

ANTIOXIDANT ACTIVITY OF OREGANO (*ORIGANUM VULGARE* L.) LEAVES

VERIFICA DELL'ATTIVITÀ ANTIOSSIDANTE DI FOGLIE DI ORIGANO
(*ORIGANUM VULGARE* L.)

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ABSTRACT

Oregano leaves (*Origanum vulgare* L.) were extracted with ethanol, diethyl ether, *n*-hexane:2-propanol 4:1 (v/v) and *n*-pentane. The highest yield was obtained with ethanol and the ethanolic extract showed the strongest inhibitory effect on lipid oxidation when added (amounts from 0.02 to 5% w/w) to a model lipid system (made up of refined bleached peanut oil), subjected to forced dynamic oxidation, using the Oxidative Stability Instrument (OSI). The hydrolyzed ethanolic extract showed a more powerful antioxidant activity than the untreated extract. Total polyphenols in

RIASSUNTO

Le foglie di origano (*Origanum vulgare* L.) sono state estratte con etanolo, etere dietilico, *n*-esano:isopropanolo 4:1 (v/v) e *n*-pentano. La resa più elevata è stata ottenuta utilizzando l'etanolo e l'estratto etanolic ha mostrato anche il più forte effetto inibitorio nei confronti dell'ossidazione lipidica, quando addizionato (in quantità variabili dallo 0,02 al 5% p/p) ad un sistema lipidico modello (costituito da olio di arachide raffinato e decolorato), sottoposto ad ossidazione dinamica forzata, mediante l'Oxidative Stability Instrument (OSI). L'estratto etanolic idrolizzato ha mo-

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this extract, expressed as gallic acid, were quantified by spectrophotometric analysis at 280 nm. The ethanolic extract was also analyzed by TLC (thin layer chromatography) and showed the presence of flavones, flavanones and dihydroflavonols, whether glycosilated or not.

strato una maggiore attività antiossidante rispetto all'estratto non trattato. I polifenoli totali di questo estratto, espressi come acido gallico, sono stati quantificati attraverso analisi spettrofotometrica, a 280 nm. L'estratto etanolicò è stato anche analizzato in TLC (cromatografia su strato sottile) e ha mostrato la presenza di flavoni, flavanoni e diidroflavonoli, sia liberi che glicosilati.

INTRODUCTION

In recent years, there has been increasing interest in the possibility of using vegetable extracts as antioxidants. The food and cosmetic industries have attempted to select vegetable extracts to replace synthetic phenolic antioxidants, such as BHA and BHT (CERUTTI, 1999; GIESE, 1996; HAMAMA and NAWAR, 1991). Furthermore, the possible use of natural antioxidants for the prevention of some human diseases has stimulated increasing interest in this field. In fact, it is well known that arteriosclerosis, ischaemia events, human cancer and inflammatory diseases are related to significant exposure of cells to oxidative stress (NAMIKI, 1990; PENG *et al.*, 1992; VINSON *et al.*, 1995; PORRINI and TESTOLIN, 1997).

Many vegetable extracts have been studied in relation to their antioxidant activity (LINDBERG and BERTELSEN, 1995; GALLINA TOSCHI *et al.*, 2000). Notably the Labiatae family is well-known for its antioxidative properties, and especially rosemary and sage have been reported to have strong effectiveness (CUVELIER *et al.*, 1990, 1994, 1996; SCHWARZ and TERNES, 1992a, b; SCHWARZ, *et al.*, 1992; CHEN *et al.*, 1992; PIZZOCARO *et al.*, 1994; RICHHEIMER *et al.*, 1996; VARELTZIS *et al.*, 1997). Oregano, thyme and marjoram extracts

have also shown a pronounced effect in stabilizing lipids against autoxidation (KIKUZAKI and NAKATANI, 1989; ECONOMOU *et al.*, 1991; CHEVOLLEAU *et al.*, 1992; VEKIARI *et al.*, 1993a, b).

This study was carried out with the aim of selecting the best extraction conditions for oregano leaves, often used in foods for its organoleptic properties, to obtain greater antioxidant power.

MATERIALS AND METHODS

Solvents and reagents were analytical or HPLC grade, from Carlo Erba (Milan, Italy) and Prolabo (Paris, France). Flavonoid standards (99% pure), gallic acid and BHT (99% pure) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Oregano leaves (*Origanum vulgare* L.) were obtained from the region of Calabria (Italy).

