Antioxidative Effect of Ajowan in a Model System

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ABSTRACT: Model conditions were utilized to test the antioxidative activity of a methanolic extract of ajowan (*Carum coptimum*) (MEA). MEA was less effective than butylated hydroxytoluene in protecting against oxidation of emulsified linoleic acid measured by coupled oxidation of linoleic acid–β-carotene, conjugated diene values, and the thiobarbituric acid values. Ajowan may have potential as a source of natural antioxidant.

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KEY WORDS: Ajowan, antioxidants, natural.

Lipids may be oxidized and hydrolyzed when food is exposed to oxygen during storage (1). Because of oxidation of lipids, several changes in food occur, such as changes in color, odor (2), texture, and nutritive value (3). Synthetic antioxidants, such as butylated hydroxyanisole, butylated hydroxytoluene (BHT), and tertiary butylhydroquinone, are used chiefly to reduce oil deterioration, rancidity, and discoloration from oxidation (4). They also prevent formation of toxic substances in food.

Approximately 5 to 10 lb of synthetic food additives are consumed each year by the average U.S. citizen (5). At the same time, there is increasing consumer concern about the use of synthetic compounds as chemical additives in foods. Consumers have become more cautious about nutritional quality and safety regarding food additives. In response to consumer demand, several natural antioxidants have been identified for possible use in foods (6). Many spice extracts may be promising sources of natural antioxidants. Also, vitamin E, rosemary (7), β-carotene (8), and ginger rhizome (9) have commercial significance. The need for widely utilizable and easily obtainable natural antioxidants continues.

Ajowan (*Carum coptimum*) seeds are cultivated in the Mediterranean and in south Asian countries. The seed is widely used as a flavoring substance for many food recipes. Ajowan seeds were utilized as a principal source of thymol and other phenolic substances (10,11). The present work was conducted with ajowan seeds to determine the antioxidative power of different ajowan seed extracts in a model system of emulsified linoleic acid.

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MATERIALS AND METHODS

Raw materials. Ajowan seeds were obtained from GSCSC Ltd. (Gandhinagar, Gujarat, India). Seeds were cleaned using a sieve set of different sizes to remove contamination particles. After cleaning, the seeds were stored under inert gas atmosphere at −25°C until utilized for extraction of substances with antioxidative properties. All glass containers were immersed in ethylenediaminetetraacetate disodium salt (EDTA) (0.5% wt/vol) for at least 24 h, rinsed several times with deionized water to minimize heavy metal contamination, sterilized, and dried at 150°C.

Chemicals and reagents. Glacial acetic acid, methanol, 1-butanol, 2-thiobarbituric acid (TBA), diethyl ether, ethanol, dichloroethane, ethyl ether, chloroform, methanol, and petroleum ether (b.p. 30–60°C) were obtained from Fisher Scientific (Itasca, IL). Linoleic acid [purest grade, 99% by gas–liquid chromatography (GLC)], Tween 20, BHT, EDTA, and crystalline *cis*-β-carotene were obtained from Sigma Chemical Company (St. Louis, MO).

Instruments. A spectrophotometer (UV-1201 spectrophotometer; Shimadzu Corporation, Kyoto, Japan) was used for absorbance measurements. A sonicator W 375 (Heat Systems; Ultrasonics, Inc., Long Island, New York) was used for emulsification, and extractions were carried out with a Soxhlet apparatus.

Analytical procedure. The extraction procedure of Taga *et al.* (12) was utilized. Combinations of solvents, extraction times, and thin-layer chromatography (TLC) tests were selected from the work of Duve and White (13). Antioxidative activities of the extracts were determined according to the procedure of Farag *et al.* (14).

