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Peroxidase activity in response to applying natural antioxidant of essential oils in some leafy vegetables

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Abstract

Based on the antioxidant property of essential oils, the effect of thyme, coriander and rosemary essential oils on reduction of peroxidase in celery (*Apium graveolens* var. *dulce*), spinach (*Spinacia oleracea* L.) and romaine lettuce (*Lactuca sativa* var. *longifolia*) were evaluated. For this reason, 50, 75, 100, 200 µl/100 ml and pure concentrations of essential oils were applied *in vivo* and *in vitro* on these leafy vegetables. The results revealed the antioxidant activity of essential oils provided significant differences among leafy vegetables ($P<0.01$). Peroxidase activity was affected by coriander concentrations and 200 µl/100ml concentration of rosemary essential oil (reductions in peroxidase activity >80%) in the *in vivo* condition, when the enzyme was obtained from celery. *In vivo* application of 200 µl/100ml of thyme and rosemary essential oils on the spinach provided 78 % and 81% antioxidant activity, respectively. *In vivo* 50 µl/100ml of thyme essential oil exerted the 73% antioxidant ability on the romaine lettuce.

Keywords: Peroxidase, Essential oils, Antioxidant, Celery, Spinach, Lettuce.

Introduction

In recent years, consumption of leafy vegetables has been increasing, especially in fresh diet for their nutritional value. For example, spinach (*Spinacia oleracea* L.) is one of the most important leafy green vegetables which contain large quantities of bioactive compounds and nutrients. Celery (*Apium graveolens* L.), on the other hand, is cultivated for its fresh-cut leaf-thickened stems (Saltveit and Mangrich, 1996). Lettuce (*lactuca sativa*) is one of the most preferred vegetables (Chen et al. 2010). Browning of the butt end cut is a limiting factor in the handling of many vegetables (Gomez and Artes, 2004; Zhang et al. 2005). Fresh-cut lettuce is very susceptible to enzymatic browning when being processed (Chen et al. 2010). For example, the antioxidant power of celery decreased after the first 7 days of storage (Vina and Chaves, 2006). Enzyme activity such as peroxidase (EC 1.11.1.7) caused enzymatic browning in fruit and vegetable tissues can cause undesirable quality changes (Nicoli et al., 1991; Nicoli et al., 1994; Ponce et al. 2004; Zhang et al. 2005). In lettuce, storing in a controlled atmosphere for 3 weeks, a remarkable increase in soluble peroxidase activity was noticed (Leja et al. 1996). Therefore, inactivation of the peroxidase enzyme is considered to be necessary to minimize the possibility of deterioration (Nicoli et al. 1991; Ponce et al. 2004). Inhibition of enzymatic browning in fruits and vegetables is generally achieved using physical or chemical treatments (Nicoli et al., 1994). Pre-cooled, and ascorbic acid treatment in celery (Johnson et al. 1974) and lettuce (Wang et al. 2004), high CO_2 controlled atmosphere (CA) over the postharvest behavior in celery (Gomez and Artes, 2004) and two packaging films (Rizzo and Muratore, 2009) were evaluated in fresh leafy vegetables for decrease of browning during storage. Indeed, ascorbic acid is an antioxidant that directly or indirectly sequesters harmful free radicals, which inhibit browning of celery (Vina and Chaves, 2006). On the other hand, the interest in the possible use of natural compounds has notably increased (Lanciotti et al., 2004). Indeed, the essential oils in crop protection are being increasingly recognized under the antimicrobial and antioxidant properties (Boyraz and Ozcan, 2005). Antioxidant properties of essential oils to reduce the enzymatic browning of fruit and vegetable and to conserve the quality have widely been studied (Lanciotti et al., 2004; Ponce et al., 2004; Alikhani et al, 2009). The effectiveness of essential oils was evaluated to reduce peroxidase activity of crude extract of leafy vegetables (Ponce et al., 2004). The antioxidant activities of edible coatings enriched with natural plant extracts such as rosemary were studied *in vitro* and *in vivo* by Ponce et al. (2008). The Chitosan did not exert any antioxidant effect when it was applied *in vitro* but when used *in vivo*, it enhanced its antioxidant properties over peroxidase (Ponce et al., 2008). Browning and processing of leafy vegetables may affect the different chemical components. Because the possible application of essential oils as natural antioxidant agents in fresh vegetables the may be a valuable alternative for preservation of physical or chemical compounds. The objective of the present work was to reduce peroxidase activity of celery, spinach and romaine lettuce with application of essential oils.