Preparation of oregano extracts

Oregano leaves (50 g) were extracted separately with 300 mL of four different solvents: ethanol, diethyl ether (not stabilized with BHT), *n*-hexane:2-propanol 4:1 (v/v) and *n*-pentane. Extractions were conducted at the boiling temperature of each single solvent, in a Soxhlet apparatus, for four hours. After each

extraction, the solvent was dried under vacuum with a Rotavapor (60°, 30°, 50° and 30°C, respectively); the solid was weighed and brought to a final volume of 25 mL with the same solvent.

Acid hydrolysis of oregano extracts

A certain volume of the solutions obtained from the extraction with four different solvents, containing amounts from 1 to 50 mg of oregano extract (calculated on the basis of the extraction yield) were dried under gentle nitrogen flow and then hydrolyzed using 1.5 mL of 1M HCl and kept in a boiling water bath for 1 h (HOLLMAN *et al.*, 1996; LU and FOO, 1997). The hydrolysis products were extracted four times with 0.5 mL of diethyl ether (not stabilized with BHT); the solvent was then dried, and the solid dissolved in 0.15 mL of the same solvent.

Preparation of the lipid model system

A refined peanut oil, from the market, was used as the model lipid system. Ten percent of bleaching earth (previously activated at 110°C for 18 h) was added and the oil was kept for 15 min at 80°C in a Rotavapor, under weak vacuum (BENDINI *et al.* 2001).

Antioxidant activity measurement

The antioxidant activity of the different oregano extracts was tested on the refined bleached peanut oil by subjecting it to forced dynamic oxidation. An eight-channel Oxidative Stability Instrument (OSI) (Omnion Inc., Decatur, Illinois) was used. Five grams (± 0.1 g) of peanut oil were weighed in each glass tube, the temperature was set at 98°C and the stream of air bubbled through the oil was 120 mL/min. The OSI measures the changes in conductivity due to the formation of ionic volatile organic acids (mainly formic acid) which are collected in a polycarbonate tube contain-

ing deionized water. The OSI time, obtained under standard conditions of temperature and air bubbling (JEBE *et al.*, 1993), corresponds to the change in slope of the volatile organic acid production, which is considered to be the end of the induction period of an oil.

Total polyphenol determination

The quantity of total polyphenols in the oregano ethanolic extract, expressed as weight percent (w/w), was determined by spectrophotometric analysis at 280 nm; the calibration curve was built with standard solutions of gallic acid, as reported by the official method (MARGHERI and FALCIERI, 1972).

Thin-layer chromatographic analysis (TLC) of oregano extracts

TLC plates, precoated with silica gel (20x20 cm, Merck, Darmstadt, Germany) were used to separate the components of the ethanolic oregano extract. The developing system used was toluene/acetone/formic acid (30:60:10, v/v/v).

The following spray reagents were used to identify the chemical compounds: (a) atomization with 5% aluminum chloride in water (w/v) (a yellow color indicates the presence of flavonoids) (VEKIARI *et al.*, 1993a), (b) exposure to ammonia fumes (observation under UV light shows a dark color that persists after exposure to ammonia fumes, if the compounds are flavones, dihydroflavonols or flavanones) (VEKIARI *et al.*, 1993a), (c) vaporization with sulphochromic mixture and carbonization at 140°C (organic compounds appear as brown color), (d) atomization with Folin-Ciocalteu reagent (phosphotungstic and phosphomolybdic acids) and, after three minutes, with 20% sodium carbonate in water (w/v) (polyphenol compounds appeared as blue color) (MARGHERI and FALCIERI, 1972).

RESULTS AND DISCUSSION

The oregano extraction using four different solvents gave yields of: ethanol 8.3% (w/w), *n*-hexane:2-propanol 4:1 6.6% (w/w), diethyl ether 5% (w/w) and *n*-pentane 3.9% (w/w). The highest yield was obtained with ethanol. Methanol was not used, even if it has been reported to be the best solvent for phenols (MØLLER *et al.*, 1999) because it is toxic and should be completely removed before the extract can be used as a food additive. The use of water, which is certainly suitable for the food industry and considered effective for the extraction of water-soluble phenolic compounds (MILOŠ *et al.*, 2000), was not considered because it requires a high temperature and a long time to be removed, during which time the antioxidant components could easily decompose.

