

Liposome-entrapped essential oils on *in vitro* and *in vivo* antioxidant activity in leafy vegetables

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RESEARCH ARTICLE

Abstract

Enzymatic browning in vegetables can cause undesirable quality changes during handling, processing and storage. Because of antioxidant properties of essential oils, effects of rosemary (*Rosmarinus officinalis* L.) and lemon (*Citrus limonum* L.), and their liposomal derivatives, were evaluated on reduction of peroxides and polyphenoloxidase activities, enzymes that promotes enzymatic browning and nutritional damage in plant tissues, in spinach (*Spinacia oleracea* L., cv. Inner), lettuce (*Lactuca sativa* L., cv. Dark green cos) and red cabbage (*Brassica oleracea* L. *capitata*, cv. Danish bull head). Multilamellar liposomes were prepared using a thin film hydration method. Antioxidant activity of the essential oils was compared with liposomal oils. Antioxidant activity of oils were maintained in the liposomal derivatives, and enhanced at low oils concentration. The highest percentage of antioxidant activity reduction was achieved *in vitro* and *in vivo* using a 0.1 mg/ml concentration of liposomal rosemary oil in all vegetables. Since essential oil is a natural compound in aromatic plants and residues have been successfully encapsulated in liposomes, the liposomal of essential oil hold potential of becoming a safe and effective method to prolong shelf-life of vegetables.

Keywords: lemon, peroxidase, polyphenoloxidase

1. Introduction

Enzymatic browning, discoloration, off-flavours and nutritional damage in vegetables result mostly from polyphenol oxidase and peroxides (Vámos-Vigyázó and Haard, 1981). Inactivation of these enzymes is necessary to minimise the possibility of deterioration. Inhibition of these enzymes is generally achieved by addition of chemical additives. Consumers are demanding less use of chemicals on products to be consumed. Essential oils are volatile, natural, complex compounds characterized by a strong odour, formed by aromatic plants as secondary metabolites (Dorman and Deans, 2000). Compounds in essential oils with phenolic structure are active against microorganisms (Dorman and Deans, 2000). Essential oils can replace traditional sanitizing agents (Cherry, 1999). The possible application of essential oils as natural antimicrobial and antioxidant agents in organically grown fresh vegetables may be an alternative to natural preservation technology.

Essential oils are sensitive materials which suffer degradation due to action of oxygen, light and temperature. They are insoluble in water, and for certain applications a controlled release is required. An adequate formulation of essential oil having these qualities is required for commercial application. Common goals in development of essential oil formulations are to protect the essential oil from degradation or loss by evaporation; to achieve a controlled release, and to facilitate handling. Possible formulations include liquid (emulsions, micelles, liquid solutions), semi-liquid (gels, liposomes), and solid forms (microcapsules or microcomposites) (Martín *et al.*, 2010). Liposomes are promising carriers for essential oils. Antimicrobial and antiviral activity of liposomes, combined with some essential oils, has been proved (Liolios *et al.*, 2009; Sinico *et al.*, 2005). Liposomal encapsulation reduces reactivity with water, oxygen, and light; decreases evaporation or transfer rate to the outside environment; promotes handling ability; masks taste, and enhances dilution to achieve a

uniform distribution in the final product when used in small amounts (Gibbs, 1999).

The study was undertaken to assess effects of essential oils of rosemary (*Rosmarinus officinalis* L.) and lemon (*Citrus limonum* L.) loaded liposomes on peroxidase (POD) and polyphenoloxidase (PPO) activities in spinach (*Spinacia oleracea* L., cv. Inner), romaine lettuce (*Lactuca sativa* L., cv. Dark green cos) and red cabbage (*Brassica oleracea* L. var. *capitata* L., cv. Danish bull head).

2. Materials and methods

Preparation of essential oils

2 kg of fresh aerial parts of vegetables were distilled in a Clevenger-type apparatus for 5 h. Essential oils were dried over anhydrous sodium sulphate and stored at 4 °C. Essential oils were dissolved in 96% ethanol and Tween 80, and diluted with distilled water (Ponce *et al.*, 2003).

