J Sci Food Agric 1998, 77, 140–146

Antioxidant Activity of Extracts Obtained by Different Isolation Procedures from some Aromatic Herbs Grown in Lithuania

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(Received 11 September 1996; revised version received 2 June 1997; accepted 23 September 1997)

Abstract: An increasing demand for natural additives has shifted the attention from synthetic to natural antioxidants. The present work examines the potential of some aromatic herbs grown in Lithuania as a source of natural antioxidants: marjoram (*Majorana hortensis* Moench), catnip (*Nepeta cataria* L), oregano (*Origanum vulgare* L), lavender (*Lavandula angustifolia* Mill), thyme (*Thymus vulgaris* L), hyssop (*Hyssopus officinalis* L), anise hyssop (*Lophantus anisatus* Benth), and sage (*Salvia officinalis* L). Dried herb samples were submitted to extraction with supercritical CO_2, acetone or methanol/water and hydrodistillation. Deodorised herb samples (after removal of essential oil) were also extracted with acetone. The antioxidant activity of these extracts, essential oils and dried deodorised aqueous extracts was assessed by the β-carotene bleaching test (diffusion and spectrophotometric methods). The highest yields of extracts were obtained using polar solvents. Thyme and sage acetone oleoresins showed high antioxidant activity in the tests performed and were regarded as the most promising sources. © 1998 SCI.

J Sci Food Agric 77, 140–146 (1998)

Key words: natural antioxidants; solvent extraction; hydrodistillation; SFE; aromatic herbs; marjoram; catnip; oregano; lavender; thyme; hyssop; anise hyssop; sage; β-carotene bleaching test

INTRODUCTION

The discovery of the inhibition of lipid oxidation by some phenolic compounds during the late 1940s has contributed to the application of synthetic antioxidants in food industry (Sherwin 1990). Synthetic antioxidants, such as gallates, 2,6-di-*tert*-butyl-4-methylphenol (BHT), *tert*-butyl hydroxyanisole (BHA) and *tert*-butyl hydroquinone (TBHQ) have been dominant since their introduction. However, some physical properties of BHA and BHT, such as their high volatility and instability at elevated temperatures, strict legislation on the use of synthetic food additives and consumer preferences have shifted the attention of manufacturers from synthetic to natural antioxidants (Porter 1980; Pokorný 1991).

Natural antioxidative substances usually have a phenolic moiety in their molecular structure. They have been found among flavonoids, tocopherols and catechines. Organic acids, carotenoids, protein hydrolysates, and tannins can act as antioxidants or have a synergistic effect when used together with phenolic antioxidants. Currently, materials which inhibit lipid

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oxidation can be obtained from plant materials, food waste, microorganisms and animal cells (Dugan 1980; Langseth 1995).

The demand for natural additives, including natural antioxidants, has grown in Lithuania during the last few years, together with the strong development of the industry and the introduction of new technologies to meet EU requirements. Therefore, it is worthwhile looking for new, local sources of natural antioxidants and developing ways to introduce them in food industries.

Some aromatic herbs that can be grown in Lithuania have been found to possess antioxidant activity: thyme, marjoram (Banias *et al* 1992), sage (Stephen *et al* 1977; Schwarz and Ternes 1992; Svoboda and Deans 1992), oregano, lavender, basil (Economou *et al* 1991; Lagouri *et al* 1993; Vekiari *et al* 1993) and catnip (Hirosue *et al* 1988). Unfortunately, the climatic conditions for the commercial cultivation of rosemary, which so far has been regarded as the most effective plant in terms of antioxidant activity, are rather unfavourable in Lithuania.

Some authors demonstrated variations in the activity of antioxidant extracts when different solvents and extraction techniques were used. Chen *et al* (1992) found that the hexane extract of rosemary possessed a higher concentration of carnosic acid and carnosol than rosemary extracts prepared with methanol or acetone. Nevertheless, due to the much lower yield of the hexane extract, it was concluded that acetone is a more suitable solvent for the isolation of antioxidant substances from rosemary leaves. Kramer (1985) analysed antioxidant extracts from clove prepared by successive percolation with petroleum ether and ethanol. The ethanol extract was concentrated and reextracted by ethyl acetate and then diethyl ether. The ethyl acetate fraction contained phenolic compounds and possessed the highest antioxidant activity. Chevolleau *et al* (1992) assessed methanol and *n*-hexane oleoresins of some Mediterranean plants. Hexane extracts of the screened plants showed much higher antioxidant activity (AA) than the corresponding methanol extracts.