The repeatability of the OSI time of the peanut oil, used as a model lipid system, was checked by analyzing sixteen samples of oil, freshly bleached each time. The mean value of the OSI time was 14.40 h, the standard deviation was ± 0.77 h (coefficient of variation was 5.36%). Before analyzing each single extract, some tests were carried out to check if small amounts (up to 3% v/v) of solvents used for the extractions could modify the OSI time of the model lipid system. Ethanol caused a small significant decrease in OSI time (-2.4%), very close to the coefficient of variation of the analysis; no significant effect was recorded for diethyl ether but *n*-pentane or *n*-hexane:2-propanol 4:1 caused a slight increase in the OSI time.

As shown in Fig. 1, the ethanolic extract gave a higher antioxidant activity than the other extracts, obtained with less polar solvents, in increasing amounts, from 0.1 to 0.6% w/w. It is also evident that the hydrolyzed ethanolic extract gave the highest antioxidant activity and this effect increased with increase in the concentration of the extract added to the oil, up to 0.4%.

The effect of the ethanolic extract was tested under a wider range of concentrations, from 0.02 to 5% w/w (Fig. 2). The OSI time of the model lipid system (14.40 h) doubled when the ethanolic extract was added at 0.5% (29.7 h) and it became almost four times as much at 5% (56.9 h). Due to the organoleptic modifications induced by adding high amounts of the extract, percentages higher than 5% were not tested. As reported in Figs. 1 and 2, the addition of 0.1% (25.70 h) hydrolysed ethanolic extract exhibited a protective effect corresponding to 97% of that shown by the addition of 0.04% (26.50 h) BHT, which is a pure standard product. The total polyphenol content of the ethanolic oregano extract was $10.17\% \pm 0.47$ (expressed as weight % as gallic acid), calculated on four determinations by UV analysis at 280 nm, near the maximum absorption of most phenols.

The ethanolic oregano extract was then analyzed by TLC (Fig. 3) and nine bands were isolated as black spots, after carbonization with a sulphochromic mixture, and were numbered starting from the top of the plate (column A). The reactions to the solution of 5% aluminum chloride in water, to ammonia fumes and to the Folin-Ciocalteu reagent were positive for bands 3-9 (R_f from 0.86 to 0.05); this response is characteristic of polyphenols, particularly of flavones, flavanones and dihydroflavonols. Bands 1 and 2 with $R_f = 0.93$ and $R_f = 0.89$, respectively, were not flavonoids (probably chlorophylls); the spot with $R_f = 0.84$ (band 4 columns A, B) could contain, as reported in the literature (VEKARI *et al.*, 1993a), apigenin (flavone, column C), eriodictyol (flavanone, column D), kaempferol (flavonol, column E), quercetin (flavonol, column G and I) or rosmarinic acid (phenolic acid, column M). Bands 6-9 corresponded to polyphenol glycosides because they disappeared when the oregano extract was hydrolyzed (column B) such as: rutin (quercetin-3-

