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Dittany (*Origanum dictamnus*) as a source of water-extractable antioxidants

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Abstract

Extracts of dittany (*Origanum dictamnus* L.) made with solvents of varying polarity were evaluated (i) by electron spin resonance (ESR) spectrometry using the spin trapping technique for their efficiencies as scavengers of free radicals, and (ii) by measurement of oxygen depletion in a methyl linoleate emulsion for their efficiencies as chain-breaking antioxidants. Aqueous extracts of dittany most efficiently scavenged hydroxyl radicals as generated by the Fenton reaction and this extract also most efficiently reduced oxygen consumption when initiated by metmyoglobin. Ethanol and acetone extracts of dittany showed less activity in both assays with methanol extract being intermediate. The high efficiencies found in the aqueous (and methanol) extracts of dittany were closely related to a high content of phenolic compounds in these extracts, while the amounts of phenolics in the ethanol and acetone extract were lower. In aqueous extracts, the antioxidative activity was confirmed in a turkey thigh meat homogenate, where development of thiobarbituric reactive substances was increasingly inhibited by increasing additions from 0.0018 mg dittany/g meat to efficient inhibition for 0.011 mg dittany/g meat and higher additions. Dittany should be further explored as a source of water-extractable antioxidants.

1. Introduction

The use of spices and herbs as antioxidants in processed foods is becoming of increasing importance in the food industry as an alternative to synthetic antioxidants (Madsen & Bertelsen, 1995; Madsen, Bertelsen, & Skibsted, 1997). It was early recognized that different plants have varying efficiencies in different types of foods (Chipault, Mizuno, Hawkins, & Lundberg, 1952; Chipault, Mizuno, & Lundberg, 1956) and a challenge today is to identify spices and herbs with optimized effects for specific types of foods. For industrial use, extracts rather than the dried leaves or seeds will be preferred for marinating meat and for use in dried products such as potato flakes, and assays have been developed to follow the antioxidative efficiencies of different fractions during extraction (Madsen, Nielsen, Bertelsen, & Skibsted, 1996). For use in foods, extracts made with water have obvious advantages in relation to certification, and we have been searching for plants not commonly in use by the food industry for which folkloristic tradition indicates potential effects. Dittany (*Origanum dictamnus* L.), only found in the Cretan mountains and already in Greek mythology associated with magic properties, has (in folk medicine) been used as a herbal tea and is further used in spiced wines (Skrubis, 1979). Dittany belongs to the Labiatae family like rosemary, thyme and oregano, but has a less penetrating taste than these spices. The use of dittany in herbal infusions indicates that the active components of this herb are readily extractable with water, and we decided to explore the antioxidative properties of dittany in more detail. The two assays previously developed were used to determine the free radical-scavenging efficiency and chain-breaking properties of antioxidants for different extracts of dittany (Madsen et al., 1996). For aqueous extracts, the ability of dittany to inhibit the development of secondary lipid oxidation products was further tested for different additions in a model food system based on cooked turkey meat.

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2. Materials and methods

2.1. Spices and chemicals

Dried leaves of dittany (*Origanum dictamnus* L.) were bought locally at the farmers market in Hania, Crete, and dried leaves of Turkish oregano (*Origanum vulgare* L.) were obtained from Paul Müggenburg, GmbH & Co. (Alveslohe, Germany). Horse heart metmyoglobin (MMb, type III), methyl linoleate and Tween 20 were obtained from Sigma (St. Louis, MO). Hydrogen peroxide (30%), iron(II) sulphate heptahydrate, methanol, ethanol, acetone, reagents for the TBARS-analysis and buffer substances were all analytical grade and were obtained from Merck (Darmstadt, Germany). Tetraethoxy-propane also from Merck, was used as standard for the TBARS analysis. 5,5-Dimethyl-1-pyrroline-*N*-oxide (97%) (DMPO) was from Aldrich Chemical Co. (Milwaukee, WI, USA). Frozen turkey thighs were bought locally. Water was purified through a Millipore Q-plus purification train (Millipore, Bedford, MA, USA).

2.2. Spice extracts

Dittany (and Turkish oregano for comparison) were extracted with the following solvents: water, methanol, ethanol or acetone. The solvent (10 ml) and the coarsely crushed leaves (0.50 g) were homogenized in an Ultra Turrax homogenizer (13 500 rpm, 1 min). After rinsing the knife of the homogenizer with 3 ml liquid and centrifugation (2300 ***g***, 10 min), the supernatant was removed and the residue resuspended in 7 ml liquid. The suspension was placed in darkness under stirring for 10 min. After repeated centrifugation (2300 ***g***, 10 min) and combination of the supernatants (corresponding to a final amount of 25 mg spices/ml solvent), the extract was filtered to yield a clear extract. The extracts were flushed with nitrogen and stored at −18°C until used. Phenolic compounds in the extracts were quantified using the Folin-Ciocalteu reagent with phenol as standard (Amerine & Ough, 1980), and each extract was analysed at two or three different concentrations, each analysis in duplicate.

