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Herbs and Spices as Potential Sources of Natural Antioxidants

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Herbs and Spices as Potential Sources of Natural Antioxidants

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C. ABSTRACT

Lipid hydroperoxides oxygen radicals and lipid oxy-radicals are related to quality deterioration in stored foods and to pathogenesis of many degenerative diseases. The chain reactions induced by those radicals are prevented in foods and in vivo by antioxidants.

Currently, the synthetic antioxidants, BHA and BHT, are used as food grade antioxidants. However, there is a growing concern about the potential involvement of these in carcinogenesis.

Therefore, there is increasing interest in the use of natural antioxidants, i.e. natural components of plant origin. Five different herbs were determined as sources for natural antioxidant, namely rosemary, sage dalmatian type, sage trifoliata, oregano and marjoram. From all these plants it was found that sage dalmatian type contains the highest amount of total antioxidants, almost 1.5-fold higher than rosemary, a well-known plant which is already utilized for extraction of a natural antioxidant.

It was found that the main antioxidative activity is extracted better with ethanol than with other apolar extractants. The ethanolic oleoresins were separated by TLC and column chromatography. The TLC patterns of the main antioxidants shown that they are phenolic compounds. Oleoresin from sage dalmatian type was separated by TLC to 4 phenolic compounds which showed the main antioxidative activity extracted from the leaves. One of the phenolic antioxidants separated by TLC was the band with R_f 0-0.01, was separated by column chromatography and identified by MS, NMR and IR spectroscopies as carnosol.

The amount of antioxidants in sage and rosemary are dependent on harvest dates being high during June for sage, and February and April for rosemary.

The most active compounds from sage and rosemary separated by TLC showed good antioxidant properties against lipid peroxidation in different model systems and in-situ in several foods.

The antioxidants isolated from sage dalmatian type showed a good antioxidant activity also against lipid oxidation by lipoxygenase and cyclooxygenase.

D. OBJECTIVES OF THE ORIGINAL RESEARCH PROPOSAL

- a. To formulate effective modern cultural practices for the cultivation and assured quality of herbs for antioxidant production.
- b. To isolate, purify and characterize antioxidants from sage, marjoram and oregano.
- c. To determine the relative activity (compared to BHT) of raw extracts and purified compounds as antioxidants in model systems.
- d. To develop strategies for the utilization of the active compounds in food products (soybean oil, muscle foods, paprika powder, and potato chips).
- e. To develop, from fresh paprika, a concentrate rich in vitamin C, vitamin E and carotenoids.
- f. To evaluate the impact of antioxidants from herb plant sources (rosemary, sage, oregano and marjoram) on a number of sensitive biochemical and physiological reactions in vitro.

- g. To conduct nutritional studies to evaluate the effectiveness of the most active ingredients on several biochemical and physiological processes, in vivo.

Due to budget changes by BARD administration, the research focus was directed particularly on objectives b., c., d. and f.

INTRODUCTION

Lipid peroxidation is one of the primary mechanism of quality deterioration in stored foods from animal and plant origin. The changes in food quality can be manifested by deterioration in flavor, color, texture, nutritive value and the production of toxic compounds (Simic and Karel 1980, Pearson et al. 1983, Ames 1983, Benedetti et al. 1984).

The consumption of polyunsaturated fatty acids (PUFA) mostly of vegetable origin has progressively increased in the U.S.A. and other developed countries. This has been in response to the widespread advocacy that PUFA may be beneficial in the reduction of coronary heart disease. The increased content of PUFA in tissues has raised concern about the increased susceptibility to peroxidation and its associated deleterious effects on tissue function and physiology, i.e. increased incidence of tumor and cancer in people of high PUFA diets (Caroll 1984).

The human diet contains a great variety of xenobiotics, natural mutagens and carcinogens. Many of these mutagens and carcinogens act through the generation of oxygen radicals and the initiation of lipid peroxidation. Oxygen radicals and lipid oxy-radicals seem to play a major role as endogenous initiators of degenerative processes, such as DNA damage, mutation, cancer, heart diseases and aging (Ames 1983).

The chain reactions induced by oxygen radicals and lipid oxy-radicals are prevented in foods, model systems and in vivo by antioxidants (Dugan 1980, Porter 1980, Pratt 1980, Kanner 1979, Tappel 1980, Dillard et al. 1983, Machlin 1980).

Antioxidants may be extremely important in regulating thromboxane synthesis. Thromboxane causes platelet aggregation and vasoconstriction and, as such, is the major cause of thrombosis, i.e. clump of aggregated platelets which occlude vital arteries to the heart and brain (Marcus 1984).

Many antioxidants were being identified also as anticarcinogens (Ames 1983, Wattenberg 1982, Draper and Bird 1984).

Synthetic antioxidants, such as BHA (butylated hydroxyanisole) and BHT (butylated hydroxytoluene) are used as food grade preservatives, therefore man is continuously exposed to them. BHA and BHT were found to protect against some mutagens and carcinogens, however, to exacerbate the toxicity of others (Rossing et al. 1985).

In recent years, there has been a high interest in the food industry and medicine to develop "natural antioxidants" from plant materials (Dugan 1980). The interest in natural antioxidants continues to grow for several reasons: a) it is tempting to utilize natural substances presumed to be safe, since they occur in foods which have been used for hundreds of years; b) thus, one could avoid the problems of proof of safety of synthetic compounds; c) the market for antioxidants is very large (in the U.S.A. alone, BHT is produced at about 4,500 tons/year - U.S. International Trade Commission, 1977).

The uses of natural antioxidants may be limited because of the lack of knowledge of the agricultural growing conditions for maximum yield of active compounds, molecular composition of the active

ingredients, the amount of active ingredients in various source materials, and the capability to impart unwanted colors or flavors to the product in which they are used.

Most of the herbs which contain natural antioxidants are collected from the wild or grown as primitive crops. They were not subjected yet to systematic breeding efforts or to modern agronomic development. Consequently, yields are still relatively low, cultivation procedures old-fashioned, and the quality of the final products frequently variable.

This proposal will address the following questions: a) What are the compounds with antioxidative activity in sage, marjoram and oregano? b) How is it possible to control lipid peroxidation in various food products by the active compounds extracted from different herbs? c) What are the implications of the natural antioxidants in a number of sensitive biochemical and physiological reactions in vitro and in vivo.

Initiation of lipid peroxidation

Lipid peroxidation has recently received much attention which was focused in the field of food, nutrition and clinical medicine (Simic and Karel 1980, Yagi 1982). This is attributed to the fact that lipid hydroperoxides are related to food quality deterioration, toxicity and a number of pathological reactions in vivo involved to the etiology of degenerative diseases.

Lipid peroxidation is in most instances a free radical chain reaction that can be described in terms of initiation, propagation, branching and termination processes.

LH = unsaturated fatty acid; $\text{L}^\cdot$ = allyl radical; $\text{LO}^\cdot$ = alkoxyl radical; $\text{LOO}^\cdot$ = peroxy radical; LOOH = hydroperoxide.

With regard to lipid oxidation, one of the most important questions concerns the source of the primary catalysts that initiate peroxidation in vivo and autoxidation in vitro.