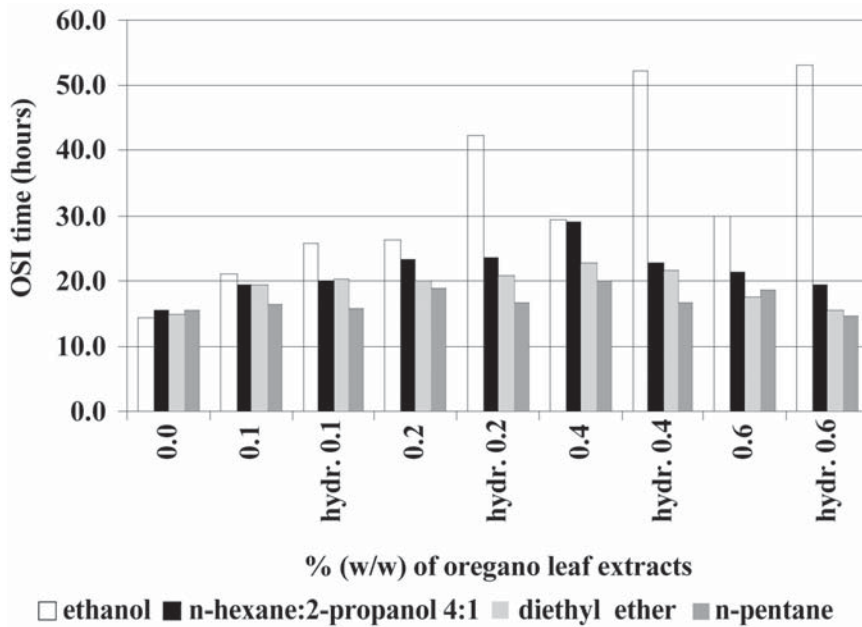


Fig. 1 - OSI time values (hours)^a of refined bleached peanut oil, with the addition of increasing amounts (from 0.1 to 0.6% w/w) of untreated and hydrolysed oregano leaf extracts, obtained using different solvents (ethanol, diethyl ether, *n*-hexane: 2-propanol 4:1, *n*-pentane). Control group was refined bleached peanut oil, with the addition of 3% (v/w) of each extraction solvent.

^aCalculated from two determinations.

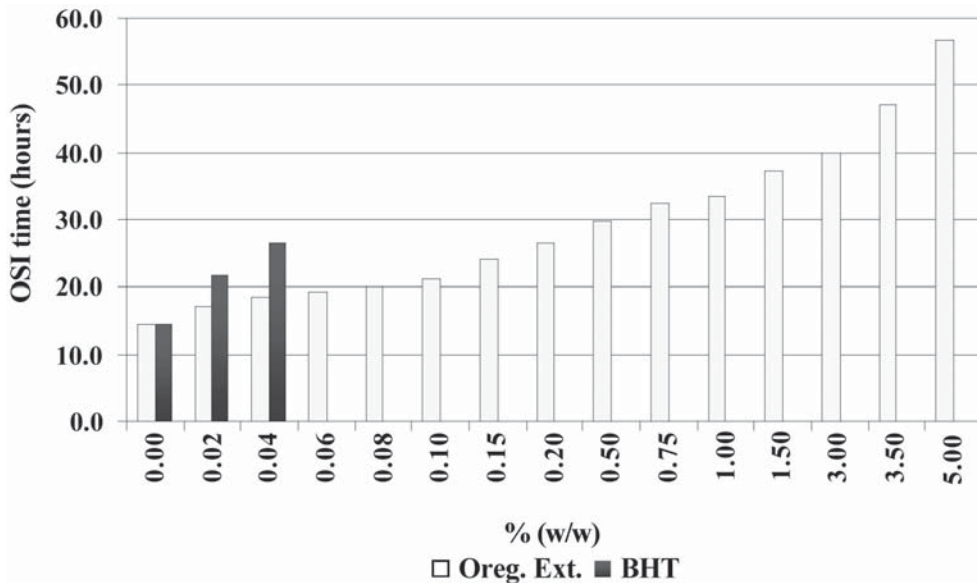


Fig. 2 - OSI time values (hours)^a of the refined bleached peanut oil, with the addition of increasing amounts (from 0.02 to 5% w/w) of untreated ethanolic oregano leaf extracts and BHT (from 0.02 to 0.04% w/w). Control group was refined bleached peanut oil, with the addition of 3% (v/w) ethanol and diethyl ether (the solvent used to dissolve BHT).

^aCalculated from two determinations.

rutinoside, R_f 0.19, spot number 12, column H) which, once hydrolyzed (column I), released its corresponding aglycone (quercetin, $R_f = 0.84$, column G).

CONCLUSIONS

To evaluate the use of vegetable extracts to protect food lipids from oxidation, the antioxidant power of the dried

oregano leaf extracts, obtained using different solvents, was measured by OSI time. The dried ethanolic extract had the highest antioxidant activity and, when added to a model lipid system at a percentage of 0.20% w/w (26.40 h of OSI time), showed the same antioxidant power as BHT added at 0.04% w/w (26.50 h of OSI time). Hydrolyzed ethanolic oregano extracts had stronger antioxidant properties, with respect to