Preparation of liposomes

Multilamellar vesicles were prepared according to a thin film hydration method (Gortzi *et al.*, 2006). Lipid solutions were prepared by dissolving 5 mg/ml of phosphatidyl choline, 1 mg/ml cholesterol and 0.05, 0.075 and 0.1 mg/ml of essential oil in 3 mg/ml chloroform. Phosphatidylcholine (PC), from fresh egg yolk, and chloroform were obtained from Sigma Chemicals (St. Louis, MO, USA.). Cholesterol was purchased from Fluka (Buchs, Switzerland). 5 ml from each solution was introduced in a 100 ml round-bottomed flask. The solvent was evaporated in a Heidolph Laborota (model 4000 rotaevaporator; Schwabach, Germany) at 35–40 °C, under reduced pressure (13–14 mm Hg). The resulting dry lipid film was hydrated with 5 ml distilled water. Mechanical stirring of lipids in aqueous medium was performed with the rotaevaporator at 37 °C and by manual stirring in the water bath, for 2 h, at the same temperature. The suspension hydrated for 2 h to anneal structural defects. Incorporated vesicles were separated from unincorporated compounds by centrifugation. Vesicular dispersions were spun in a laboratory centrifuge Hettich Universal 320 R (London, UK), at 10 °C, 3,000×g, for 60 min. The supernatant was removed and liposomes reconstituted with distilled water.

Determination of antioxidant activity

Fresh vegetables were obtained from the local market of Esfahan city. Spinach, romaine lettuce and red cabbage were used as raw materials for POD and PPO assays. For POD assay, 10 g of each vegetable were cleaned and washed to reduce contaminants. Vegetables were chopped and homogenized with 30 ml water in a commercial blender (Stomacher 400 Circulator Homogenizer; Seward, KS, USA) for 3 min. The slurry was filtered through 2 layers

of cheesecloth and centrifuged at 10,000×g for 15 min. All steps were carried out at 4 °C. The supernatant, which contained POD activity, was used as the enzyme source for the experiment (Ponce *et al.*, 2004). POD activity was determined spectrophotometrically at 25 °C with an UV-1601 PC UV-vis spectrometer (Shimadzu Corporation, Tokyo, Japan) at 470 nm using guayacol as the substrate and H₂O₂ as the hydrogen donor. The substrate mixture contained 10 ml of 1% guayacol, 10 ml of 0.3% hydrogen peroxide and 100 ml of 0.05 mol/l sodium phosphate buffer (pH 6.5). The reaction cuvette contained 2.87 ml substrate mixture, 0.1 ml POD crude vegetable extract and 0.03 ml antioxidant solution (essential oils of rosemary and lemon with concentrations of 0.05, 0.075 and 0.1 mg/ml and liposomes with essential oils of rosemary and lemon loading concentrations of 0.05, 0.075 and 0.1 mg/ml). One unit of activity was defined as a change in absorbance of 0.06 s.

For PPO assay, 10 g of vegetables were homogenized at a 1:2 ratio with 0.5 mol/l phosphate buffer (pH 7.0) in presence of 50 g/l polyvinylpyrrolidone (ICN Biomedicals, Inc., Toledo, OH, USA) with a commercial blender (Stomacher 400 Circulator Homogenizer) and centrifuged at 12,700×g for 30 min. The supernatant, which contained PPO activity, was used as the enzyme source. Crude extract samples were divided into aliquots and frozen; after thawing, samples were used immediately. The substrate mixture contained 20 mmol/l catechol as substrate in 5 mmol/l sodium phosphate buffer (pH 7). The reaction cuvette contained 2.9 ml substrate mixture, 0.1 ml PPO crude vegetable extract and 0.03 ml antioxidant solution (essential oils of rosemary and lemon – concentrations of 0.05, 0.075 and 0.1 mg/ml and liposomes with essential oils of rosemary and lemon loading concentrations of 0.05, 0.075 and 0.1 mg/ml). The rate of catechol oxidation was followed at 25 °C at 400 nm for 60 and 120 s. An enzyme activity unit was a 0.001 change in absorbance between 60 and 120 s under the assay conditions (Ihl *et al.*, 2003). For POD and PPO the reference cuvette contained only substrate mixture. For each enzyme source, a reagent blank was prepared with 0.03 ml deionized water instead of antioxidant solution (control).

Studies *in vivo*

Spinach leaves were immersed in essential oils, liposomal essential oils, or water for 180 s and dried at room temperature. Fresh raw spinach immersed in distilled water was the control. Peroxides and PPO activities were determined as described above.

Statistical analysis

The experiment was established with 2 factors (antioxidant agents and concentration) for *in vitro* and *in vivo* studies, using a completely randomized design in duplicate on 3 independent lots. The ANOVA in SPSS (version 15.0, SPSS

Inc., Woking, Surrey, UK) was performed to analyse data for each experiment and means were separated by least significant difference.