Unsaturated fatty acids (PUFA) oxidation by direct interaction with triplet oxygen ($^3\text{O}_2$) is spin-forbidden. This barrier could be eliminated by changing the electron spin state of the molecules during reactions with free radicals of high oxidation potential such as hydroxyl radical ($\text{HO}^\cdot$) (Borg et al. 1978), perhydroxyl radical ($(\text{HO}^\cdot)_2$) (Gebicki and Bielski 1981), carbon trichloride radical ($\text{Cl}_3\text{C}^\cdot$) (Poyer et al. 1980), nitrogen dioxide radical ($\text{NO}_2^\cdot$) (Pryor and Lightsey 1981) and halide radicals ($\text{Cl}^\cdot$) (Kanner and Kinsella 1983). NADPH-dependent microsomal lipid peroxidation seems to be initiated by an activated ferryl ion (Tien et al. 1981). The change of the electronic spin state of oxygen from triplet to singlet could also initiate peroxidation of PUFA molecules (Clements et al. 1973). Recently, we discovered that an H_2O_2 -activated metmyoglobin and methemoglobin, probably a porphyrin cation radical, $\text{P}^+-\text{Fe}^{\text{IV}}=\text{O}$, could initiate the oxidation of membranal lipids (Kanner and Harel 1985) and several other molecules of biological interest (Kanner and Harel 1985).

Plant and animal lipoxygenases and cyclooxygenase are also catalyzers which stimulate the production of bioactive hydroperoxides

(Smith and Lands 1972, Hamberg and Samuelsson 1974, Eskin et al. 1977).

The control of lipid peroxidation by antioxidants

Mechanism and synthetic antioxidants. Several excellent reviews of antioxidant types and mechanism were published (Uri 1961, Scott 1965, Stuckey 1968, Labuza 1971, Porter 1980). For convenience, antioxidants are divided into two classes. Preventive (or primary) antioxidants, which reduce the rate of initiation of free radical chains most commonly by converting the free radical-producing hydroperoxide products, ROOH, to innocuous products, such as alcohols. Nickel and cobalt chelates (Betts and Uri 1966) and large amounts of ascorbic acid, in the presence of traces of cupric ions (Kanner et al. 1977), work as hydroperoxide decomposers for a rapid termination by the following reactions:

Chain-breaking (or secondary) antioxidants trap the chain-propagation peroxy radical, ROO $\cdot$, and other free radicals and thereby reduce the length of the autoxidation chains. The majority of chain-breaking antioxidants, AH, are phenols. The mechanism of most phenolic antioxidants could be described by the following reactions:

A good antioxidant must usually be effective as a hydrogen, electron or radical donor. The resulting antioxidant must be

sufficiently stable or in a low redox potential which does not initiate a new chain.

The major synthetic antioxidants in common foods and cosmetics are BHA, BHT, TBHQ (t-butyl hydroxyquinone), propyl galate and ascorbyl palmitate. About 20 million pounds of synthetic antioxidants are used annually by food manufacturers in the United States. In recent years, BHA and BHT were found to work as promoters of carcinogens (Malkinson 1983, Rossing et al. 1985).

Natural antioxidants

Vitamin E (α -tocopherol) is a natural fat soluble molecule that concentrates in the hydrophobic interior of biological membranes. Tocopherol primary role in biological systems is that of a lipid-soluble chain-breaking antioxidant (Tappel 1982). It was found by the Burton group (1983) that α -tocopherol interacts with peroxy radical in apolar solvents at a rate constantly higher than BHT, however in water emulsions and in foods its antioxidative effects were found to be lower than synthetic antioxidants (Porter 1980). A "repair" of α -tocopherol radical by ascorbic acid was postulated for many years, a direct observation of a free radical interaction between vitamin E and vitamin C was demonstrated (Parker et al. 1979).

Ascorbic acid. Because its activated orthodihydroxyl group has a low reducing potential, it is therefore a useful hydrogen donor and electron donor in solution. Ascorbic acid is autoxidizable in the presence of trace metals and oxygen and generates hydrogen peroxide and hydroxyl radicals (Kanner et al. 1985). The production of these compounds could explain its prooxidative effect in foods (Kanner et al. 1977, 1978, Fisher and Deng 1977). Ascorbic acid, however, was found to work as an antioxidant at high concentration (see primary

antioxidants). It was also found that in the presence of EDTA or other chelating agents, ascorbic acid works as an antioxidant at low concentration probably as a secondary-type antioxidant (Kanner et al. 1985). In vivo, where the concentration of "free metals" is controlled, ascorbic acid seems to impart an antioxidative effect (Kunert and Tappel 1983).

Spices and herbs. Observation that foods prepared with various spices and herbs for flavor enhancement seem to have longer keeping times led to examination of these and the extracts as antioxidants for food (Dugan 1980). Chipault et al. (1957) evaluated the antioxidant properties of many plants and found that rosemary and sage were the most effective extracts. Similar results were published by others (Watanabe and Ayano 1974, Jacobson 1973, Chang et al. 1977). Only more recently was it found that marjoram and oregano extracts have very significant antioxidative activity (Gerhardt and Sehroter 1983).

Herbs and spices are generally used to flavor and season food. In some cases, they have been used for their antiseptic activity. Traditionally, some herbs have been used which are of antioxidant properties, however their application is limited because of their characteristic flavor. Rosemary extract is known to contain antioxidants (Tateo et al. 1988, Klingenberg and Verstrynge 1989, Nakatani et al. 1984, Wu et al. 1982, Inatani et al. 1982, Nakatani and Inatani 1981, 1983, 1984, Houlihan et al. 1984). The essential oils have no antioxidant activity while the oleoresins of rosemary and of some other Labiatae herbs show an antioxidative effect (Hartmann et al. 1980, Change et al. 1977, Bracco et al. 1981, Inatani et al. 1983, Berner et al. 1973).

A pure clone of Dalmatian sage with high thujone content in its essential oil (Putievsky et al. 1986) was chosen as a source for antioxidants production. The factors of variation and different climate conditions were eliminated by growing the pure sage clone under cultivated conditions.

Antioxidants and lipoxygenase/cyclooxygenase. Because of the important biological effects of the products of LO and CO, there has been considerable interest in the effects of antioxidants on these enzymes. Early studies revealed that BHA at low concentrations inhibited cyclooxygenase while apparently stimulating lipoxygenase in human platelets (Agradi et al. 1981).

Both vitamin E and propylgallate also affect in vivo activity of these enzymes (Carpenter 1981).

Quercetin inhibits 12-LO in human platelets and research in our laboratory showed that tannic acid was an effective inhibitor of CO and LO. Inhibition of lipoxygenase, particularly in platelets, may reduce production of lipid peroxides which inhibit the synthesis of the aggregatory prostacyclin and thereby permits aggregation. Thus, conceivably it would be desirable to discover antioxidants which reduce LO induced hydroperoxides in platelets.

A diverse group of chemicals can inhibit LO and CO (Lands 1981, Rome and Lands 1975), however very limited research has been conducted evaluating naturally-occurring antioxidants. There is a need for a lipoxygenase inhibitor for the control of anaphylaxis and for both CO and LO in controlling inflammatory responses (Higgs and Vane 1983. In addition, in view of the role of both products of CO and LO in cell-mediated immunity and in tumorigenesis, the need for selective inhibitors with minimal side effects is apparent.

Because prostaglandin synthetase contains cyclooxygenase activities that require hydroperoxides for maximum activity (Kulmacz and Lands 1982), the antioxidant capacity of different tissues may markedly affect the amount and types of prostaglandins and/or leukotrienes produced in tissues. Thus, naturally-occurring antioxidants like flavonoids and plant tissues may be effectively in selectively reducing the generation of thromboxane by blood platelets.