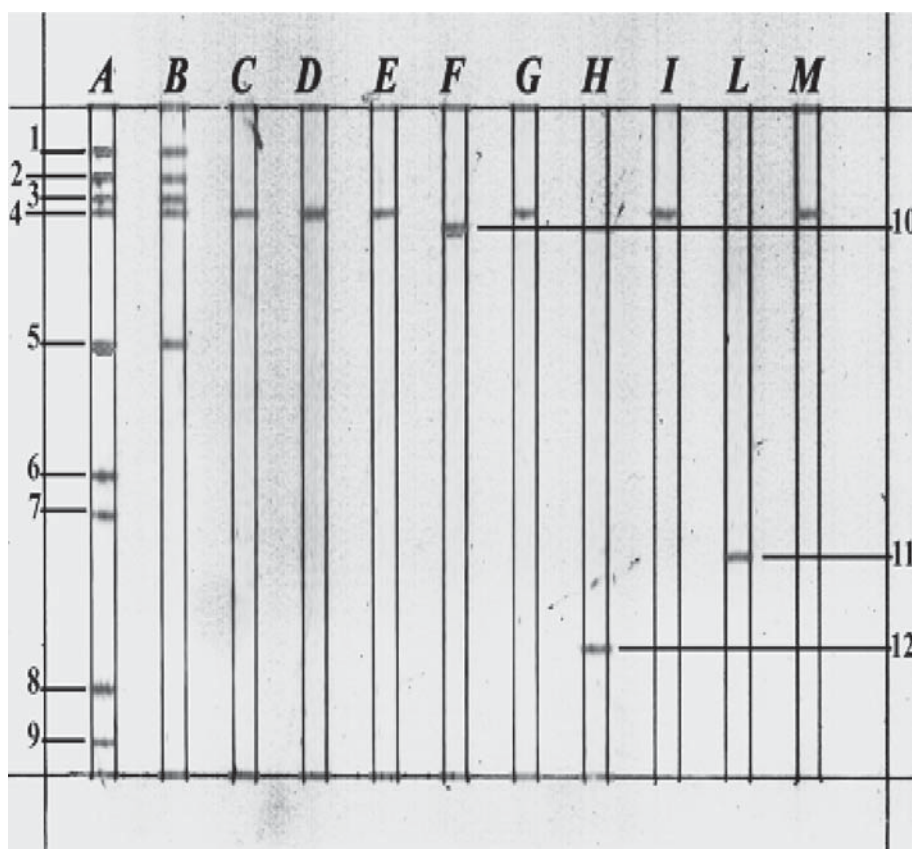


Fig. 3 - Thin layer chromatography of: A = Untreated ethanolic extract of oregano leaves; B = Hydrolysed ethanolic extract of oregano leaves; C = Apigenin; D = Eriodictyol; E = Kaempferol; F = Taxifolin (dihydroquercetin); G = Quercetin; H = Rutin (quercetin-3-rutinoside); I = Hydrolysed rutin; L = Hesperidin (hesperetin-7-rhamnoglucoside); M = Rosmarinic acid.

The bands visualized as black spots after carbonisation with sulphochromic mixture, are numbered from 1 to 9, starting from the top of the plate. The R_f (retention factor) of the bands are:

A1,B1 $R_f = 0.93$; A2,B2 $R_f = 0.89$; A3,B3 $R_f = 0.86$; A,B,C,D,E,G,I,M-4 $R_f = 0.84$;

A5,B5 $R_f = 0.64$; A6 $R_f = 0.45$; A7 $R_f = 0.39$; A8 $R_f = 0.13$; A9 $R_f = 0.05$;

F10 $R_f = 0.82$; L11 $R_f = 0.33$; H12 $R_f = 0.19$.

Mobile phase: toluene/acetone/formic acid 30:60:10 v/v/v.

the non-hydrolyzed ones. In fact 0.20% (42.30 h of OSI time) of the hydrolyzed extract showed an antioxidant power 1.6 times higher than the untreated extract. TLC analysis of the untreated and hydrolyzed extracts showed the presence of polyphenolic compounds in both types of extracts and the lack of phenolic glycosides (spots with low R_f) in the hydrolyzed ones. This evidence suggests that free phenolic aglycones have a higher antioxidant power, as confirmed by the structure activity relationship studies of HERRMANN (1976) and DAS and PEREIRA (1990) and as showed for oregano volatile aglicone, tested by measuring peroxide values of lard, stored at 60°C (MILOŠ *et al.*, 2000). This evidence merits further investigation on the use of phenolic hydrolyzed oregano extracts as a source of natural antioxidants for the food and cosmetic industries.

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