Materials and methods

Leafy vegetables as raw materials

The mature celery (*Apium graveolens* var. *dulce*), spinach (*Spinacia oleracea* L.) and romaine lettuce (*Lactuca sativa* var. *longifolia*) were used as raw materials for peroxidase

Table 1. ANOVA of antioxidant treatment on peroxidase activity of celery, spinach and lettuce.

S.O.V	df	Sum of squares (peroxidase activity)		
		Celery	Spinach	Lettuce
Stage (a)	1	21888.02**	31517104.69**	7634360.5**
Antioxidant (b)	2	6571.66**	9056606.67**	3963440**
Concentration (c)	4	37005**	13254771.67**	4342811.66**
a × b	2	83821.66**	818240*	1296446.66**
a × c	4	4705**	6857795**	3393575**
b × c	8	30020**	7643418.33**	2369243.3**
a ×b × c	8	47770**	5566085**	2152370**
error	99	23025	9522550	6332425
CV %		17.73	15.14	15.11

** (P<0.01)

assay. The vegetables were obtained from local markets during experimentation. Leaves with evident physiological damage were discarded.

Antioxidant application

For peroxidase assay, two types of antioxidant application were utilized: (1) Extract of crude vegetables prepared with no treatment then 0.03 ml of antioxidants solution (essential oils, ascorbic acid or water) were added to the extract (*in vitro* samples). (2) Crude vegetables immersed in antioxidant solution (essential oils, ascorbic acid or water) and was dried by exposure to room temperature (*in vivo* samples) and afterwards prepared for peroxidase measuring (Ponce et al., 2008).

Antioxidant agents

The essential oils utilized for antioxidant source were thyme (*Thymus vulgari*), coriander (*Coriandrum sativum*) and rosemary (*Rosmarinus officinalis*). Essential oils extracted with hydro-distillation by Clevenger. Essential oils were dissolved in ethanol 96% (v⁄v) and Tween 80, and then diluted with distilled water to attain pure concentration, 50, 75, 100 and 200 µl/100ml of essential oils. Essential oils concentrations were used based on minimum inhibitory concentration (MIC). (Ponce et al., 2003; Moreira et al., 2005).

Extracts preparing (Source of enzyme)

10 grams of leafy vegetables was chopped and then 30 ml of distilled water added during homogenation. The slurry was centrifuged (SIGMA-3K30) at 10000g for 15 min at 4°C. The supernatant, which contained peroxidase activity, was used as the enzyme source for the experiment (Ponce et al., 2004).

Substrate preparing

The substrate mixture contained 10 ml of 1% guaiacol, 10 ml of 0.3% hydrogen peroxide and 100 ml of 0.05M sodium phosphate (pH 6.5) buffer (Ponce et al., 2004).

Reaction cuvette

The reaction cuvette contained 2.87 ml substrate mixture, 0.1 ml crude extract, and 0.03 ml treatment solution (essential oils, ascorbic acid and water) in a total volume of 3 ml.

Determination of enzyme activity

Peroxidase activity was determined at 25°C with a spectrometer (PD-303UV) at 470 nm using guaiacol as the substrate and H_2O_2 as the hydrogen donor (Ponce et al., 2004).

Comparison of essential oils antioxidant activity

In order to make comparison of essential oils antioxidant activity, 0.03 ml deionized water as a control sample or ascorbic acid as a chemical control (0.017 g / 100 *g)* were added to reaction cuvette (Ponce et al., 2004).

Peroxidase activity measure

One unit of activity is defined as a change in absorbance of 0.001 min^{-1}.

Statistical analysis

For data collection, the experiment was established in factorial with 3 factors (antioxidant application, antioxidant source, concentration) using complete randomized design in 4 replications. Antioxidant application was defined 2 levels *in vitro* and *in vivo*. Antioxidant source subjected in 5 levels (Thyme, Coriander, Rosemary, Ascorbic acid and Water) and concentration supplemented in 7 levels (Pure, 50, 75, 100 and 200 µl/100ml for essential oils, 0.017 g/100 g for Ascorbic Acid and Pure concentration for water). The ANOVA was performed for analysis of the data obtained for each experiment and the means were done by least significant difference (LSD) test (P<0.01). Group comparison was used for analysis of these comparisons: C_1- Comparison applying thyme, coriander and rosemary essential oils with control. C_2- Comparison between applying thyme with coriander. C_4- Comparison between applying thyme with rosemary. C_3- Comparison between applying coriander with rosemary (Soltani, 2007). Data analyzed by SAS software (2001).