MATERIALS AND METHODS

Growing conditions. Seeds of S. officinalis, received from: 1) Slano region, Dalmatia, Yugoslavia, in 1981, 2) Split region, Dalmatia, Yugoslavia, in 1981, 3) Switzerland, unknown origin, in 1988, 4) Kew Gardens, London, in 1988, were grown at the Neve Ya'ar Experiment Station located in northern Israel. These plants were examined for yield components, and one plant was selected and propagated vegetatively, by stem cuttings, 35 x 15 cm between cuttings. Approximately, 1 month before planting 800 kg potassium fertilizer and 500 kg phosphate fertilizer ha^{-1} were applied to the entire experimental field. Ammonium nitrate fertilizer was applied immediately before planting and after each harvest, 300 kg ha^{-1} on each occasion. From the end of March and until November, the field was irrigated weekly with 300 m^3 water ha^{-1} .

Seven different treatments of harvest frequencies were examined. The experimental design was a Latin square with seven replicates, each 5 m x 2 m. All harvesting was done with a motorized cutter. From each replicate, all the fresh matter was weighed, and a sample of 400-500 g was oven-dried for 48 h at 40°C. The leaves were separated manually

and weighed. Other drying experiments were carried out by spreading the plants in the shade. Dry material was calculated after drying the fresh matter for 24 h at 100°F.

Fresh and dry material from sage (Salvia fruticosa), marjoram (Majorana hortensis) and oregano (Origanum vulgare) were chosen as the raw materials for isolation, purification and characterization of natural antioxidants. In addition, we extracted active compounds from rosemary (Rosmarinus officinalis) as a comparable herb with antioxidative activity.

Isolation, purification and characterization of antioxidants.

Fresh and dry plant material from sage, marjoram and oregano were extracted with various solvents (Hexane, ethane). The oleoresins were separated by flash chromatography on a column packed with silica gel or florisil, using polar and non-polar eluents. Fraction was analyzed by analytical TLC, the antioxidative compounds were detected using ethanolic solution of linoleate and β -carotene (Harel and Kanner 1985). Further separation was carried out by preparative TLC or HPLC chromatography.

Extraction of sample. One g of dried sage or rosemary was homogenized in a homogenizer (Polytron[®], Switzerland) with 15 ml of ethanol, then incubated for 30 min in the dark at room temperature. The mixture was separated by centrifuging for 10 min at 10,000 rpm. The residue was twice more extracted with 15 ml ethanol. The ethanolic extracts were evaporated to dryness under vacuum and then dissolved with 25 ml ethanol for further analysis.

Quantification of total phenolic compounds. Soluble phenolic content in the ethanolic extracts obtained from sage was determined by the folin ciocalteu assay, as described by Singleton and Rossi (1965).

Quantification of chlorophyll. The amount of chlorophyll was determined according to Arnon (1949).

Thin layer chromatography. TLC plates with silica gel were also used to separate constituents of the ethanolic extract. The plates [(Merk F₂₅₄ in Israel. Analtech UNIPLATE preparative plates, 20 x 20 cm, 500 μ m, or EM-precoated analytical plates, 10 x 10 cm, 200 μ m in U.S.A.)] were activated at 100°C for 1 h. The ethanolic extracts (1 ml) were applied to TLC plates. The mixtures were separated using hexane:ethyl ether:glacial acetic acid (82-12-6 in Israel) (8:2:1, by volume) in the first direction, and/or butan-1-ol:glacial acetic acid:water [4:1: (upper layer) by volume] in the second direction. Detection of the antioxidants was done by an ethanolic solution of linoleate β -carotene. Antioxidants prevent the bleaching of β -carotene. Yellow spots after 10 h of plate storage demonstrate the presence of antioxidants. A solution of 20% sodium carbonate and further folin ciocalteu reagent were used for the detection of phenolic compounds. Bands of interest were scraped from TLC plates, soaked for 30 min in 20 mL ethanol, filtered and evaporated on an analytical evaporator (Meyer N-EVAP, Organomation Associates, Inc., MA) at 45°C. The residue was redissolved in 1.0 ml ethanol and then stored at -70°C until required for analysis.

Hydrodistillation. Essential oils were obtained from a laboratory essential oil determination apparatus (a modified Clevenger type). After hydrodistillation - a period of 1 h proved to be sufficient for an almost complete isolation - the oil was cooled to room temperature, separated from the distillation water and stored in a refrigerator at 2-6°C.

Chromatography and analysis:

GLC. A hexane solution of oleoresin (0.1 μ l) or a neat sample (0.1 μ l) of essential oil was injected on the following GLC columns:

- a) 5% carbowax 20 M on an acid-washed silanized Chromosorb W 80-100 mesh, 2.5 m x 4 mm (ID) glass, 30 ml/min. N_2 flow rate. Temperature was programmed at 4 $^\circ$ /min, from 80 $^\circ$ initial temperature to 200 $^\circ$.
- b) Supelcowax 10 fused silica capillary column, 30 m x 0.25 mm ID, 0.25 mm film. Operating conditions: temperature program 80-200 $^\circ$ (5 $^\circ$ /min), carrier gas, nitrogen, 5 ml/min.

Chromatograms and post-integration reports were obtained on a Varian 3700 gas chromatograph with a flame-ionization detector and a Hewlett-Packard 3390A integrator.

HPLC. HPLC was performed at room temperature with a Varian model 5000 liquid chromatograph equipped with an UV-50 variable wavelength detector and a Hewlett-Packard 3390A integrator. A Regis spherisorb S50DS II analytical column (0.46 x 25 cm) was used for analytical HPLC. Elution was effected isocratically using acetonitrile:water 35:65 (pressure 85 AT, flow rate 1 ml/min).

Flash chromatography. Flash chromatography assembly consists of a set of three chromatography columns, flow controllers, pinch clamps and solvent reservoirs, as described by Still et al. (1978). The chromatography was performed on a silica gel 60 (230-400-mesh) ("Merck") or silica gel 60A (200-245-mesh) ("Aldrich"), under nitrogen pressure (1-2 AT). Three eluents were generally used:

- a. Petrol ether 60-80 $^\circ$ (75%-25%) - chloroform (25%-75%).
- b. Chloroform (95%-80%) - methanol (5%-20%).

c. Benzene (98%-85%) - acetone (2%-15%).

TLC (test of antioxidant after column chromatography). The fractions obtained were concentrated under vacuum and checked by analytical TLC.

The crude extract, the fractions and the pure compounds isolated by flash chromatography were applied to analytical silica gel 60 ("Merck") pre-coated aluminum sheet (20 x 20 cm, layer thickness 0.2 mm). Preparative TLC was performed with 20 x 20 cm, 2 mm-thickness plates. Eluent: chloroform-methanol 97:3. After drying, the plates were sprayed with 5% solution of iron(III) chloride in chloride in hydrochloric acid, 0.5 mol/l.

Spectroscopy. 1) NMR: ^1H -NMR spectra was obtained at 200 MHz with a Bruker Model WP-200 SY Fourier transform spectrometer. The samples were dissolved in deuterated acetone or deuterated methanol. 2) MS: MS were obtained on a Finnigan 4000 system apparatus. 3) FTIR: Spectra were obtained in Nujol mull on a Perkin-Elmer Model 1600 Fourier transform infrared spectrophotometer.