Extraction. Cleaned ajowan seeds were air-dried at 40°C for 48 h to ensure dryness, then finely ground (1-mm screen) in a Wiley mill (12). Each ground sample (100 g) first was continuously extracted for 6 d in a Soxhlet extractor, with 250 mL of solvent. Ethyl ether was used for extraction number 1, and petroleum ether was used for extraction numbers 2–7, as shown in Figure 1. Fresh solvent was added to make up non-significant evaporation losses during extraction. The residues were air-dried and reextracted for each method with solvents. Fresh solvent was added, if needed, during extraction. Crude, viscous, concentrated extracts were obtained after rotary evaporation at 45°C. The whole extraction procedure was carried out in the dark to protect the extract from light-induced

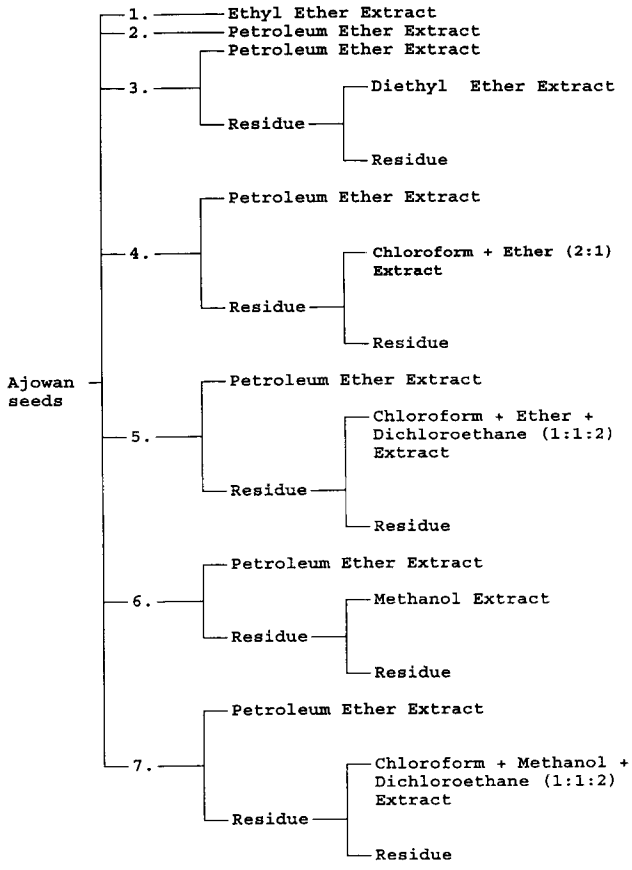

FIG. 1. Scheme for extraction of antioxidants from ajowan seeds.

isomerization. All extracts were stored under nitrogen at −18°C until tested.

TLC tests. TLC tests were carried out for rapid evaluation of antioxidative activity among extracts. In this procedure (15,16), TLC plates (0.25 mm), precoated with Silica Gel G (Fisher Scientific), which had been activated at 100°C for 1 h, were streaked with 200 μL of extract, and developed by a mixture of chloroform/ethanol/acetic acid (98:2:2). Plates were sprayed with a solution of 9 mg β-carotene dissolved in 30 mL chloroform, to which two drops of linoleic acid and 60 mL of ethanol were added. The intensity and persistence of the orange color resulting from daylight exposure for 6 h corresponded to the relative antioxidant activity of the extracts (12,13,17).

Testing of ajowan in a model system with linoleic acid. The experimental procedures of Farag *et al.* (14) were followed. In this method, 10 mL β-carotene (dissolved in chloroform, 0.05%) was pipetted and mixed with linoleic acid (*ca.* 1.4 g) and 1 mL Tween 20 (0.02%). The solvent was evaporated, then 500 mL deionized water was added and emulsification was achieved with agitation by using an ultrasonic bath. The stock solutions of emulsified solvent extracts had been prepared by the following procedure. Methanolic extracts of ajowan (MEA), with the highest antioxidative activity in TLC tests, were mixed with Tween 20 at different levels (0.02, 0.05, 0.1, and 0.2% on w/w basis) in a 50-mL volumetric flask. The mixture was filled to volume with deionized water and emulsified by agitation in an ultrasonic bath for 15 min. Similarly, BHT (0.02% on w/w basis) was mixed with Tween 20, made up to 50 mL with deionized water and emulsified in the same manner as the MEA.