Results

Peroxidase content

Based on ANOVA (Table 1) and group comparison (Table 3), the effects of essential oils on the activity of peroxidase provided significant differences among the celery, spinach and lettuce (P<0.01). As shown in Table 1, the trilateral interaction of experimental factors (antioxidant applying stages × antioxidants kind × antioxidant concentrations) is significant in the celery, spinach and lettuce (P<0.01). Table

Table 2. Content of peroxidase (Units/min/gr) and percentage of peroxidase activity reduction (Numbers in bracket) in the presence of essential oils (thyme, coriander and rosemary) and ascorbic acid as antioxidant in celery.

stage	antioxidant	Concentrations (µl/100ml)	Celery	Spinach	Lettuce
In vivo	Thyme	50	120 (68) ^{e}	1925 (58) ^{ghij}	630 (73) ^{p}
		75	135 (64) ^{e}	1975 (57) ^{ghi}	1472.5 (36) ^{hijkl}
		100	180 (52) ^{bc}	1532.5 (66) ^{jklm}	800 (56) ^{op}
		200	185(51) ^{b}	990 (78) ^{o}	1378.5 (40) ^{ijklm}
		pure	75 (80) ^{fg}	1855 (59) ^{ghij}	1512.5 (34) ^{ghijkl}
	Coriander	50	60 (84) ^{fg}	1800 (60) ^{ghijk}	1232.5 (46) ^{klmn}
		75	60 (84) ^{fg}	2157.5 (52) ^{gh}	1017.5 (56) ^{mno}
		100	60 (84) ^{fg}	1365 (70) ^{klmn}	1135 (51) ^{lmno}
		200	60 (84) ^{fg}	1355 (70) ^{lmn}	1342.5 (42) ^{jklm}
		pure	60 (84) ^{fg}	1032.5 (77) ^{no}	1015 (56) ^{mno}
	Rosemary	50	90 (76) ^{f}	1092.5 (60) ^{no}	1912.5 (17) ^{cdefg}
		75	163(57) ^{cd}	1235 (73) ^{mno}	2087.5 (9) ^{bcd}
		100	90 (76) ^{f}	1172.5 (74) ^{mno}	1665 (28) ^{efghij}
		200	45 (88) ^{hi}	860 (81) ^{o}	1342.5 (42) ^{kljm}
		pure	30 (92) ^{i}	1105 (76) ^{mno}	1792.5 (22) ^{defgh}
	Ascorbic acid	0.017	60 (84) ^{fg}	1155 (75) ^{mno}	855 (63) ^{nop}
In vitro	Thyme	50	45 (88) ^{hi}	2625 (42) ^{f}	1770 (23) ^{defghi}
		75	30 (92) ^{i}	3457.5 (24) ^{b}	2152.5 (6) ^{bcd}
		100	30 (92) ^{i}	3230 (29) ^{bcd}	1935 (16) ^{cdef}
		200	30 (92) ^{i}	3005 (34) ^{cdef}	2052.5 (11) ^{bcde}
		pure	60 (84) ^{fg}	2667.5 (41) ^{ef}	1195 (48) ^{lmn}
	Coriander	50	90 (76) ^{f}	3365 (26) ^{bc}	1620 (30) ^{fghijk}
		75	90 (76) ^{f}	2927.5 (36) ^{def}	2380 (0) ^{ab}
		100	157.5 (58) ^{d}	3017.5 (34) ^{cdef}	2135 (7) ^{bcd}
		200	75 (80) ^{fg}	1735 (62) ^{hijkl}	1885 (18) ^{defg}
		pure	30 (92) ^{i}	1352.5 (70) ^{lmn}	1195 (48) ^{lmn}
	Rosemary	50	67.5 (82) ^{g}	3075 (32) ^{bcde}	1852.5 (19) ^{defgh}
		75	90 (76) ^{f}	1690 (63) ^{ijkl}	2650 (0) ^{mno}
		100	60 (84) ^{fg}	3097.5 (32) ^{bcde}	1890 (18) ^{defg}
		200	60 (84) ^{fg}	2177.5 (52) ^{g}	1922.5 (16) ^{cdef}
		pure	30 (92) ^{i}	1032.5 (77) ^{no}	1672.5 (27) ^{efghij}
	Ascorbic acid	0.017	45 (88) ^{hi}	1970 (57) ^{mghino}	1290 (44) ^{jmkl}
	Control	0	375 (0) ^{a}	4552.5 (0) ^{a}	2300 (0) ^{abc}
	LSD		21.39	435.14	309.3

Means followed by the same letter are not significantly different at 1% by LSD. Numbers in bracket: percentage of peroxidase activity reduction.