Authentic samples: Two types of commercial rosemary extract with strong antioxidant activity were available - rosemary extract powder, 0.05-0.1% on glucose, and rosemary liquid oil soluble, 0.25-0.5% on mono/diglycerides of fatty acids as carrier ("Visuvia"). Both samples were dissolved in methanol and used as references in TLC and HPLC.

Mechanism of antioxidants action. Raw and purified polar and non-polar soluble fraction, isolated using well-known biochemical methods was evaluated for antioxidative activity in different model systems and foods.

The evaluation of antioxidants activity was determined by the inhibition of β -carotene co-oxidation (provitamin A) in the presence

of linoleate. This system is closely related to the possible damage of peroxy and alkoxyl radicals to nutritional compound in foods, and the production of off-flavors during lipid peroxidation (Kanner et al. 1976). The inhibition of β -carotene co-oxidation was carried out by a method previously published by us (Kanner et al. 1976, Kanner 1979). Briefly, the technique consists of following the decrease in absorbance at 460 nm in the cuvette of a double-beam recording spectrophotometer. The test sample contains 1.5 ml buffered carotene-linoleate, 0.1-0.4 antioxidant extract in ethanol solution and distilled water to a final volume of 2.0 ml. Concentration in the final reaction mixture will be β -carotene 1.4×10^{-5} M, linoleate 2×10^{-3} M, Tween-20 0.5%, buffer phosphate pH 7.0, 0.1 M at 25°C. The stimulation of the oxidation will be conducted by ferric chloride or hemin. The initial rate of decrease in absorbance will be computed from a recorded graph and converted into rate of carotene oxidation in nmoles.

The inhibition of membranal lipid peroxidation by three different pathways which represent possible initiation of lipid peroxidation in biological tissues were determined using muscle tissues microsomes. Microsomes were prepared as previously described by us (Kanner and Harel 1985). a) Enzymatic microsomal lipid peroxidation was initiated by NADPH and ADP-Fe⁺⁺ as described by Apgar and Hultin (1982). b) Microsomal lipid peroxidation was initiated by redox cycle using iron and ascorbate (Kanner and Harel 1985). Microsomal lipid peroxidation will also be initiated by H₂O₂-activated metmyoglobin (Kanner and Harel 1985). Lipid peroxidation and the inhibition by antioxidants will be determined by the accumulation of malondialdehyde

(Kanner and Harel 1985). The effectiveness of the natural antioxidants were compared with well-known antioxidants, such as BHT and TBHQ.

Evaluation of antioxidant activity in foods

The inhibition of soybean oil (free from antioxidants added in industry) oxidation by various antioxidants extracted from herbs was determined using a method by which the oil is heated to 110°C and the breakdown of low molecular volatile acids was collected in distilled water. The change in the potential of water-acid solution was determined by a potentiometer electrode and recorded. This method evaluates the potential of the natural antioxidants to inhibit lipid oxidation in high temperature, relatively very similar with frying.

Lipid peroxidation is one of the primary mechanism of quality deterioration in stored muscle tissues. Ground dark turkey muscle tissues were chosen as a food where natural antioxidants could prevent lipid peroxidation during storage or cooking. Isolates with antioxidative activity in ethanolic solution were mixed with muscle emulsions and stored at 2°C. The measurement of lipid peroxidation in muscle in situ was determined by accumulation of MDA and conjugated dienes (Kanner and Juven 1980).

Oxidation of the oil during frying was tested by the accumulation of free fatty acids (Berman (1980). Lipid peroxidation during storage of potato chips, tightly sealed in mini jars at water activity of 0.01 at 25°C, was determined.

Determination of the effectiveness of plant extracts on several sensitive biochemical reactions.

The antioxidative effectiveness of various plant extracts were determined on the activity of 5-lipoxygenase (potato), 12-lipoxygenase (trout-gill), 15-lipoxygenase, soy-lipoxygenase and cyclooxygenase.

5-Lipoxygenase was purified from freshly obtained red potato tubers by a modification of the procedure of Shimmizu et al. (1984). 12-Lipoxygenase was purified from trout-gill by a method developed by us (Ksieh et al. 1988). Soy-lipoxygenase was obtained from Sigma (Type V, purified by affinity chromatography). Cyclooxygenase was purified by the procedure of van Ouderra and Baytenbek (1982) as modified by Burgess et al. (1987).

Lipoxygenase assay. To vary the activity and specificity of the lipoxygenase reactions and to determine inhibitory effects of flavonoids, the enzyme preparation (1 mg protein/ml) was incubated at 25°C with arachidonic acid (50 μ M) using labeled [1-¹⁴C]arachidonic acid as tracer. The extent of enzymatic reaction was calculated from the conversion of radioactive substrate fatty acid into its hydroxy analog following 10 min incubation. The reaction was quenched by acidification with formic acid to pH 3.0 and the products were extracted twice with 1 ml ethyl acetate as described. Extracted lipids were separated by thin layer chromatography (TLC) using a solvent system consisting of chloroform/methanol/acetic acid/water (90:8:1:0.8, v/v/v/v). The radiolabeled product, 12-hydroxy-5,8,10,14-eicosatetraenoic acid, from labeled arachidonic acid, was generated due to the activity of gill lipoxygenase and was detected qualitatively by autoradiography as reported. The extent of product formation during the reaction period was quantified by locating, and scraping the corresponding bands and measuring by liquid scintillation counting the radioactivity in each band.

Calculations of enzyme velocity. The rate of lipoxygenase-catalyzed reaction was determined by oxygen consumption using a polarographic cell (Hansatech oxygen electrode, Hansatech Ltd.,

Norwich, England). For enzyme assays, the lipoxygenase preparation (1 mg protein/ml) was equilibrated in phosphate buffer (0.05 M, pH 7.4) in a water-jacketed oxygen electrode chamber at 25°C for one min. Then the flavonoid or specific inhibitor in ethanol (1 μ l) was introduced into the enzyme assay mixture and incubated for 10 min. The enzyme activity then was initiated by the addition of arachidonic acid (25 nmol) in ethanol (1 μ l) and the oxygen concentration was monitored by the electrode and recorded continuously using a microcomputer. The experimental data were smoothed to eliminate random electronic noise. The first differential of the oxygen concentration vs. time data (dO_2/dt) was calculated. The maximal rate and lag time required to reach this rate were obtained from these first different plots. The appropriate programs for performing these analyses were written in Forth for the Apple IIe.

Relative inhibitory efficacy of flavonoids and antioxidants on gill lipoxygenase. Various antioxidants and flavonoids were tested to compare their inhibitory effects on gill lipoxygenase. The gill lipoxygenase was prepared as described above. The enzyme was preincubated with an inhibitor at 25°C for 10 min. The radiolabeled substrate, arachidonic acid, then was added to initiate enzyme reaction. The reaction was stopped after an additional 10 min incubation; the products were separated by thin layer chromatography and detected by autoradiography as mentioned above. The inhibitory effect of each inhibitor was estimated by measuring the radioactivity in the product by liquid scintillation counting as stated above. At least five concentrations of each inhibitor were employed to determine its inhibitory potency. The IC_{50} value, the concentration of inhibitor

causing 50% reduction of enzyme activity, was calculated by linear regression analysis.