2.3. Free radical scavenging assay

Determination of free-radical scavenging capacity was based on competition between the spin trap DMPO and the antioxidants present in the extracts in their reaction with hydroxyl radicals. Hydroxyl radicals were generated by the Fenton reaction (H_2O_2/Fe(II)), and the fraction reacting with DMPO determined by ESR-spectrometry.

A 0.10 M aqueous solution of DMPO was prepared by direct dissolution and was stored for up to 1 week at 5°C. The DMPO was purified through active coal and the DMPO concentration was controlled prior to use by measuring the absorbance at 228 nm (HP 8452A diode array spectrophotometer, Hewlett–Packard, Palo Alto, CA). The DMPO solution itself was found to be ESR-silent. 0.010 M H_2O_2 was used as stock solution. 0.010 M $FeSO_4$ was freshly prepared and used within 4 h. DMPO solution (1.00 ml) and Fe(II) solution (5 μl) were mixed and diluted to 10.00 ml. 2.95 ml of this solution was mixed with spice extract (50 μl), and the reaction was initiated by addition of H_2O_2 (30 μl), to give [DMPO] = 9.9 mM, $[Fe^{2+}]$ = 5.0 μM and $[H_2O_2]$ = 99 μM in the final solution. The spice extracts were evaluated for 16.5 μl extract/ml, corresponding to 0.413 mg dried spice/ml in comparison with a reference without spice extract. Immediately after mixing, the reaction mixture was transferred to a flat quartz cell (WG-813-TMS) for measurement of aqueous solutions (0.3×13×115 mm, Wilmad Glass Company Inc., Bueno, NJ) in the cavity of a Bruker ECS 106 spectrometer (Bruker, Karlsruhe, Germany). Measurements were carried out at room temperature (20 ± 1°C) after 2 min with the following spectrometer settings: centre field 3475.60 G; sweep width 55 G; microwave power 20 mW; modulation frequency 100 kHz; modulation amplitude 1.01 G; receiver gain 1.00×10^5; conversion time 81.92 ms; time constant 163.84 ms and sweep time 83.89 s.

The degree of inhibition (I_{ESR}) was calculated by:

$$I_{ESR} = \left(1 - \frac{\text{Peak height}_{\text{Sample}}}{\text{Peak height}_{\text{Reference}}}\right) \times 100\% \quad (1)$$

Measurement of reference and sample containing spice extract was in each case performed on the same day, using the same stock solutions.

2.4. Oxygen consumption assay

Fifty-microlitre spice extracts of spice (12.5 mg dried spice/ml) were added to 5.00 ml of a 1.33 mM methyl linoleate emulsion in 5.0 mM aqueous phosphate buffer with pH = 5.8 and 0.2 w/w% Tween 20 as emulsifier (Jørgensen & Skibsted, 1993). In order to initiate oxidation, 50 μl of a 0.20 mM MMb aqueous solution was added. The final concentrations in the phosphate buffer were 0.123 mg dried spice/ml and 2.0 μM MMb. Immediately after MMb addition, measurement of the oxygen consumption was started by injection of the sample into a thermostated (25.0 ± 0.1°C) 70 μl measuring cell (Chemiware, Viby J., Denmark) with no head space. Oxygen concentration was measured with a Clark electrode (Radiometer, Copenhagen, Denmark) and recorded for approximately 20 min at time intervals of 30 s with a PC-based data collecting system.

The initial oxygen consumption rate $v(O_2)$ in μmol l^{-1} s^{-1} was calculated from:

$$v(O_2) = \frac{-\text{slope} \times [O_2]_{\text{initial}} \times 10^6}{100} \quad (2)$$

where the slope (per cent O_2 per s) was calculated from the oxygen consumption in the 90 to 70% interval relative to the initial 100% oxygen concentration corresponding to water saturated with air, $[O_2]_{\text{initial}} = 2.6 \times 10^{-4}$ M at 25°C. The influence of each of the spice extracts on the initial rate of oxygen consumption was expressed as an antioxidative index relative to the rate in the absence of spice extract:

$$I_{\text{oxygen}} = \frac{v(O_2)_{\text{with spice present}}}{v(O_2)_{\text{without spice present}}} \quad (3)$$