Table 3. Group comparison of applying essential oils as a peroxidase reducing agent in the celery, spinach and lettuce.

C	Treatment							Sum of squares		
	Thyme *in vivo*	Thyme *in vitro*	coriander *in vivo*	coriander *in vitro*	rosemary *in vivo*	rosemary *in vitro*	control	celery	spinach	lettuce
C_1	+1	+1	+1	+1	+1	+1	-6	6957.5*	10633900.1**	7018229.4**
C_2	+1	+1	-1	-1	0	0	0	292410**	26822250.6**	3614578.1**
C_3	+1	+1	0	0	-1	-1	0	290702**	21638410**	1148523.6**
C_4	0	0	+1	+1	-1	-1	0	5^{ns}	556111.25^{ns}	946125*

** (P<0.01), * (P<0.05), ^{ns} (P>0.05) C = comparison, C_1 = Comparison among applying thyme, coriander and rosemary essential oils with control. C_2 = Comparison between applying thyme with coriander. C_4= Comparison between applying thyme with rosemary. C_3= Comparison between applying coriander with rosemary.

2 shows that control of celery, spinach and lettuce has 375, 4552.5 and 2300 units/min/g peroxidase content, respectively. In celery, *in vivo* application of 100 and 200 µl/100ml concentrations of thyme had 180 and 185 units/min/g peroxidase content, respectively (Table 2). As results show, *in vitro* thyme and rosemary and *in vivo* coriander exerted the highest reduction of peroxidase activity on the celery (Table 2, Fig. 1). *In vivo* application of thyme, coriander and rosemary exhibited a grand ability to minimize the peroxidase content on the spinach (Table 2, Fig. 2). Group comparison results (Table 3) revealed the reduction of proxidase activity was significantly different among thyme, coriander and rosemary in the lettuce. The activity of the peroxidase obtained from *in vitro* romaine lettuce was not significantly affected by the application of 75 µl/100ml of coriander and rosemary essential oils (Table 2, Fig. 3). The romaine lettuce peroxidase activity was strongly influenced by *in vivo* thyme and coriander (table 2, Fig. 3).

Antioxidant activity

As shown in Table 2, the celery *in vivo* antioxidant activity was most affected by the pure and 200 µl/100ml concentrations of rosemary and coriander treatments and the