Reaction mixture. Reaction mixtures were prepared by mixing emulsified MEA solution with emulsified linoleic acid–β-carotene solution. Similarly, BHT solution (0.02%) was added to emulsified linoleic acid–β-carotene to compare the antioxidant efficiency with MEA. Six beakers of each concentration (0.02, 0.05, 0.1, and 0.2%) of methanolic extracts of ajowan and six beakers of BHT along with six beakers of control (without extract or synthetic antioxidant) were kept in a water bath at 50°C. Aliquots were taken from each of the beakers of all treatments (0.02, 0.05, 0.1, 0.2% MEA, 0.02% BHT and control) at the intervals shown in the following three methods and analyzed to follow the oxidation of linoleic acid.

(i) Coupled oxidation with β-carotene. In the method of coupled oxidation with β-carotene (17), β-carotene was a marker for linoleate oxidation, and the bleaching of β-carotene resulted from oxidation by degradation products of linoleic acid (12). An aliquot (0.2 mL) of the reaction mixture was taken at 1-h intervals from each beaker, then 3 mL ethanol (99%) was added to the aliquot and the mixture was vortexed for 30 s. Absorbance was recorded with a spectrophotometer at 462 nm against a blank containing emulsified linoleic acid–β-carotene.

(ii) Conjugated diene (CD) values. This method is used to trace lipid oxidation by measuring CD formation. To measure CD values (18), an aliquot (0.1 mL) of the reaction mixture was taken from each beaker at one-day intervals, diluted with methanol (3 mL), and vortexed. Absorbance was measured at 232 nm by using the spectrophotometer against a blank containing the reagents (MEA, β-carotene), except linoleic acid.

(iii) TBA test. An aliquot (100 mg) of the reaction mixture was taken from each beaker and made up to 25 mL with 1-butanol in a 25-mL volumetric flask, and the solution was mixed (19). A 5-mL aliquot of this diluted reaction mixture and 5 mL TBA reagent were pipetted into a dry, stoppered test tube and placed in the water bath at 95°C for 120 min. TBA reagent was made as follows: 200 mg of TBA was added to 100 mL 1-butanol, the mixture was filtered, and stored at 4°C for not more than seven days. After samples were cooled, light absorbance was measured at 532 nm against a blank containing all the reagents except linoleic acid. Samples were drawn at one-day intervals. The extinction coefficient of the TBA–malonaldehyde product (1.56×10^5) was used to convert the absorbance values into concentration of secondary oxidation products.

Statistical analyses. The experimental design was completely randomized, and six replications (six beakers) for each treatment were randomly and independently processed. Data analysis and graphic plotting were done with SAS programs (20). Differences were determined by comparing treatment

means with the least significant difference multiple comparison method and then the procedures of Newman (21) and Keuls (22). Analysis of variance and regression analysis were also used to analyze the data (23).

RESULTS AND DISCUSSION

TLC test for rapid evaluation of antioxidative activity among extracts. Concentrated ajowan extracts as shown in Figure 1 were evaluated for antioxidant activity by using TLC tests. Methanolic extracts produced the darkest orange spots (13) and showed the greatest antioxidative activity among all extracts. These methanolic extracts were utilized for further study in a model system which was simplified to minimize variables and obtain reproducible results. The variables were restricted to the type of antioxidants.

Coupled oxidation of linoleic acid–β-carotene method. Six replications of each treatment were run and averaged. The trend of the disappearance of β-carotene coupled with oxidation of the linoleate system is shown in Figure 2. The data obtained showed that all levels of MEA produced an antioxidative effect, and the extent of antioxidant activity was largely dependent on the concentration of the crude extracts. As shown in Figure 2, the time for bleaching of β-carotene was the fastest for the control, was slower for treatments of MEA at different concentrations, and was the slowest for BHT. The greater time required for the complete bleaching of β-carotene indicated lower deterioration of lipid. Bleaching of β-carotene resulted from oxidation by degradation products of linoleic acid (13). The antioxidant indexes (time required for complete bleaching of β-carotene) for treatments with MEA were significantly ($P < 0.05$) greater than that of the control. There were significant differences among the four tested MEA samples. Increasing concentrations of MEA, up to tenfold, showed greater antioxidative activity. BHT exhibited higher antioxidative power than the four samples of MEA added at levels from 200 to 2000 ppm.