The manufacturing procedures for obtaining crude and refined extracts of rosemary usually consists of two successive steps: distillation of volatiles and extraction of active antioxidant substances with different solvents (Löliger 1991). The references surveyed do not suggest, however, the existence of one universal extraction procedure for all aromatic herbs. This is also not expected due to differences in the nature of their antioxidant compounds. Therefore, it is important to select the most efficient isolation method for each particular herb that could be a source of natural antioxidants.

The present study is aimed at assessing the antioxidant activity of some aromatic herbs which can be commercially cultivated in Lithuania and evaluating several isolation techniques in order to select the most promising sources of natural antioxidants and the most efficient routes to extract them.

EXPERIMENTAL

Plant material

The following aromatic herbs were collected in the experimental garden of the Lithuanian Institute of Horticulture on the second day of July 1994 (stage of vegetation defined):

- marjoram (*Majorana hortensis* Moench): blossom formation;
- catnip (*Nepeta cataria* L): full bloom;
- oregano (*Origanum vulgare* L): full bloom;
- lavender (*Lavandula angustifolia* (Mill): full bloom;
- hyssop (*Hyssopus officinalis* L): full bloom;
- thyme (*Thymus vulgaris* L): full bloom;
- anise hyssop (*Lophantus anisatus* Benth): full bloom;
- sage (*Salvia officinalis* L): was collected from plants that did not form blossoms that year.

As a reference plant, dried rosemary (*Rosmarinus officinalis* L) obtained from the Superior Institute of Agronomy, Technical University of Lisbon, Portugal (harvested in Arrábida, Portugal, July 1994) was used. The harvested herbs were dried in a drying cabinet with forced ventilation at ambient temperature for 2–3 days. The decrease in mass during the drying process varied from 64% (hyssop) to 80% (thyme). The loss of essential oil under these conditions is negligible (Venskutonis 1995). After the plants had been sorted out, only leaves and blossoms were used for the assessment of AA. The samples were packed in double walled paper bags and stored for ~3–4 months at ambient temperature before use.

Isolation techniques

Six different types of extracts were prepared for each of the dried herbs (Fig 1):

- essential oils (EO) hydrodistilled from 50 g of herb;
- deodorised acetone oleoresins (DAO) obtained by reextracting all dried solid retentate remaining after hydrodistillation with 250 ml freshly redistilled acetone (99+%, Acros Organics, 17.717.63) under continuous shaking for 24 h;
- deodorised water extracts (DWE) concentrated from the liquid retentate remaining after hydrodistillation using a Vacuubrand Diaphragm Pump CVC 24, a Büchi Rotavopor R-114 with a