3. Results and discussion

In vitro assays: antioxidant properties

Indigenous activity of enzymes differed among vegetable crude extracts (Table 1 and 2). The highest POD and PPO activities were in red cabbage. The source of the enzyme affected POD and PPO susceptibility to the antioxidant agents. Activities of the POD and PPO from each vegetable were affected by antioxidant agent. Activities of the enzymes were influenced by liposome-entrapped essential oils. Most liposomal oils at 0.075 and 0.1 mg/ml concentrations affected activity of the enzymes, regardless of enzyme source, and had the highest antioxidant activity on the 3 vegetable extracts. Lemon essential oil had the highest antioxidant effect on POD and PPO from the 3 vegetable extracts. The effect of lemon essential oil was dependent on source of the enzyme. Liposomal and essential oil of lemon was less effective in reduction of POD and PPO activities from the 3 vegetables than liposomal and essential oil of rosemary. Liposomal incorporation of rosemary oil was effective in minimizing activities of 2 enzymes in the 3 vegetables; also PPO activity in romaine lettuce extracts had the lowest resistance to action of all essential oils. Hemeda and Klein (1990) stated that differences in peroxide activity against antioxidant agents could be related to presence of isoenzymes.

Ponce *et al.* (2004, 2008) concluded when the concentration of essential oils increased, the percentage of peroxides activity remaining tended to decrease in romaine lettuce and spinach extracts; rosemary oil reduced PPO activity obtained from butter lettuce (*Lactuca sativa* L., var. bib), but combinations of chitosan with rosemary oil did not enhance reduction of PPO activity from butter and romaine lettuce extracts.

In vivo assays: antioxidant properties

Greater antioxidant effects on POD and PPO were obtained in spinach treated with liposomal incorporation rosemary oil; however, lemon oil was not effective enough as an antioxidant agent (Table 3). Effects of antioxidant agents on *in vivo* antioxidant activity in spinach were lower compared to *in vitro* antioxidant activity. Essential oil interacts with components on vegetable surfaces, enhancing antioxidant properties. When oils are incorporated in liposomes, the active antioxidant principles of oils were slowly released onto the product surface, and effects were enhanced. Also, Liolios *et al.* (2009) reported that antioxidant activity of thymol and carvacrol improved after their encapsulation in liposomes, indicating that liposomal oil is more useful than essential oil.

Essential oils were successfully incorporated into liposome to form liposomal oils. After loading oils into liposome, the antioxidant activity was maintained, and enhanced at low oil concentration. Liposomal oils exhibited complete reduction of POD and PPO activities of rosemary oil at low concentration, while free lemon oil had negligible antioxidant effects. The antioxidant activity of liposomal

Table 1. Content of peroxidase (units/min/g) in presence of essential oils and liposomal oils (rosemary and lemon) as antioxidant in crude vegetable extracts (*in vitro*).^{1,2}

Antioxidant	Antioxidant agent concentration (mg/ml)	Spinach	Red cabbage	Romaine lettuce
Crude extract (control)	0	1,166 (0) a	27,174 (0) a	983 (0) a
Rosemary	Essential oil (0.05)	810 (-30.51) efg	23,474 (-13.61) d	625 (-36.42) ef
	Essential oil (0.075)	849 (-27.21) def	23,774 (-12.52) cd	697 (-29.07) cd
	Essential oil (0.1)	742 (-36.35) fg	22,524 (-17.08) d	583 (-40.71) fg
	Liposomal essential oil (0.05)	572 (-50.9) i	12,574 (-53.43) h	586 (-40.42) fg
	Liposomal essential oil (0.075)	631 (-45.9) hi	15,499 (-42.74) g	498 (-49.31) g
	Liposomal essential oil (0.1)	568 (-51.3) i	14,474 (-46.49) g	372 (-62.11) h
Lemon	Essential oil (0.05)	952 (-18.31) cd	25,524 (-6.12) b	903 (-8.18) b
	Essential oil (0.075)	1,012 (-13.17) bc	24,249 (-7.13) bc	736 (-25.12) c
	Essential oil (0.1)	1,095 (-6.07) b	23,949 (-11.88) bcd	934 (-5.02) ab
	Liposomal essential oil (0.05)	873 (-25.11) def	17,474 (-35.53) f	590 (-40.01) f
	Liposomal essential oil (0.075)	892 (-23.52) de	15,774 (-41.74) g	686 (-30.22) cde
	Liposomal essential oil (0.1)	786 (-32.6) fgh	19,449 (-28.31) e	657 (-33.11) def

¹ Values between brackets = percentage of peroxidase activity reduction (-).

