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