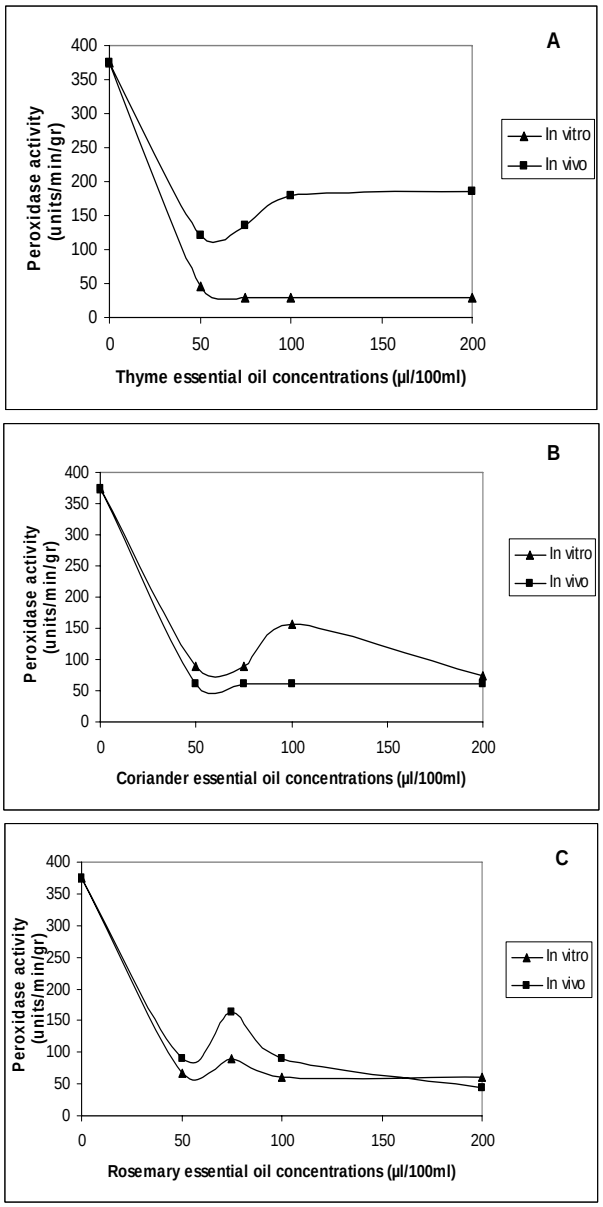

Fig 1. Change of peroxidase activity in presence of thyme (A), coriander (B) and rosemary (C) *in vitro* and *in vivo* application of essential oils in the celery ($LSD_{0.05}$ = 21.39).

reduction of peroxidase was attained >80 %. Peroxidase activity was affected by thyme and rosemary (except for 75 µl/100ml concentration) and pure concentration of coriander (reductions in peroxidase activity above 80 %) when the enzyme was obtained from *in vitro* celery. *In vitro* and *in vivo* pure concentration of essential oils exerted the grand antioxidant activity on the celery (P<0.01). as well as *in vitro* and *in vivo* applying of ascorbic acid showed a high antioxidant activity (>80 %) on the celery (Table 2). The spinach *in vitro* and *in vivo* peroxidase activity was most affected by pure concentration of coriander and the antioxidant activity was attained 70 % and 77 %, respectively. *In vivo* application of 200 µl/100ml of thyme (reductions in peroxidase activity 78 %) and rosemary (reductions in peroxidase activity 81 %) exerted the highest antioxidant activity on the spinach (Table 2). A great antioxidant activity was obtained under *in vivo* treatments of rosemary on the spinach (Table 2, Fig. 2). On the other hand, the spinach *in vivo* and *in vitro* antioxidant ability of ascorbic acid was attained 75 % and 57 %, respectively (Table 2). *In vivo* applying of 50 µl/100ml of thyme attained the highest antioxidant activity (73 %) on the romaine lettuce (Table 2). Likewise, the romaine lettuce *in vivo* antioxidant activity was most affected by pure, 75 and 100 µl/100ml concentrations of coriander and 100 µl/100ml concentration of thyme and the reduction of peroxidase was obtained >50 % (Table 2). The romaine lettuce *in vivo* and *in vitro* application of ascorbic acid provided 63 % and 44 % antioxidant activity, respectively (Table 2).

Discussion

Peroxidase content and antioxidant activity of essential oils

The results revealed the activity of the peroxidase obtained from crude celery, spinach and romaine lettuce was different. Hemeda and Klein (1990) and Ponce et al. (2004) also reported differences in Peroxidase activity of different crude vegetable extracts. The results revealed peroxidase content of celery is 375 units/min/g that is lower than spinach (4552.5 units/min/g) and lettuce (2300 units/min/g). Ponce et al. (2008) revealed that peroxides activity of romaine lettuce extract was 2.12 AUkg-1 ×106. The peroxidase activity was 1400-1800 (U/min/ml) in the fresh-cut asparagus lettuce (Chen et al. 2010). Ponce et al. (2004) found large differences between the peroxidase activities in leafy vegetables. They reported that cabbage had the highest peroxidase activity (29081.88 Units/min/g) in leafy vegetables. Our results showed, the antioxidant activity of essential oils provided significant differences among leafy vegetables peroxidase activity (P<0.01). Some reports concluded the significant difference between vegetables in essential oils and antioxidant activities (Ponce et al., 2004; Alikhani et al, 2009). The effectiveness of essential oils as natural antioxidants is also varied with the enzyme sources (Ponce et al., 2004). Hemeda and Klein (1990) revealed that differences in the peroxidase activity against antioxidant agents could be related to the presence of isoenzymes. In this research, *in vivo* application of thyme, coriander and rosemary was shown a grand ability to minimize the peroxidase content on the spinach. Ponce et al. (2008) were observed peroxidase inhibition during the first storage day of butternut squash when chitosan coatings enriched *in vivo* with rosemary. Peroxidase activity was affected by pure, 75 and 100 µl/100ml concentrations of coriander and 100 µl/100ml concentration of thyme (reductions in activity >50 %) when the enzyme was obtained from *in vivo* romaine lettuce. Ponce et al. (2004) concluded that action of the essential oils at 0.05 % and 0.075 % on butter lettuce extracts is an indication of a poor effect over the peroxidase activity. In addition, when the concentration of essential oil increased the peroxidase activity remained tended to decrease in the romaine lettuce and spinach extracts (Ponce et al., 2004). *In vivo* application of 50 µl/100ml of thyme on lettuce provided grand antioxidant activity. On the other hand, pure and high concentrations of thyme, coriander and rosemary essential oils had remarkable antioxidant power in the spinach with high peroxidase content (Table 2). When the concentration of essential oil increased the percent of peroxidase activity tended to decrease in the spinach extracts (Ponce et al., 2004). Alikhani et al. (2009) concluded that application of more than 75 and 100 µl thyme and rosemary essential oils will decrease the peroxidase activity in the squash and lettuce extracts. Therefore the vegetable with high peroxidase would present the great resistance to the action of all the essential