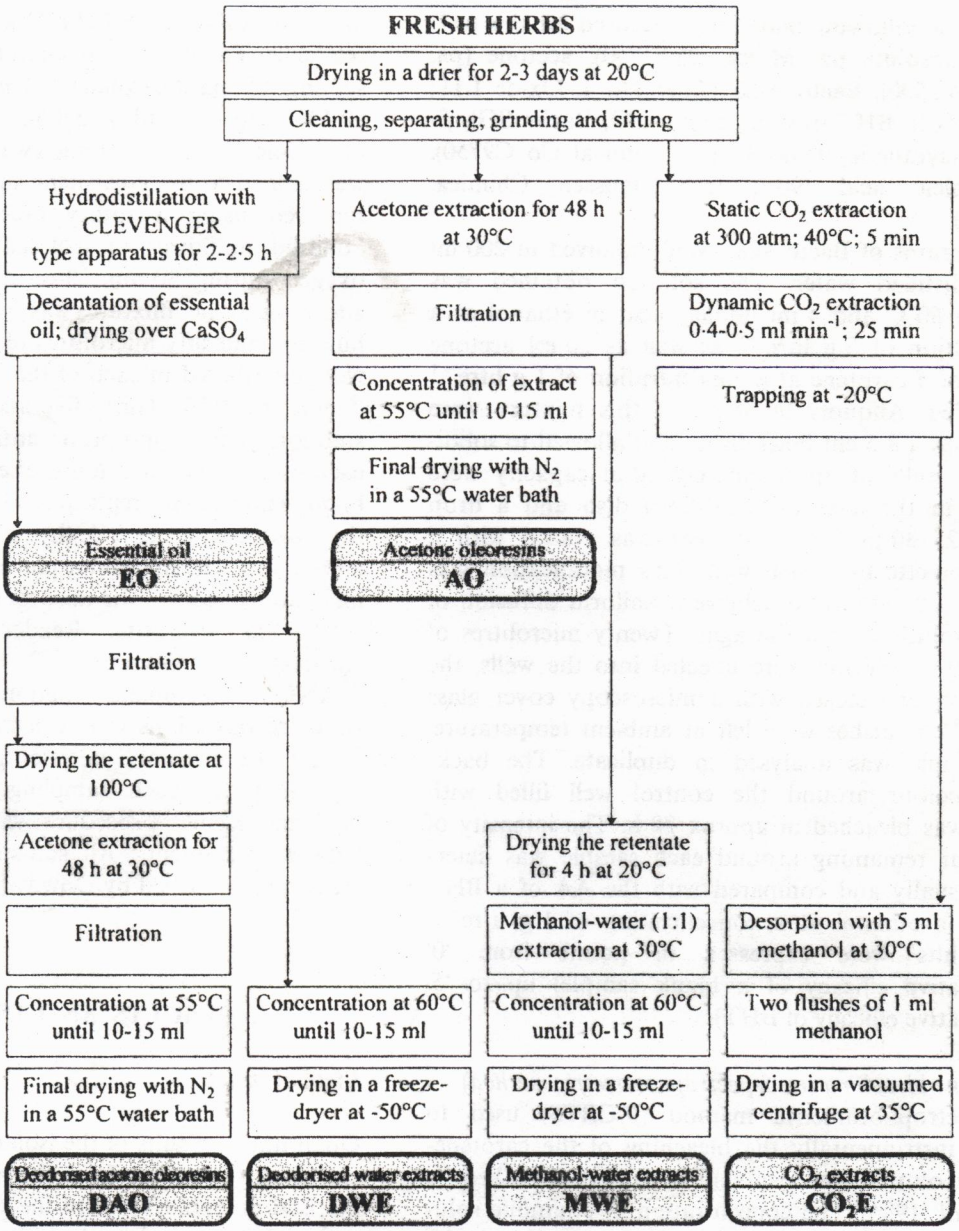

Fig 1. Flow scheme of the isolation techniques used for the preparation of extracts from Lithuanian aromatic herbs for screening their antioxidant activity.

Büchi Waterbath B-480; the concentrate was subsequently freeze-dried at −40°C in a Virtis freeze-drier;

- CO_2 extracts (CO_2E) extracted in a 10 ml extraction vessel from 2–3 g of herb material using a Suprex PrepMaster™ extractor coupled with an AccuTrap™ collecting device. The trap-collector was filled with Ottawa Sand Standard (20–30 mesh, Fisher Chemicals, S 23-3). The restrictor and the trap-collector were set at 35°C and −30°C, respectively;
- acetone oleoresins (AO) obtained by extracting 50 g of herb material with 250 ml freshly redistilled acetone by applying continuous shaking for 24 h;
- methanol/water extracts (MWE) obtained by reextracting the plant material remaining after the acetone extraction by applying continuous shaking for 24 h.