RESULTS AND DISCUSSION

Extraction and separation of active compounds by TLC

The antioxidants from sage and rosemary were extracted with hexane and ethanol. It was demonstrated that ethanol is a better extractant than hexane. The results indicate that the antioxidants are relatively polar compounds (Table 1). The separation of the antioxidants was performed by TLC using β -carotene-linoleate as reagents for identification of the active compounds. The antioxidants prevent the oxidation and bleaching of β -carotene. Orange spots indicate the location of antioxidants. It was shown that sage dalmatian type could be separated by TLC to nine different compounds with antioxidative activity (Fig. A). The TLC plates were sprayed also with folin-ciocalteu reagent which is specific for phenolic compounds. The main antioxidants identified previously by the β -carotene-linoleate reagent also gave positive results with folin-ciocalteu reagent. Fraction #6 in sage dalmatian type gave a significant antioxidative effect and it was found not to be a phenolic compound. Separation antioxidants from rosemary on TLC, results in eight bands of active compounds. Bands 1 and 4 contain the main active compounds (Table 2). The bands of the active compounds were scraped from the TLC plates, soaked in ethanol, filtered and diluted for determination of the antioxidative activity. It was found that the total activity, or dilution factor for 50% inhibition of β -carotene-linoleate oxidation from rosemary TLC plate, is 9,519 (Table 2). The total activity from

sage dalmatian type is 15,916 (Table 3), almost 1.7-fold higher than rosemary extract.

Table 1. The effect of solvent extraction on the antioxidant activity from rosemary and sage.

Treatment	β -carotene-linoleate oxidation DF- I_{50}
Rosemary (ethanol)	1:10,000
" (hexane)	1: 7,000
Sage (ethanol)	1:15,000
" (hexane)	1:10,000

DF- I_{50} = Dilution factor for 50% inhibition of β -carotene-linoleate oxidation by hemin.

Extraction, separation and dilution of the active compounds. One g of dried leaves of sage or other herbs were homogenized with 15 ml ethanol, then incubated for 30 min in the dark at room temperature. The mixture was separated by centrifugation and diluted to 25 ml ethanol. One μ l of the extract was applied to a TLC plate. The mixture was separated using hexane, ether, and acetic acid (80,12:6, v/v). The active compound was identified by spraying with β -carotene-linoleate or with folin ciocalteu reagent on the margin of the plate. The lines of the antioxidant were scraped, dissolved in ethanol, extracted, evaporated to dryness and redissolved in 1 ml ethanol. This fraction was diluted by ethanol to give an inhibition of 50% of lipid peroxidation in different model systems.

The reaction mixtures contained, in a final volume of 2 ml, the

following reagents added in that order to give the final concentration; 0.1 M buffer tris pH 7.0, 14 μ M β -carotene, 2 mM linoleate, 0.05% Tween-20, 20 μ M hemin, and 0.1 ml antioxidants.

Fig. A. TLC patterns of antioxidants from sage dalmatian.

O, spray with β -carotene-linoleate reagent; ●, spray with folin ciocalteu reagent. Development element (hexane/ether) acetic acid (8:1:0.5).

A₁ and A₂ are fractions separated from sage (A₁=carnosol).

Fig. 1. Inhibition of membrane lipid peroxidation by the active fraction #V from sage D.

The reaction mixture contained, in a final volume of 2 ml, the following reagents added in that order to give the final concentration: acetate (50 mM) pH 6.5; ascorbic acid (200 μM); microsomes (1 mg protein); antioxidants (0.1 ml in ethanol); FeCl_3 (10 μM).

Fig. 2. Inhibition of membrane lipid peroxidation by the active fraction #IV from rosemary and sage trifoliolate #V. $\square$, rosemary; $\blacksquare$, sage trifoliolate.

The reaction mixture - see Fig. 1.

Table 2. Separation of antioxidant active fraction from rosemary by TLC.

Fraction No.	DF - I ₅₀
1	3,000
2	416
3	625
4	4,000
5	500
6	500
7	166
8	312
Total	9,519

Table 3. Active antioxidants from sage D separated by TLC.

Fraction No.	R _f	DF - I ₅₀
1	0.01	3,500
2	0.08	1,470
3	0.10	250
4	0.16	1,785
5	0.24	5,555
6	0.30	2,702
7	0.33	156
8	0.36	280
9	0.39	290
Total	-	15,916
BHT		7.75 μ M

הספריה המרכזית
למחקר ב-1978
בית-הספר

Sage trifoliate was found to contain active antioxidants of dilution factor of 5,282, which is almost 40% from those of sage dalmatian. Low antioxidative activity was demonstrated in marjoram and oregano.

The inhibition of membrane lipid peroxidation by herbs most active compound separated on TLC, was determined using several catalyzers as initiators of lipid peroxidation. We found that membrane lipid peroxidation by an Iron Redox Cycle initiator could be inhibited (I_{50}) by fraction #V from sage D extracted from 50 μ g dry matter (Fig. 1). The same inhibition was achieved using 50 μ g dry matter of rosemary and 70 μ g dry matter from sage trifoliate (Fig. 2).

Membrane lipid peroxidation was also determined using initiators such as enzymic NADPH reductase and H_2O_2 -activated myoglobin. The enzymic NADPH reductase-dependent membrane peroxidation was inhibited by almost the same amount of antioxidants (Figs. 3,4) however this was dependent on H_2O_2 -activated myoglobin and was inhibited by only 20-30 μ g dry matter (Figs. 5,6).

As the antioxidants are phenols it may be possible that they act not only to trap free radicals but also to deactivate and reduce the initiator, ferrylmyoglobin, to metmyoglobin.

The active compound from sage dalmatian type (carnosol) after separation and purification was determined as an antioxidant and compared to the well-known antioxidant BHT. It was found that I_{50} of carnosol is 0.1 μ M compared to 0.32 μ M of BHT (Fig. 7).

The inhibitory effect of fraction #V from sage D toward lipid peroxidation was also demonstrated in other systems, such as turkey muscle homogenate (Fig. 8), minced muscle (Fig. 9) and heated soybean oil (Fig. 10).

Table 4. Active antioxidants from sage T separated by TLC.

Fraction No.	DF - I ₅₀
1	625
2	250
3	1,000
4	1,000
5	1,470
6	271
7	416
8	250
Total	5,282

Table 5. Active antioxidants from marjoram separated by TLC.

Fraction No.	DF - I ₅₀
1	208
2	208
3	312
4	250
5	312
6	625
Total	1,915

Table 6. Active antioxidants from oregano separated by TLC.

Fraction No.	DF - I ₅₀
1	173
2	290
3	290
Total	753

Fig. 3. Inhibition of membrane enzymic lipid peroxidation by the active fraction #V from sage D., sage.

The reaction mixture contained, in a final volume of 2 ml, the following reagents added in that order to give the final concentration: histidine (5 mM) pH 7.0; KCl (0.12 M); ADP (0.4 mM); 0.1 ml antioxidant in ethanol; microsomes (1 mg protein).

Fig. 4. Inhibition of membrane enzymic lipid peroxidation by fraction #IV of rosemary.

Fig. 5. Inhibition of membrane lipid peroxidation in a system containing H_2O_2 -myoglobin, by active fraction #V from sage.

The reaction mixture contained, in a final volume of 2 ml, the following reagents added in that order to give the final concentration: histidine (5 mM); KCl (0.12 M); microsomes (1 mg protein); 0.1 ml antioxidants; H_2O_2 -myoglobin (30 μM each).

Fig. 6. Inhibition of membrane lipid peroxidation in a system containing H_2O_2 -myoglobin, by active fraction #IV from rosemary and #V from sage trifoliolate. $\square$, rosemary; $\blacksquare$, sage trifoliolate.

The reaction mixture - see Fig. 5.

