Oregano Flavonoids as Lipid Antioxidants

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Oregano (Origanum vulgare L.) leaves were successively extracted with hexane, ethyl ether, ethyl acetate and ethanol. The ethanol extract was reextracted in a separatory funnel with petroleum ether, ethyl ether, ethyl acetate and butanol. The ethyl ether layer was the most effective in stabilizing lard against oxidation, with activity equal to butylated hydroxytoluene. It also showed antioxidant activity when tested on vegetable oils under storage or frying conditions. The main antioxidant factors isolated from the ethyl ether layer consisted of flavonoids. Chromatographic and spectrophotometric analysis demonstrated the presence of the flavone apigenin, the flavanone, eriodictyol and the dihydroflavonols, dihydrokaempferol and dihydroquercetin.

KEY WORDS: Antioxidant activity of flavonoids, antioxidants, flavonoids, lipid oxidation, natural antioxidants, oregano extracts, oregano flavonoids, plant extracts.

Flavonoids are widely distributed in the plant kingdom. Several relevant compounds have been isolated from plant extracts and found to possess antioxidant activity (1–4). The efficiency of polyhydroxyflavonoids in relation to their structure also has been investigated (5–7).

Oregano extracts have shown a pronounced effect in stabilizing lipids against autoxidation (8,9). In a recent study we extracted oregano leaves with solvents of increasing polarity, namely hexane, ethyl ether (EE), ethyl acetate (EA) and ethanol, and found all the extracts effective in stabilizing lard. We were able to isolate the main antioxidant factors of the hexane extract (10). The present work was undertaken to isolate and identify the antioxidant compounds of the ethanol extract of oregano leaves.

EXPERIMENTAL PROCEDURES

Preparation of the extracts. Oregano leaves (200 g) were successively extracted with hexane, ethyl ether, ethyl acetate and 95% ethanol as described by Vekiari et al. (10). The ethanol extract was further processed for the isolation of antioxidants as follows: the extract was filtered, and the filtrate was dried under vacuum at 40°C in a rotary evaporator. The residue was thoroughly mixed with 400 g boiling water and filtered. The filtrate was successively extracted in a separatory funnel with petroleum ether, b.p. 40–60°C (PE), EE, EA and butanol. Several 100-mL portions of each solvent were used until the solvents were colorless. The obtained layers were dried and kept in sealed dark bottles until use.

Column chromatography. The EE layer was further fractionated by column chromatography. A glass column (i.d. 2.5 cm, length 50 cm) was used, packed with silica gel (7734 Merck, Darmstadt, Germany). Six fractions were obtained by stepwise gradient elution with 1, 5, 10, 50 and 100% methanol in dichloromethane, as presented in Figure 1. The fraction Fr2 was refractionated on the same column by stepwise gradient elution with 5, 10 and 20% methanol in dichloromethane, to three subfractions, namely 2A, 2B and 2C (Fig. 1).

Thin-layer chromatography (TLC). TLC plates precoated with silica gel (5715 Merck) were used to separate the constituents of the subfraction 2B. The plates were developed with chloroform/acetic acid/formic acid (9:2:1, vol/vol/vol) (CAF). Bands of interest were scraped from TLC plates, soaked overnight in 50 mL methanol, filtered, evaporated in vacuum to near dryness in a rotary evaporator at 40°C and redissolved in 1 mL methanol. Further separation was conducted on polyamide sheets (11F 254 Merck) with benzene/methyl ethyl ketone/methanol (4:3:3, vol/vol/vol) (BMM) as solvent. The eluted components were further separated by two-dimensional technique on cellulose plates (5716 Merck). Chromatograms were developed in the upper phase of n-butanol/acetic acid/water (4:1:5, vol/vol/vol) (BAW), air dried and redeveloped in the second dimension in 15% acetic acid. Other developing systems used were ethyl acetate/acetic acid/water (8:2:4, vol/vol/vol).

FIG. 1. Isolation of antioxidants from the ethyl ether layer. Percent refers to % methanol in dichloromethane. Fr, fraction; TLC, thin-layer chromatography; CAF, chloroform/acetic acid/formic acid; BMM, benzene/methyl ethyl ketone/methanol.

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(EAW) on cellulose plates and methanol/acetic acid/water (72:4:4, vol/vol/vol) (MAW) on polyamide sheets.

The following spray reagents were used to identify chemical compounds: (i) aluminum chloride 5% solution in water. A yellow color indicates the presence of flavonoids (11,12); (ii) naturstoff reagent 2-aminoo-thylenester-di-phenylboric acid (0.75% in methanol). Flavonoids present characteristic fluorences when sprayed with naturstoff-reagent. A yellow-green fluorescence is indicative of 4'-OH flavonoids (12). Especially kaempferol derivatives have a bright, yellow-green color (13). Orange-yellow fluorescence indicates the presence of quercetin derivatives (13).