² Values in columns followed by the same letter are not significantly different at $P < 0.05$ (least significant difference).

Table 2. Content of polyphenoloxidase (units/min/g) in presence of essential oils and liposomal oils (rosemary and lemon) as antioxidant in crude vegetable extracts (*in vitro*).^{1,2}

Antioxidant	Antioxidant agent concentration (mg/ml)	Spinach	Red cabbage	Romaine lettuce
Crude extract (control)	0	1,351 (0) a	23,050 (0) a	1,003 (0) de
Rosemary	Essential oil (0.05)	1,160 (-14.1) c	19,892 (-13.7) d	983 (-2) defg
	Essential oil (0.075)	1,241 (-8.12) b	18,645 (-19.11) e	1,083 (+7.98) ab
	Essential oil (0.1)	1,064 (-21.23) ef	17,905 (-22.32) e	943 (-6.01) ghi
	Liposomal essential oil (0.05)	1,071 (-20.71) ef	13,565 (-41.15) h	952 (-5.11) fgh
	Liposomal essential oil (0.075)	954 (-29.41) g	13,072 (-43.29) hi	912 (-9.07) i
Lemon	Liposomal essential oil (0.1)	849 (-37.17) h	12,120 (-47.42) i	920 (-8.32) hi
	Essential oil (0.05)	1,146 (-15.20) cd	21,413 (-0.1) bc	982 (-2.12) defg
	Essential oil (0.075)	1,118 (-17.22) cdef	21,847 (-5.22) ab	1,052 (+4.93) bc
	Essential oil (0.1)	1,076 (-20.32) def	20,906 (-9.3) bcd	1,102 (+9.84) a
	Liposomal essential oil (0.05)	1,070 (-20.78) ef	16,107 (-30.12) f	961 (-4.22) efgh
	Liposomal essential oil (0.075)	1,062 (-21.42) ef	16,273 (-29.4) f	1,007 (+0.41) cd
	Liposomal essential oil (0.1)	984 (-27.14) g	14,676 (-36.33) g	989 (-1.41) def

¹ Values between brackets = percentage of polyphenoloxidase activity decrease (-) or increase (+).

² Values in columns followed by the same letter are not significantly different at $P < 0.05$ (least significant difference).

Table 3. Content of peroxidase and polyphenoloxidase activities (units/min/g) in spinach coated with water, essential oils and liposomal oils (rosemary and lemon) as antioxidant (*in vivo*).^{1,2}

Antioxidant	Antioxidant agent concentration (mg/ml)	Enzymes activities in spinach	
		Peroxidase	Polyphenoloxidase
Water (control)	0	1,246 (0) a	1,201 (0) a
Rosemary	Essential oil (0.05)	1,030 (-17.31) efg	948 (-21.1) h
	Essential oil (0.075)	1,063 (-14.71) de	1,099 (-8.46) cd
	Essential oil (0.1)	1,007 (-19.2) g	991 (-17.51) g
	Liposomal essential oil (0.05)	871 (-30.1) hi	1,017 (-15.29) fg
	Liposomal essential oil (0.075)	817 (-34.42) i	872 (-27.36) i
Lemon	Liposomal essential oil (0.1)	882 (-29.18) h	815 (-32.12) j
	Essential oil (0.05)	1,112 (-10.72) cd	1,138 (-5.21) bc
	Essential oil (0.075)	1,138 (-8.63) bc	1,100 (-8.41) cd
	Essential oil (0.1)	1,177 (-5.51) b	1,150 (-4.23) b
	Liposomal essential oil (0.05)	1,019 (-18.18) fg	1,048 (-12.72) ef
	Liposomal essential oil (0.075)	1,054 (-15.4) ef	1,061 (-11.63) de
	Liposomal essential oil (0.1)	1,055 (-15.36) e	1,026 (-14.61) efg

¹ Values between brackets = percentage of peroxidase and polyphenoloxidase activities reduction (-).

² Values in columns followed by the same letter are not significantly different at $P < 0.05$ (least significant difference).

oils mainly depended on oil loading concentration per liposome. Liposomal encapsulation of essential oils at optimal formulation can result in an increased availability of oils and lead to elimination of enzymatic browning.

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