Fig. 7. Inhibition of membrane lipid peroxidation by carnosol and BHT. A=carnosol; A₂=phenolic compound on TLC R_f=0.08.

The reaction mixture - see Fig. 1.

We had examined and compared the antioxidant effectiveness of extractions from several herbs towards lipid peroxidation of heated soybean oil. At a level of 16 μ g dry matter extract, the best antioxidative effect was achieved from sage dalmatian type (Fig. 12).

The antioxidative effect of the extract obtained from 16 μ g dry matter of sage dalmatian type toward lipid peroxidation of soybean oil was compared to other synthetic antioxidants (Fig. 13).

Purification of 5-lipoxygenase and cyclooxygenase are demonstrated in Tables 7 and 8.

Thin-layer chromatography for the active compounds in sage was developed using precoated silica gel plates (Uniplate Preparative, Analtech). The separation of sage-active compound by the Dr. Kinsella group showed a pattern which differed from that demonstrated by us. It seems that the differences were obtained because of the utilization of different silica gel plates and detecting agents. Nevertheless, the most active compound was found to be carnosol and three other phenol compounds.

The bands of interest were scraped from TLC plates, soaked for 30 min in 20 ml ethanol, filtered and evaporated to dryness. The residue was redissolved in 1 ml ethanol and then used for analysis. Carnosol (purified by the group in Israel) gave the band #IV and was found to be an important antioxidant. It is important to note that chlorophyll a and b showed a I_{50} higher than that of carnosol. The antioxidative effects of chlorophyll against lipoxygenase was also shown by others (Cohen et al. 1989, Fig. 14).

Figure 15 showed the I_{50} of the total ethanolic extract from sage on the basis of gallate equivalent. Figures 16 and 17 showed the

Fig. 8. Inhibition of lipid peroxidation in turkey muscle homogenate at pH 6.5, 37°C.

□, muscle homogenate (3:1); ■, muscle homogenate (3:1) in the presence of 2% NaCl; ◇, muscle homogenate in the presence of fraction #V extracted from 32 µg dry matter of sage D; △, muscle homogenate in the presence of 2% NaCl and fraction #V extracted from 32 µg dry matter of sage D.

Fig. 9. Lipid peroxidation of minced turkey muscle stored at 4°C, as affected by the active fraction #V extracted from sage D.

□, control; △, 1 µg DM/g; ◇, 0.125 µg DM/g; ■, 0.06 µg DM/g.

Fig. 10. Lipid peroxidation of soy-bean oil (20 ml) heated to 110°C, as affected by the active fraction #V extracted from sage D.
 □, control; Δ, 8 µg DM; x, 16 µg DM; ◇, 32 µgDM.

Fig. 11. Lipid peroxidation of soy-bean oil (20 ml) heated to 110°C, as affected by the active fraction #IV extracted from rosemary. mho-units of conductivity.
 □, control; +, 8 µg DM; ◇, 16 µg DM; Δ, 32 µg DM.

Fig. 12. Lipid peroxidation of soy-bean oil (20 ml) heated to 110°C, as affected by herb antioxidants; active fractions from herbs at a concentration of 16 μ g DM/20 ml oil. fraction #V extracted from sage D.

□, control; ▽, sage D; +, rosemary; Δ, sage T; ◇, marjoram; x, oregano.

Fig. 13. Lipid peroxidation of soy-bean oil at 110°C, as affected by synthetic antioxidants compared to fraction #V from sage D.

□, control; ▽, sage D, 16 μ g DM/20 ml; x, BHT (100 μ M); +, TBHQ (100 μ M); Δ, AP (100 μ M).

Table 7. Purification of 5-lipoxygenase from red potato

Purification step	Total protein mg	Total activity units	Specific activity units/mg	Yield %	Fold purified
Crude extract	4496.8	482.2	0.10	100	1
15-45% (NH ₄) ₂ SO ₄ fraction	486.6	414.7	0.85	86	9
DEAE-cellulose chromatography	110.2	203.8	1.85	42	22

Table 8. Purification of cyclooxygenase from ram seminal vesicle

Purification step	Total protein mg	Total activity units	Specific activity units/mg	Yield %	Fold purified
Crude extract	15.40	550.2	35.7	100	1
Microsome fraction	6.28	787.3	127.8	143	3.6
DEAE-cellulose chromatography	1.47	461.5	315.2	84	8.8

Fig. 14. TLC patterns of phenolic compounds and chlorophylls in sage. Main compound of Band IV on TLC is carnosol.

Table 9. Contents and IC_{50} of each phenolic compound or chlorophyll in sage.

	Contents (mg/g of dried sage)	IC_{50} (10^{-5} M)
Band I	7.73 (50.1%)	1.39
Chl a	0.46 (3.0%)	0.15
Band II	1.82 (11.8%)	1.25
Chl b	0.37 (2.4%)	0.12
Band III	2.39 (15.5%)	0.48
Band IV (carnosol)	2.65 (17.2%)	0.26

effect of each phenolic compound concentration and chlorophyll concentration on the inhibition of lipoxygenase.

Various flavonoids and related compounds have been tested as inhibitors for cyclooxygenase and lipoxygenase (Table 10). It was found that some are more effective as lipoxygenase inhibitors and some more effective as cyclooxygenase inhibitors. Carnosol showed a high inhibitory activity toward 5-lipoxygenase. Several other researches were published concerning the potential antioxidative effects of flavonoids.

Table 11 shows the changes of the inhibitory intensity of sage ethanolic extract on soybean LO activity during storage. The inhibitory activity was almost stable even at room temperature, while was slightly decreased under light irradiation.

The content of chlorophyll a, which is a main colour compound in sage, also did not decrease in the dark, while under light irradiation was rapidly decreased during storage (Table 12).

Table 13 shows the changes of total phenolic compounds content in sage ethanolic extract during storage. The value behavior was almost similar to that of chlorophyll shown above. However, in case of decreasing rate under light irradiation, the total phenol compounds were less than that of chlorophyll.

Table 14 shows the changes of contents of each phenolic compound and chlorophylls in the ethanol extract during storage of sage. All phenolic compounds were more stable than those of chlorophylls during storage. Although the content of chlorophyll in sage was rapidly decreased under light irradiation storage, the inhibitory activity was almost sustained (Tables 11 and 12). It was assumed because the

phenolic compounds, which are the main inhibitor on LO activity in sage, was stable even under light irradiation storage.

Fig. 15. Inhibitory effect of total ethanolic extract from sage on soy-lipoxygenase activity at pH 6.6.

Fig. 16. Inhibitory effect of each phenolic compound in sage ethanolic extract on soy-lipoxygenase activity at pH 6.6.

Fig. 17. Inhibitory effect of chlorophyll 2 and 6 in sage ethanolic extract on soy-lipoxygenase activity at pH 6.6.