2.5. Inhibition of development of TBARS in a model food

Frozen turkey thighs were thawed and skin, bone and visible fat were removed prior to mincing (Beem Gigant CS-M, type EF 5-10). Minced meat was vacuum-packed (99%) in bags with 20 g and stored at −25°C until used. The minced meat was heated in the bags in a water bath (80°C for 5 min) and 5.0 g samples homogenised (8000 rpm for 30 s in an Ultra Turrax) after addition of 50 ml aqueous Tris-maleate buffer (pH 7.4). Samples of 3.7 ml of the homogenate (corresponding each to approximately 0.34 g of meat) were transferred to test tubes which, after addition of aqueous dittany extract (0.025, 0.050, 0.100, 0.150 or 0.200 ml, or no addition as control), were incubated in a shaking water bath at 37°C for 0, 10, 30, 60 or 90 min. After incubation, during which a gentle flow of air was forced through the samples, the entire content of each tube was subjected to analysis for thiobarbituric acid reactive substances (TBARS) using a procedure previously described (Madsen, Sørensen, Skibsted, & Bertelsen, 1998), and using the difference in absorbance, $A_{532\ \text{nm}} - A_{600\ \text{nm}}$, measured on a Shimadzu UV-1201 Spectrophotometer (Shimadzu, Kyoto, Japan), where $A_{600\ \text{nm}}$ is used to correct for sample turbidity. TBARS were measured in triplicate and expressed as moles malondialdehyde (MDA)/kg meat.

2.6. Statistical analysis

The results from the measurement of phenolic compounds were analysed statistically by a two-way analysis of variance, the solvents and the herbs (dittany or Turkish oregano) being the two parameters and concentration of phenolic compounds the response variable, with two or three repetitions.

The ESR results (I_{ESR}) reported are the average values of triplicate measurements. Results were analysed for differences between solvents with I_{ESR} being the response variable. A similar analysis was performed with the data for inhibition of oxygen consumption, I_{oxygen}. The analyses were performed by using the statistical software SAS ver. 6.10 (1990).

3. Results and discussion

In the preparation of spice extracts a number of solvents have been used. Chang, Ostric-Matijasevic, Hsieh, and Huang (1977) reported that, for rosemary and sage, the highest antioxidative capacity was obtained by using polar solvents, methanol being the most effective. As a confirmation of this finding, methanol has been the most often used solvent in spice extraction procedures (Madsen & Bertelsen, 1995). The use of water as a solvent is rare, although a patent using an aqueous carbonate buffer (pH ~8.5) describes a procedure to obtain spice extracts with a low content of aromatic compounds (Viani, 1977). In the present study, analysis of phenolic content showed phenol equivalents in the extracts in the range 7 to 22 meq/l (see Table 1). Statistical analysis showed a significant interaction between the herbs and the solvents ($p < 0.001$). The aqueous extract of dittany had a significantly ($p < 0.001$) higher amount of phenolic compounds compared to the dittany extracts obtained using methanol, ethanol or acetone as solvents. Analysis of the extracts of Turkish oregano revealed a significantly ($p < 0.001$) lower amount of phenolic compounds in the ethanol and acetone extracts compared to the water and methanol extracts, the methanol extract having significantly ($p < 0.05$) higher amount of phenolic compounds (see footnote to Table 1). These results confirm that any unique properties of dittany are not specially related to a higher content of phenolics, as the value 21.7 meq/l in aqueous extract of dittany is comparable to the value, 20.7 meq/l, found in the methanol extract of Turkish oregano. Such properties are rather related to the fact that the phenolics in dittany are more extractable by water in contrast to the finding for other spices (Chang et al., 1977) including Turkish oregano, as is evident from the results presented in Table 1.

The ESR spin trapping assay shows that the free radical scavenging capacity of the dittany extracts, at least qualitatively, correlates with the content of phenolics. The extracts rich in phenolics, i.e. the methanol and in particular the water extracts, were able to scavenge the hydroxyl radicals generated in the Fenton reaction, as shown by a significant ($p < 0.05$) reduction in the ESR signal. The excellent free radical scavenging property of the water and methanol extracts found by the ESR method was paralleled in the chain-breaking properties as documented in the oxygen depletion assay. The aqueous and methanol extracts most effectively reduced the oxygen consumption, while the effects of

Table 1
Extracts of dittany as radical scavengers (I_{ESR}) and chain-breaking antioxidants (I_{oxygen})

Solvent	Water	Methanol	Ethanol	Acetone
Phenol	21.7	13.8	7.7	6.7
[meq/l][a]	±0.7	±0.6	±2.1	±1.5
I_{oxygen}[b]	0.036	0.042	0.091	0.074
I_{ESR}[c]				
0.413 mg spice/l	72%	68%	43%	14%
Dielectric constant[d]	78.5	32.6	24.3	20.7

[a] Results for Turkish oregano were 17.9 (water), 20.7 (methanol), 10.9 (ethanol) and 8.5 (acetone), all with a standard deviation of 1.0.
[b] Defined in Eq. (3).
[c] Defined in Eq. (1).
[d] From Weast (1986).