Evaluation of antioxidant activity

β-Carotene bleaching test (agar diffusion method)

The β-carotene bleaching test, diffusion method, (β-CBTD) was chosen for the initial screening of a large number of samples because of its simplicity and visual evidence of the results. The test procedure used was similar to the one described by Taga *et al* (1984). The extracts and oils obtained were dissolved in ethanol (1 g

litre^{-1}). The following materials were used for this test: ethanol (absolute pa, Merck, 983.2500), acetone (pa, Merck, 14.2500), Bacto Agar (Agar No 1, Oxide L11; Unipath Ltd), BHT (purum, Fluka EG, Buchs SG), β-carotene (synthetic, 95%, Sigma Chemical Co C9750), and linoleic acid (90% tech, Janssen Chimica, 22.724.26).

Three grams of Bacto agar were dissolved in 200 ml boiling distilled water. The solution obtained was cooled to 50°C and 4 ml linoleic acid in ethanol at a concentration of 5 g litre^{-1} as well as 20 ml acetone solution of β-carotene at a concentration of 1 g litre^{-1} were added. Aliquots of 35 ml of this mixture were poured into a 8·5 cm Petri dishes and allowed to solidify. Three wells of approximately 50 µl capacity were punched in the agar of each Petri dish and a drop (approx 25–30 µl) of liquid agar was placed with a Pasteur pipette into each well. This modification was made by the authors to achieve a uniform diffusion of the test solutions into the agar. Twenty microlitres of each sample solution were injected into the wells, the wells were then closed with a microscopy cover glass and the Petri dishes were left at ambient temperature. Each sample was analysed in duplicate. The background colour around the control well filled with ethanol was bleached in approx 28 h. The intensity of the colour remaining around each sample was determined visually and compared with the AA of a BHT solution in ethanol at a concentration of 1 g litre^{-1}. The results were expressed in points from '0' (antioxidative efficacy of a blank sample) up to '5' (antioxidative efficacy of BHT).

β-Carotene bleaching test (spectrophotometric method)

The spectrophotometric method (β-CBTS) used to monitor instrumentally the bleaching of the carotene-linoleate solution was also similar to that described by Taga *et al* (1984). The concentration of ethanolic solutions of isolates used in this test was 2 g litre^{-1}. An ethanolic solution of BHT (2 g litre^{-1}) was used as a reference. The model test mixture was prepared by dissolving 0·5 mg β-carotene in 1 ml chloroform (ar grade, 99·5%, Labscan Ltd, A 3505E). Twenty five microlitres of linoleic acid and 200 mg Tween 40 (SIGMA, P-1504) were added to the β-carotene solution. Chloroform was removed using a rotary evaporator at 50°C. One hundred millilitres of distilled water saturated with oxygen during 30 min, flow rate 100 ml min^{-1} were added and the mixture was vigorously shaken. Two hundred and fifty microlitres of this model test mixture were distributed in each of the wells of multiwell plates (Sigma, M-0156). Thirty five microlitres of an ethanolic solution of the appropriate antioxidant were added to each well. An equal amount of ethanol was used for the blank samples. Six replicates were prepared for each of the treatments (sage AO, thyme AO, oregano AO, BHT and blank). The microtiter plates were then placed in an incubator at 55°C. The absorbance was measured in an EAR 400 Microtiter Reader (SLT-Labinstruments, Austria) at $\lambda = 490$ nm.

ANOVAs (completely randomised design), means and standard deviations were calculated for the data of the β-CBTS experiment. Effects of treatments were analysed separately for each sampling period. The Student–Newman–Keul's procedure was used to distinguish between treatments. All statistical analyses were performed as described by Dowdy (1983).

RESULTS AND DISCUSSION

Yield of isolates

Quantitative results of the isolation of essential oils and extracted substances from the analysed herbs are summarised in Table 1. All of the plants were quite rich in essential oil compared with some published data

TABLE 1

Yields of the isolates obtained from dried aromatic herbs (g kg^{-1} dry matter) and their antioxidant activity as evaluated by the β-carotene bleaching test (diffusion method)[a]