Table 10. The relative inhibitory effects of various phenols, flavonoids and antioxidants on 5-lipoxygenase, 12-lipoxygenase, 15-lipoxygenase and-cyclooxygenase.

ph for reaction	IC ₅₀ (μM) on			
	5-LOX (pH 6.3)	12-LOX* ¹ (pH 7.0)	15-LOX (pH 8.0)	Ram-COX (pH 8.0)
Flavonone	18,197	-	NI	28,184
Flavanone	5,248	-	1,047	NI
Chalcone	4,074	-	347	316,228
Chrysin	1,000	30	6,761	4,169
Apigenin	167	5	5,248	575
Fistin	20	0.25	15.8	50
Quercetin	10	0.40	2.5	63
Myricetin	20	2	145	159
Rutin	17,783	7	191	32
Kaempferal	79	-	5,754	79
Naringinin	550	-	115	1,202
Morin	100	-	24	18
Catechin	6,310	-	251	2.7
Epicatechin	3,802	-	214	1.4
Carnosol	1.9	-	3,311	21
Esculin	50,118	500	5,012	16,982
Esculetin	589	0.02	25	16
Chlorogenic acid	NI* ²	-	141	28
Tannin	1.3	-	0.6	1.6
Caffeic acid	27,542	-	6,457	7.1
Indomethacin	5,000	-	NI	0.8
NDGH	4.0	-	3.2	55
L-Ascorbate	63.0	-	NI	26
α-Tocopherol	25.1	-	58	NI
BHA	6.3	5	1,514	159
BHT	3.5	1,000	83	NI
Propyl gallate	104.7	-	50	661
Tert.-butylhydroquinone	39.8	-	794	525

*¹Rudy's data on trout-gill lipoxygenase.

*²NI: No inhibited.

Table 11. Changes of inhibitory potency of the sage extract on soy-lipoxygenase activity (pH 6.6) during storage at several conditions.

(%)				
Stored Days (Month)				
	0	1	3	5
Control*	69.2 ± 4.2	-	-	-
-20°C		58.8 ± 3.7	68.6 ± 2.7	67.9 ± 3.2
5°C	-	67.4 ± 1.7	68.2 ± 3.9	66.9 ± 3.0
25°C	-	68.2 ± 3.3	67.9 ± 3.3	67.2 ± 1.8
25°C + Light	-	64.2 ± 1.0	65.2 ± 1.6	64.6 ± 2.7

*Stored at -80°C

Table 12. Changes of chlorophyll a content in sage ethanolic extract during storage.

µg/g of dried sage				
Stored Days (Month)				
	0	1	3	5
Control*	459.0 ± 12.7	-	-	-
-20°C		449.4 ± 2.5	473.9 ± 10.7	463.7 ± 13.2
5°C	-	405.5 ± 7.8	425.4 ± 15.5	484.1 ± 6.4
25°C	-	425.2 ± 1.2	427.3 ± 5.4	439.7 ± 5.0
25°C + Light	-	337.4 ± 5.4	311.0 ± 3.8	279.6 ± 0.6

*Stored at -80°C

Table 13. Changes of total phenolic compounds content in sage extract during storage.

		mg/g of sage			
		Stored Days (Month)			
		0	1	3	5
Control*	18.8 ± 0.6	-	-	-	-
-20°C		17.7 ± 0.4	15.8 ± 1.1	16.5 ± 0.2	
5°C	-	15.7 ± 0.5	15.5 ± 0.2	15.6 ± 0.3	
25°C	-	15.5 ± 0.4	15.1 ± 0.2	14.9 ± 0.2	
25°C + Light	-	14.5 ± 0.2	14.3 ± 0.4	13.8 ± 0.6	

*Stored at -80°C

Fig. 18. TLC of purified antioxidant from Salvia officinalis, Dalmatian type.

Isolation of two antioxidants from the leaves of *Salvia officinalis*,
Dalmatian type.

Fine ground dry leaves of sage (Dalmatian type) (783 g) were extracted with methanol (7 l) at room temperature for 24 h. The filtrate was concentrated under vacuum to 2 l, bleached with active carbon (50 g) at 40°C and filtered again. The methanolic solution was evaporated to 400 ml. A white amorphous precipitate (15 g) was separated from the solution by filtration and the solution was reconcentrated to dryness (33.2 g of oleoresin; 4.2% from dried leaves). The oleoresin (8 g) was first separated into 15 fractions using a glass column (i.e. 3.8 cm; length 52 cm) packed with silica gel (270 g) (column A). The column was eluted in the following order: petrol ether - chloroform 75% - 25%, 50% - 50%, 25% - 75%, 0% - 100%, and chloroform-methanol 95% - 5%, 90% - 10%, 80% - 20%, respectively.

The fractions were analyzed by TLC and 5 fractions (8-12) with antioxidant activity were rechromatographed on column B (i.d. 3.2 cm; length 42 cm) packed with silica gel (130 g). The column was eluted with the following solvents: benzene-acetone 90% - 10%, 85% - 15%, 80% - 20%, and 75% - 25%, respectively. Eleven fractions were collected and analyzed by TLC. Fractions 7-9 were combined, the solvents were evaporated and the antioxidant A₁ was crystallized few times from methanol [TLC: R_f 0.63 (Fig. 18); HPLC: rt. 6.85; 95% pure by HPLC (Fig. 19)]. Fractions 10 and 11 were rechromatographed on column B. Twelve fractions were separated and analyzed by HPLC. Fraction 4 was found to contain 75% of the antioxidant A₂ [TLC: R_f 0.77 (Fig. 1); HPLC: rt. 6.57]. The compound was purified by recrystallization from methanol (96% purity by HPLC, Fig. 20).

Identification of the carnosol (A₁) isolated from *Salvia officinalis* (Dalmatian type).

TLC and HPLC experiments of diluted solutions of the white precipitate of A₁ show that the compound is of high purity. The color reaction between the acidic ferric chloride spraying solution and the tested TLC spot show the presence of phenolic function in the compound. Natural and synthetic antioxidants are known to have one or more phenolic groups attached to aromatic rings.

The IR spectrum of A₁ (Fig. 21) revealed the presence of phenolic OH groups by the sharp absorptions at $3,491\text{ cm}^{-1}$. The indication of an aromatic ring is the absorption at $1,585\text{ cm}^{-1}$. The vibration of the C=O group of the bridged δ -lactone is at $1,711\text{ cm}^{-1}$. The frequency of the J C-O vibration occurs at $1,199\text{ cm}^{-1}$.

The mass spectrum of A₁ shows a very small peak of the molecular ion at m/e 330. Elimination of CO₂ gave the base peak at m/e 286. The peak observed at m/e 284 (35% from the base peak) is a result of loss of a hydrogen molecule from the ion at m/e 286. Loss of a methyl group from fragment m/e 286 forms a tertiary carbonium ion of fragment m/e 271 (30%).

Loss of two methyl groups and of isopropyl group from the parent molecular ion resulted in formation of an ion, m/e 213 (12%). Other fragmentation ions appear at m/e (relative intensity) 216 (24), 215 (77), 204 (31), 202 (34), 201 (28), 173 (20).

The ¹H-NMR spectrum of A₁ (Fig. 22) shows the presence of aromatic proton (6.67 ppm, s) at c-14, oxymethine proton at c-7 (5.39 ppm, dd), and four methyl protons at 1.15, 1.11 and 0.81 ppm.

Fig. 21. IR spectrum of the antioxidant A₁.

Fig. 22. ¹H-NMR spectrum of A₁.

Fig. 23. Gas chromatogram of the essential oil of Dalmatian sage on Supelcowax 10 fused silica capillary column (B).

Antioxidants content in the oleoresins of salvia and rosemary at different harvest dates and from various sources.

Dried leaves of Salvia officinalis (Dalmatian type), S. fruticosa and Rosmarinus officinalis were extracted with methanol in the usual procedure (see: "Materials and Methods"). Two antioxidants A₁ and A₂ were detected in the oleoresins by HPLC. The purified antioxidants from Dalmatian sage were used as external standards.