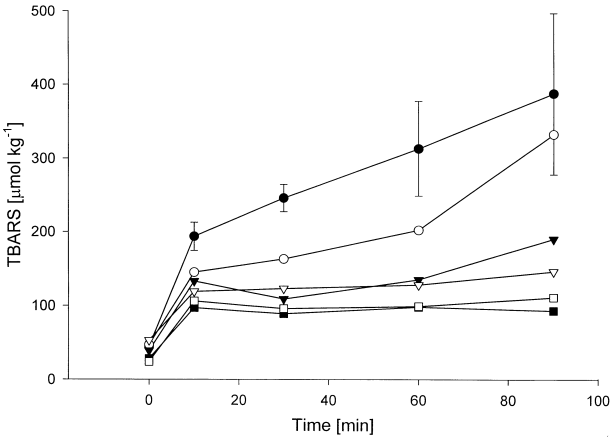

Fig. 1. Development of secondary oxidation products measured as thiobarbituric acid-reactive substances (TBARS) in a meat slurry made of cooked turkey thighs at 37°C during constant air-supply in the absence or presence of increasing amounts of aqueous dittany extract (0.025 g spice/ml): ● no addition; ○ 0.025 ml; ▼ 0.050 ml; ▽ 0.100 ml; ■ 0.150 ml; □ 0.200 ml extract added to 3.7 ml meat homogenate made from 5.0 g cooked turkey meat in 50 ml of aqueous buffer (see section: Inhibition of development of TBARS in a model food).

the ethanol and acetone extracts were less significant (Table 1).

A general conclusion is that the antioxidative activity, seen for the different extracts of dittany, is closely related to the content of phenolic compounds, confirming the key role of phenolic compounds as scavengers of free radicals and as primary, chain-breaking antioxidants. For the aqueous extract of dittany an unexpectedly high amount of phenolic compounds was detected (21.7 meq/l), while the extracts of dittany obtained from the organic solvents had a lower content of phenolic compounds (7–14 meq/l). The amounts of phenolic components were lowest in the extracts made with the low-polarity solvents, ethanol and acetone, and these extracts also had a lower antioxidative activity. In a comparison of dittany with Turkish oregano used as a standard spice, previously investigated in detail (Madsen et al., 1996), a higher level of phenolic compounds was observed in all extracts *except* for the water extracts.

Based on the results obtained by the ESR and the electrochemical assay, the antioxidative properties of aqueous extracts of dittany were tested in a food model system using cooked turkey thighs. Secondary lipid oxidation products were measured as TBARS in a meat homogenate under mildly accelerated temperature conditions. Aqueous dittany extract clearly had antioxidative properties in the meat system as is evident from Fig. 1. For the non-protected meat homogenate, the TBARS-value rose rapidly, while as little aqueous extract as 0.0018 mg dried dittany/g meat (0.025 ml of extract with 0.025 mg dittany/ml water) added to 3.7 ml of homogenate) clearly limited the oxidation. For increasing additions, the effect became more significant and, at additions corresponding to 0.011 mg dried dittany/g meat or higher, oxidation was efficiently halted.

The composition of dittany appears only to have been sparsely investigated. The essential oil of dittany has been analysed and the main components were β-phellandrene and carvacrol (Skrubis, 1979), but these aromatic compounds hardly contribute to the high amount of phenolic compounds in the water extract, due to their low solubility. Methanol extracts of numbers of spices belonging to the Labiatae family, including Turkish oregano and dittany, have previously been investigated to characterize the antioxidative activity. For the methanol extracts, Turkish oregano was found to be the most effective spice, closely followed by dittany (Economou, Orepoulon, & Thomopoulus, 1991). However, the high amount of water-soluble phenolic compounds present in dittany, and the corresponding high antioxidative activity, provide interesting perspectives. The use of water as an extraction solvent is certainly suitable for the food industry. An investigation, with the aim of identifying water-soluble antioxidants in the fabled Cretan herb, should be initiated.

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