CD formation method. To compare the antioxidative behavior of different concentrations of MEA with BHT, data obtained for six replications of each treatment were averaged and plotted over time as shown in Figure 3. The speed of formation of CD decreased with increasing concentrations of MEA. The times required to reach a value of 1.5 mM (millimolar) of the CD products for each treatment were also measured. These times were significantly ($P < 0.05$) greater for the MEA samples than for the control, which indicates good antioxidative properties and a marked preventive effect on the oxidative stability of linoleic acid in aqueous media treated

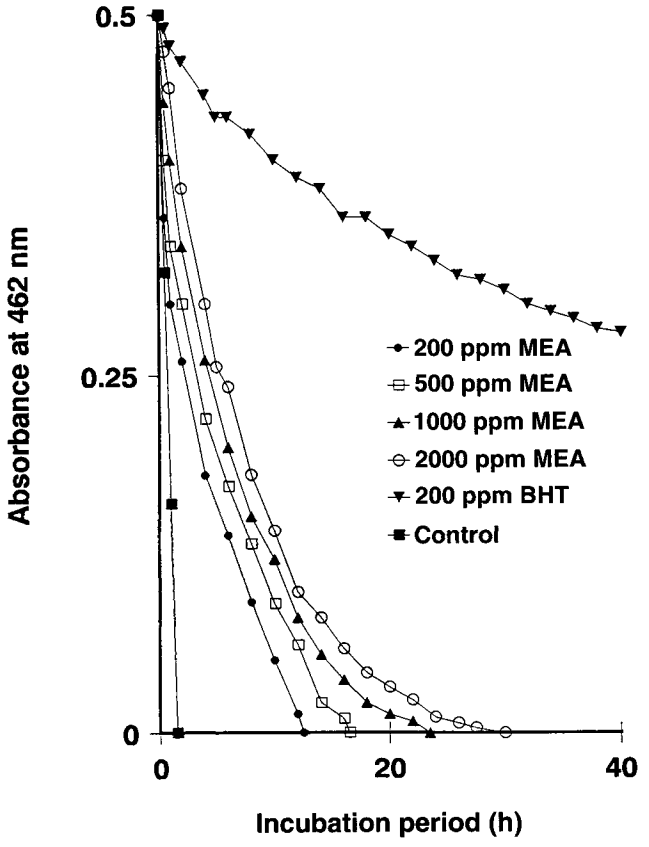

FIG. 2. Coupled oxidation of emulsified linoleic acid–β-carotene with added methanolic extracts of ajowan (MEA) and butylated hydroxytoluene (BHT), as shown by bleaching of β-carotene.