The two antioxidants were not detected in the oleoresins of marjoram and oregano. The content of the antioxidants was high in rosemary (7.5-13.7%) and low in S. fruticosa (1.7-3.9%) (Table 15). However, we should note that in sage there are other phenolic compounds with antioxidative activity and they were not determined.

The maximum content of antioxidants was detected in Dalmatian sage in June and April, with A₂ predominant. A₁ was the major antioxidant in the other analyses. The content of camphor, an essential oil constituent, was also high in June, while the content of 1.8-cineole and borneol was high in April (Putievsky et al. 1986).

The essential oils and yield components of Dalmatian sage (Putievsky et al. 1986; Ravid and Putievsky 1985; Putievsky and Ravid 1987) and rosemary (Werker et al. 1985) were also analyzed.

The antioxidants content in the oleoresins of S. officinalis from Switzerland and England, and of the Salvia hybrid were compared to commercial rosemary extracts (Table 17). The highest content of the two antioxidants was detected in the hybrid of Dalmatian sage x S. fruticosa (7.3%) with A₁ predominant.

Our results demonstrated that sage dalmatian type have antioxidants at a higher activity than rosemary. The antioxidants extracted from sage were good stabilizers of lipid peroxidation in a

Table 15. Antioxidants content (%) in the oleoresins of Dalmatian sage, *Salvia fruticosa* and rosemary at different harvest dates.

	Dalmation sage		<i>Salvia fruticosa</i>		Rosemary	
	A ₁	A ₂	A ₁	A ₂	A ₁	A ₂
February	3.9	0.7	1.6	0.7	9.5	4.3
April	2.8	4.2	1.5	0.2	9.4	3.4
June	2.9	7.4	1.4	0.3	6.0	3.6
August	5.4	1.1	2.9	1.0	7.1	2.9
October	3.8	0.8	2.6	0.6	5.1	2.4

*Stored at -80°C

Table 16. Total antioxidants in sage (Dalmatian type) and rosemary at different harvest dates.

	Sage	Rosemary
February	9,000*	9,000
April	11,000	9,000
June	13,000	8,000
August	11,000	7,500
October	8,500	5,000
Total	52,500	38,000
X	10,500	7,700

*DF - I₅₀ = Dilution factor for 50% inhibition of β -carotene-linoleate oxidation by hemin.

Table 17. Antioxidants content (%) in the oleoresins of sage from different sources, from sage hybrid and commercial rosemary extracts.

Herb oleoresin	source	A ₁	A ₂
<i>Salvia officinalis</i>	Swiss, unknown origin	0.7	traces
" "	England ⁽¹⁾	1.1	0.1
Hybrid of Dalmatian sage x <i>S. fruticosa</i> ⁽²⁾		6.1	1.2
Rosemary extract powder	"Visuvia"	2.8	traces
" " liquid			
oil soluble	"Visuvia"	1.2	0.3

⁽¹⁾For details on genetic affinities and essential oil composition see Putievsky et al. (1990) and Diwan-Rinzler (1987).

⁽²⁾Details on agricultural yield components and essential oil analysis see Putievsky et al. (1984).

Table 18. Composition of Dalmatian sage essential oil.

Peak No.	Compound	%
1	α -pinene	0.2
2	camphene	t
3	β -pinene	0.1
4	linonene	t
5	1.8-cineole	5.0
6	-terpinene	t
7	p-cymene	0.4
8	α -thujone	56.9
9	β -thujone	7.5
10	copaene	t
11	camphor	0.5
12	linalool	1.0
13	linalyl acetate	0.3
14	bornyl acetate	1.4
15	β -caryophyllene	1.3
16	terpinen-4-ol	0.1
17	α -humulene	0.8
18	borneol	0.3
19	-cadinene	0.1
20	caryophyllene oxide	0.8
21	viridoflorol	14.4

t < 0.1%

The main components in the essential oil of Dalmatian sage are α -thujone (56.9%), viridoflorol (14.4%), β -thujone (7.5%) and 1.8-cineole (5.00%). The thujone content (64.4%) is an important factor for the high quality of the oil. Growing a pure sage clone under cultivated conditions is important for the production of an improved essential oil for the flavor industry and of an oleoresin with excellent antioxidative properties.

broad spectra of foods and goods model system prepared and stored at low but also at high temperature. The active compounds do not only inhibit prooxidants like hemoproteins and free iron but also lipoxigenas and cyclooxygenase. Rosmanol was found to have an antioxidative activity of about four times higher than BHT (Nakatani and Inatani 1983). Our results showed that carnosol is almost three-fold more active than BHT. Because of the involvement of lipoxigenase and cyclooxygenase products in the control of several pathophysiological diseases, the results showed that antioxidants from sage inhibit these enzymes, may help to control the activity of these enzymes in the future.

Summing up:

- 1) The antioxidants from sage and rosemary are relatively polar compounds, they were extracted better with ethanol than with hexane.
- 2) The ethanolic extracts from rosemary, sage dalmatian, sage trifoliolate, marjoram and oregano were separated by TLC and the antioxidants were detected by β -carotene linoleate spray and folin-ciocalteu reagent. The highest antioxidative compounds extracted from sage dalmatian, were separated into nine antioxidants. Between the five most active compounds, four gave positive reactions with folin-ciocalteu reagent and most probably are phenols.
- 3) Sage dalmatian type contains almost 1.7-fold higher antioxidative activity than rosemary, a well-known herb which is utilized for extraction and production of a market natural antioxidant.

- 4) The antioxidants from sage and rosemary separated by TLC showed high antioxidant properties in different model systems of lipid peroxidation and in-situ several foods.
- 5) The amount of antioxidants in sage and rosemary are dependent on harvest dates, being high during June for sage dalmatian type and February and April for rosemary.
- 6) The antioxidants isolated from sage dalmatian type showed a good antioxidant activity against lipid oxidation by lipxygenase and cyclooxygenase.
- 7) One of the natural phenolic antioxidants, separated by TLC as the band with RF 0-0.01, was separated by column chromatography and identified by MS, NMR and IR spectroscopes as carnosol.
- 8) Carnosol was found to inhibit 50% of membrane lipid peroxidation at a concentration of 0.1 μ M and 3-fold better than BHT.

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F. Description of cooperation

Dr. J. Kanner the principal investigator in Israel was responsible for the experiments connected with the evaluation of the natural antioxidants in biological systems and foods. Dr. Kanner was also responsible for the primary separation and purification of the antioxidants from sage, rosemary and other spices.

Dr. E. Putievsky was responsible for the agricultural experiments and Dr. V. Ravid for the separation purification and the detection of the antioxidants, NMR and mass spectroscopy.

Dr. J. E. Kinsella contribute the results connected with the stability of the antioxidants from sage and the determination of them activities toward lipid oxidation by lipoxygenase.

During the first and second years Drs. J. Kanner and V. Ravid exchanged visits with Dr. Kinsella in order to adjust methods on antioxidant activity, separation and purification.

G. Evaluation of the research achievements with respect to the aims of the original research proposal.

Due to budget changes by BARD administration, the research focus was directed particularly on objectives b,c, d and f.

The proposed research contribute to the evaluation of the effectiveness of several natural antioxidants extracted from spices as inhibitors of lipid peroxidation in biological systems and foods.

The studies demonstrated the possible utilization of natural antioxidants as food stabilizers and the potential of these antioxidants to replace synthetic antioxidants. Enlarging utilization of herbes for the extraction of natural antioxidants will be beneficial for agriculture in terms of economy of crop production.