Tests of antioxidant activity. Storage tests were conducted on lard and refined corn oil, soybean oil and olive residue extracted oil. The oils were obtained from a commercial refining plant and contained no additives. Lard was melted at 85°C and filtered before use. A calculated quantity of the antioxidant (to obtain a concentration of 0.01 or 0.02%, w/w, on a dry basis) was added to 25 g of the substrate and dissolved by stirring. Comparative experiments were conducted with the commercial antioxidants butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) or tertiary butylhydroquinone (TBHQ) at the same concentration as the oregano antioxidants. Control samples with no additives were prepared under the same conditions. The samples were incubated in the dark in 50-mL open beakers at 35 and 65°C. All experiments were run in duplicate and the presented results are the average of two trials. Significance of treatments was estimated by the Student's t-test at the probability level of 0.05 (14). At definite time intervals the peroxide values were determined according to Official Method Cd 8-53 of the American Oil Chemists' Society (15).

The EE layer was tested at frying temperature (180°C) in refined olive residue extracted oil at a concentration of 0.05%. TBHQ at the same concentration was tested for comparison. The samples (50 g) were heated in 100-mL open beakers on electric hot plates for 8-h periods daily, followed by overnight cooling at room temperature. The temperature was adjusted at 180 ± 3°C by a rheostat. All experiments were run in duplicate and the presented results are the average of two trials. Samples were withdrawn and analyzed at definite time intervals. The absorbance at 232 nm (E232), the iodine value (IV) and the refractive index (RI) were determined according to Standard Methods 2.505, 2.205 and 2.102, respectively, of the International Union of Pure and Applied Chemistry (16). Analysis of fatty acid composition was conducted on the starting oil and after nine days of frying by Official Method Ce 1-62 of the American Oil Chemists' Society (15). A Hewlett-Packard series 5700 gas chromatograph (Hewlett-Packard Co., Avondale, PA) was used, equipped with a flame-ionization detector and a packed column (15% DEGS, Chrom Pack, Middelburg, The Netherlands). The temperature of the injector and detector was adjusted to 250°C and the temperature of the oven to 180°C.

The bands separated by TLC were tested for antioxidant activity according to the carotene spray procedure of Philip as described by Pratt and Miller (4). β-Carotene (9 mg) was dissolved in 30 mL chloroform. Two drops of purified linoleic acid and 60 mL ethanol were added to the β-carotene-chloroform solution. The solution was sprayed on chromatograms streaked with the antioxidant solution, and the chromatograms were exposed to daylight until background color was bleached. Bands in which yellow color persisted possessed antioxidant activity.

Spectral identification of flavonoids. Spectra, 200–450 nm, were obtained by using a Varian DMS 80 ultraviolet (UV)-VIS spectrophotometer (Varian, Springvale, Australia). A solution of the antioxidant (3 mL at a concentration of 100 ppm) in methanol (spectral grade) was used as the test solution. The sodium methoxide spectrum was measured immediately after three drops of stock solution (2.5 g sodium dissolved in 100 mL methanol) were added to the test solution (12,17).

For the sodium acetate spectrum, coarsely powdered anhydrous sodium acetate was added to a cuvette containing the antioxidant solution until a 2-mm layer of sodium acetate remained at the bottom. The spectrum was recorded two minutes after the addition of the sodium acetate. The boric acid-sodium acetate spectrum was recorded after mixing with the addition of boric acid to the cuvette containing the sodium acetate-antioxidant solution (12,17).

The aluminum chloride spectrum was recorded immediately after the addition of six drops of aluminum chloride stock solution (5.0 g aluminum chloride dissolved in 100 mL methanol) to a cuvette containing the antioxidant solution. Three drops of hydrochloric acid-aluminum chloride stock solution (5.0 mL hydrochloric acid and 5.0 mL aluminum chloride solution) were added to the cuvette, and the hydrochloric acid-aluminum chloride spectrum was recorded immediately (12,17).

RESULTS AND DISCUSSION

Antioxidant activity of the extracts. Table 1 presents the antioxidant activity of the ethanol extract and the obtained layers in lard, compared to BHA and BHT. The PE layer was too dilute (total solids content 0.02%, wt/vol) and was not tested. The EE layer was the most effective, with activity equal to BHT and much greater than the parent ethanol extract.

The results of the addition of the EE layer to vegetable oils stored at 65 and 35°C are given in Tables 2 and 3, respectively. The stability of the oils toward autoxidation was considerably increased with the addition of EE. Compared to BHT, the EE layer was a better antioxidant in most cases, but it was not as effective as TBHQ at 35°C.

TABLE 1

<table>
<thead>
<tr>
<th>Additive</th>
<th>Peroxide value (meq/kg) after days</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
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<tr>
<td>None</td>
<td></td>
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<tr>
<td>Ethanol extract</td>
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<tr>
<td>Ethyl ether layer</td>
<td></td>
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<td>Ethyl acetate layer</td>
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<td>Butanol layer</td>
<td></td>
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<td>Butylated hydroxyanisole</td>
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<td>Butylated hydroxytoluene</td>
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aValues within a column followed by different superscript letters are significantly different (P < 0.05).