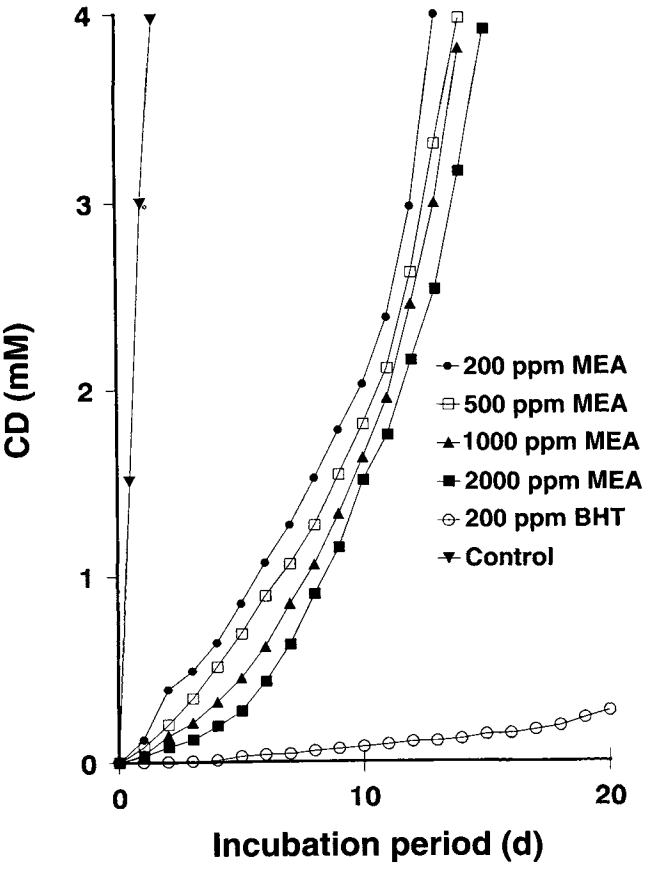

FIG. 3. Antioxidative effect of MEA and BHT on oxidation of emulsified linoleic acid, as shown by measuring conjugated diene (CD) values (millimolar). See Figure 2 for other abbreviations.

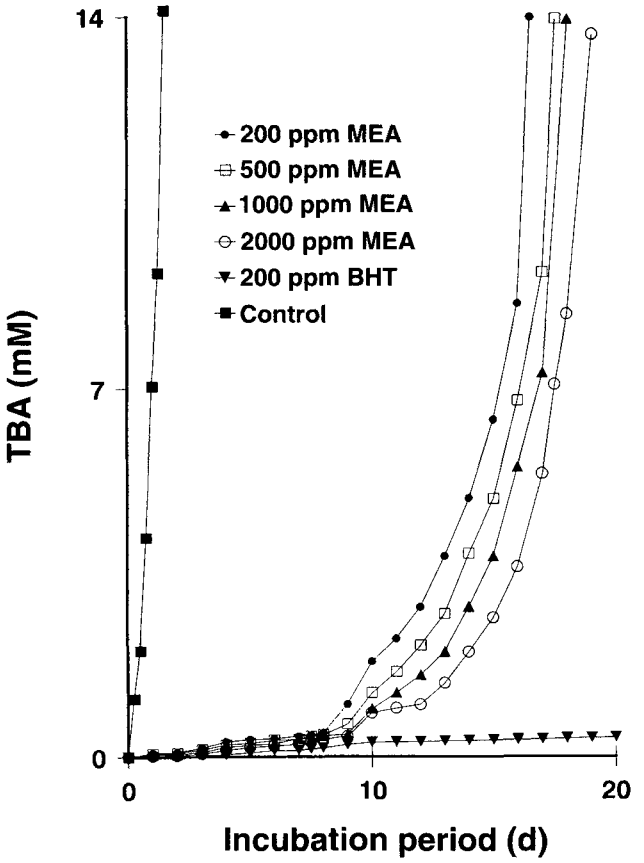

FIG. 4. Antioxidative effect of MEA and BHT on oxidation of emulsified linoleic acid, as shown by measuring thiobarbituric acid (TBA) values (millimolar). See Figure 2 for other abbreviations.

with MEA. An increase in concentration of MEA from 200 to 2000 ppm increased the antioxidative power of the system. There was a significant difference in antioxidative properties between 0.1 and 0.2% MEA samples. The speed for formation of secondary oxidation products was the greatest for the control, indicating its poor oxidative stability. BHT showed the slowest formation of CD among all treatments, and was found to be the best antioxidant.

TBA test. The data obtained from six replications of each treatment were averaged and plotted against time as shown in Figure 4. Increased concentrations of MEA decreased the oxidation of linoleic acid as measured by formation of secondary oxidation products. The speed of oxidation was greatest for the control and was lowest for BHT. Comparisons of different treatments were carried out by measuring time required to reach TBA values of 7 mM (millimolar) of the TBA–malonaldehyde product. The MEA extracts were significantly ($P < 0.05$) more effective than was the control, and effectiveness increased with increased MEA concentrations from 200 to 2000 ppm. There were significant differences between four tested MEA samples, indicating the antioxidative activities of the crude extracts.

Mild antioxidative properties of MEA were demonstrated in a model system. The MEA is a mixture of methanol-soluble materials, which may include phenolic and hydroxy phenolic antioxidant compounds with acids, alcohols, sugars, or glycerides. Extraction and identification of phenolic and hydroxy phenolic antioxidant compounds are recommended. Thus, ajowan may have potential as a source of natural antioxidants.

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