Plant	*EO*	*DAO*	*DWE*	*AO*	*MWE*	*CO_2E*
Sage	58·0	**62·3 (4)**	**90·8 (1)**	**66·3 (4)**	**87·3 (2)**	**50·2 (4)**
Oregano	25·5	16·2	155·1	30·1	**71·7 (1)**	53·9
Rosemary	69·1	**39·7 (4)**	**58·8 (1)**	**113·3 (4)**	**60·2 (3)**	**71·5 (5)**
Catnip	28·2	29·6	119·5	82·7	126·1	22·8
Thyme	**62·7 (2)**	**4·4 (3)**	73·2	**35·0 (4)**	**73·9 (3)**	**54·6 (4)**
Lavender	42·4	9·8	135·1	105·3	105·5	26·7
Hyssop	45·0	13·8	127·9	30·9	96·0	37·1
Anise hyssop	60·4	36·2	141·6	44·7	123·8	59·6
Marjoram	36·4	6·0	115·3	**31·3 (2)**	**108·1 (1)**	37·4

[a] Yields of isolates possessing antioxidant activity are printed in bold. In parentheses: antioxidant activity, scale 0 (low)–5 (high).

(Senatore 1996; Venskutonis *et al* 1997). For instance, in the present study, the concentration of EO in dried matter of thyme, anise hyssop and sage was approx 60 g kg^{-1}. It should be noted, between, that only leaves and flowering parts were used for the hydrodistillation.

The total amount of deodorised extracts (DAO + DWE) in the dry mass of analysed herbs varied from 78 g kg^{-1} (thyme) to 178 g kg^{-1} (anise hyssop). In most of the herbs, DWE yield was considerably higher than that of DAO, ie from 19·2 times (marjoram) to 3·9 times (anise hyssop). In sage and rosemary, however, DAO and DWE yields were more similar to each other, with DWE 1·4 times and 1·5 times higher than DAO, respectively. These results indicate that the processes occurring during the hydrodistillation and distribution of extractives in filtrate and retentate are specific for each particular herb.

The yields of extracts obtained by acetone were lower than those isolated by methanol-water, except for lavender (almost equal) and rosemary (AO > MWE approx 1·9 times). Extremely low yields of CO_2E were obtained for catnip and lavender.

In assessing the peculiarities of the extraction by different method, it is worthwhile comparing the yield of EO, DAO and DWE (procedures involving hydrodistillation) with that of AO and MWE (no hydrodistillation). The results obtained show that the sums of the extractives isolated during and after hydrodistillation (EO + DAO + DWE) were generally higher than those obtained excluding the hydrodistillation step (AO + MWE). A striking example is oregano extracts in which EO + DAO + DWE yield was 1·9 times higher than that of AO + MWE. Boiling in water and the concomitant physical changes in the plant cell structure may account for these differences. In the case of catnip and lavender the yield of EO + DAO + DWE was slightly lower than that of AO + MWE. This could be attributed to a higher affinity of extractives from these two herbs for the methanol used in the latter extraction procedure.

The differences in the yields obtained using various solvents could be caused by several factors, ie composition of each particular herb, differences in the solubility of extractives and their polarity, extraction technique, etc. It is, however, difficult to give a definite explanation for the results obtained within the scope of the present study.

β-Carotene bleaching (agar diffusion method)

The products of linoleic acid oxidation affected the β-carotene agar medium in 28 h in the blank. The discoloration process around the wells with antioxidant solutions was retarded and isolate solutions differed in their protective efficacy. The results of the visual assessment of antioxidant containing wells are summarised in Table 1. In order to compare the efficiency of different isolation techniques and herb materials, both in terms of AA and yield of each particular isolate, BHT-equivalent yields Eq_{BHT} were introduced.

$$Eq_{BHT} = Y_i \times \frac{AA_i}{AA_{BHT}}$$

where: Y_i is the yield of the particular isolate (g kg^{-1}); AA_i is the antioxidant activity of the particular isolate according to β-CBT (scale 0–5 points); and AA_{BHT} is the antioxidant activity of BHT in β-CBT (5 points).

Eq_{BHT} can be defined as the amount of isolate obtained from 1 kg of a particular herb possessing AA equal to that of BHT.

As can be seen from Table 1, active isolates were obtained from five of the nine herb species tested. The Eq_{BHT} values for the different types of isolates obtained for each of these herbs are summarised in Fig 2.

The results show that rosemary isolates possess the highest antioxidant efficiency, eg Eq_{BHT} of rosemary AO and MWE was 90·6 g kg^{-1} and 36·1 g kg^{-1}, respectively. The same type of isolates from two other plants, thyme and sage, were also very effective. These results agree with those obtained by Banias *et al* (1992) and Herrmann *et al* (1981), who found that methanolic oregano extracts possessed high AA on lard. However, in this study the effect of oregano MWE on the β-carotene model system was not so evident (Eq_{BHT} = 14·3 g kg^{-1}, 1 point). AO (Eq_{BHT} = 12·5 g kg^{-1}, 2 points) and MWE (Eq_{BHT} = 21·6 g kg^{-1}, 1 point) solutions of marjoram also slightly retarded β-carotene bleaching. It is interesting to note that the isolates obtained from this herb during hydrodistillation (EO, DAO and DWE) were not effective in the test performed, possibly due to the breakdown of active constituents during the prolonged thermal stress. The same tendency for procedures involving hydrodistillation to result in isolates of lower Eq_{BHT} when compared with isolates obtained by cold extraction methods is observed in extracts from other herbs. The sum of Eq_{BHT} values for EO, DWE and DAO of thyme and

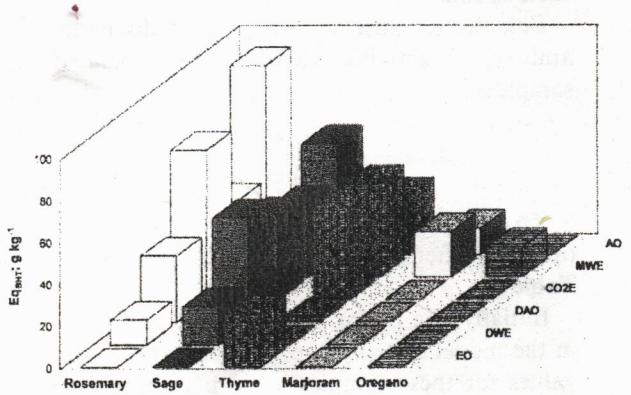

Fig 2. Antioxidant efficiency (Eq_{BHT}) of different isolates obtained from Lithuanian aromatic herbs (see text for abbreviations).

rosemary result in 27·7 and 43·6 g kg^{-1}, respectively, whereas the Eq_{BHT} values for AO and MWE of the same plants totals 72·3 and 126·7 g kg^{-1}, respectively.

In general, herb isolates prepared with less polar solvents (CO_2E, DAO and AO) possessed higher AA than DWE or MWE (Table 1).

In most cases, EO of the assessed herb did not retard oxidation of β-carotene linoleate model system. Thyme EO, however, displayed some antioxidant activity (2 points, Table 1). Similar findings were also reported by other authors (Farag *et al* 1989; Chevolleau *et al* 1992).

The activity of CO_2E was almost similar to that of AO, except for marjoram. However, the considerably lower yields of CO_2E when compared to the yields of AO + MWE indicate that a considerable part of the antioxidant substances may remain in the plant material after SFE. For instance, Eq_{BHT} of the sage CO_2E was more than twice as low as that of AO + MWE. Therefore, the conditions of CO_2-extraction need to be further investigated.

The results of the preliminary β-CBTD pointed out sage and thyme as the most promising herbs among the ones that are commonly grown in Lithuania (Fig 2). For these herbs DAO, AO, MWE and CO_2E resulted in isolates of some antioxidant efficiency. Among these, AO and CO_2E displayed antioxidant activity scores higher than 4 points (Table 1). When making the decision on the most effective way of processing a particular herb, not only yield and properties of the isolates, but also economical aspects should be considered. Having this in mind, AO of sage and thyme were used in subsequent experiments. Oregano AO and BHT were also included for comparative purposes.

β-Carotene bleaching (spectrophotometric method)

As a result of β-carotene bleaching caused by the oxidation of linoleic acid, the absorbance of the test solutions decreased with time (Fig 3). The discoloration process in the model system progressed differently for the various samples.

For the purpose of the present discussion, relative antioxidant activities (RAA) were calculated for each sample as follows:

$$\text{RAA} = \frac{\text{Absorbance of sample}}{\text{Absorbance of BHT (reference)}}.$$

RAA values (averages ± standard deviations) obtained after 28 and 56 h of oxidation are given in Table 2.

Initially, the performance of sage and BHT solutions in the model system were very similar (Fig 3). The RAA values for these samples at 28 h were not significantly different ($P > 0·01$). After the same period, thyme and oregano displayed significantly ($P < 0·01$) lower absorbance values than sage and BHT. After 56 h, however, all

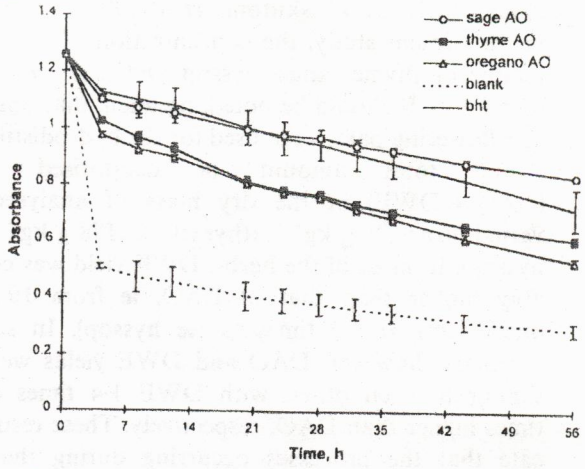

Fig 3. Antioxidative effects of ethanolic solutions of herb acetone oleoresins and BHT in the β-carotene linoleic acid model system at 55°C.

samples differed significantly ($P < 0·01$) among themselves. The sage AO solutions were the most efficient in inhibiting oxidation in the model system used at the end of the experimental period. The absorbance for BHT samples was, by then, 1·2 times lower than the corresponding value for sage AO. Although the performance of thyme AO and oregano AO did not differ after 28 h, at the end of the experiment, the absorbance of thyme samples exceeded that of oregano with 10%. These differences in antioxidant efficiency after 28 h and 56 h may be partly explained by the thermal instability of BHT and the active compounds in oregano AO. Thyme AO, although less effective in this model system than sage, is worth attention due to having a more prolonged inhibitory effect on the discoloration of the reaction mixture than BHT and oregano (Fig 3).

When comparing the β-carotene bleaching results obtained by spectrophotometric and the diffusion methods, the higher activity is associated with sage and thyme AO in both tests. For both methods, the activity of sage AO is comparable to that of BHT. The only discrepancy observed was in the lack of antioxidant

TABLE 2

Relative antioxidant activity (RAA) of herb acetone oleoresins assessed at 55°C by the β-carotene bleaching test (spectrophotometric method)[a]

Sample	*28 h*	*56 h*
Sage AO	1·01 ± 0·049a	1·16 ± 0·057a
Thyme AO	0·81 ± 0·059b	0·85 ± 0·061b
Oregano AO	0·80 ± 0·056b	0·75 ± 0·061c
BHT (reference)	1·00a	1·00d
Blank	0·41 ± 0·043c	0·41 ± 0·039e

[a] The values shown represent the average of six determinations ± standard deviations. Within the same column, averages marked with the same following letter are not significantly ($P > 0·01$) different.

activity of oregano AO when assessed by the diffusion method. Limitations of the agar diffusion test, such as the visual assessment and interference of the diffusion of antioxidants through the agar matrix, may account for this. Another explanation may be provided by the difference in experimental conditions between both tests (temperature, light, reaction time).

The data on extraction procedures and antioxidant activity assessment obtained in these experiments single out thyme and sage as the most promising sources of natural antioxidants. AO appears to be an appropriate technique for the isolation of substrates containing natural antioxidative compounds from the herbs studied. Purification and identification of the active compounds in these herb isolates is required for a better understanding of the protective mechanisms involved and for possible application in foods.

ACKNOWLEDGEMENTS

The authors wish to thank Mr J L Cozijnsen for his technical support. The contributions of Mrs M Baranauskiene (Lithuanian Institute of Horticulture, Lithuania) and Dr T Vasconcelos (Dept. of Botanics, Technical University of Lisbon, Portugal) in provided the herb samples are gratefully acknowledged.

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