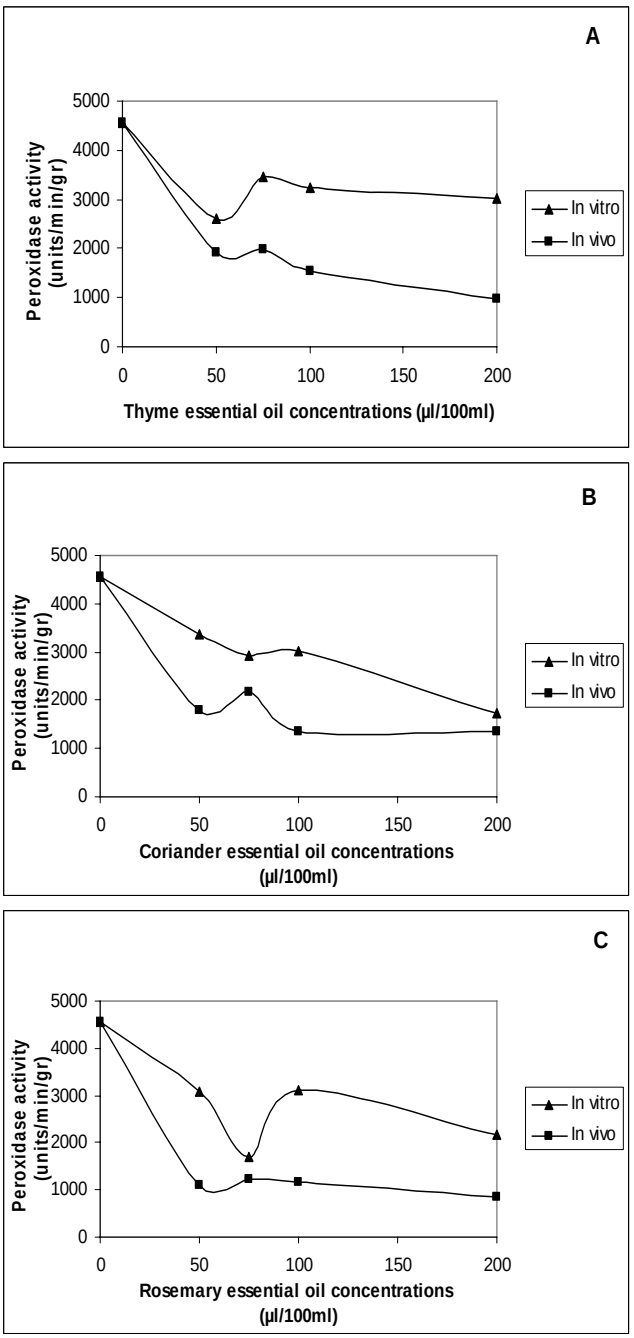

Fig 2. Change of peroxidase activity in presence of thyme (A), coriander (B) and rosemary (C) *in vitro* and *in vivo* application of essential oils in the spinach ($LSD_{0.05}$ = 435.14)

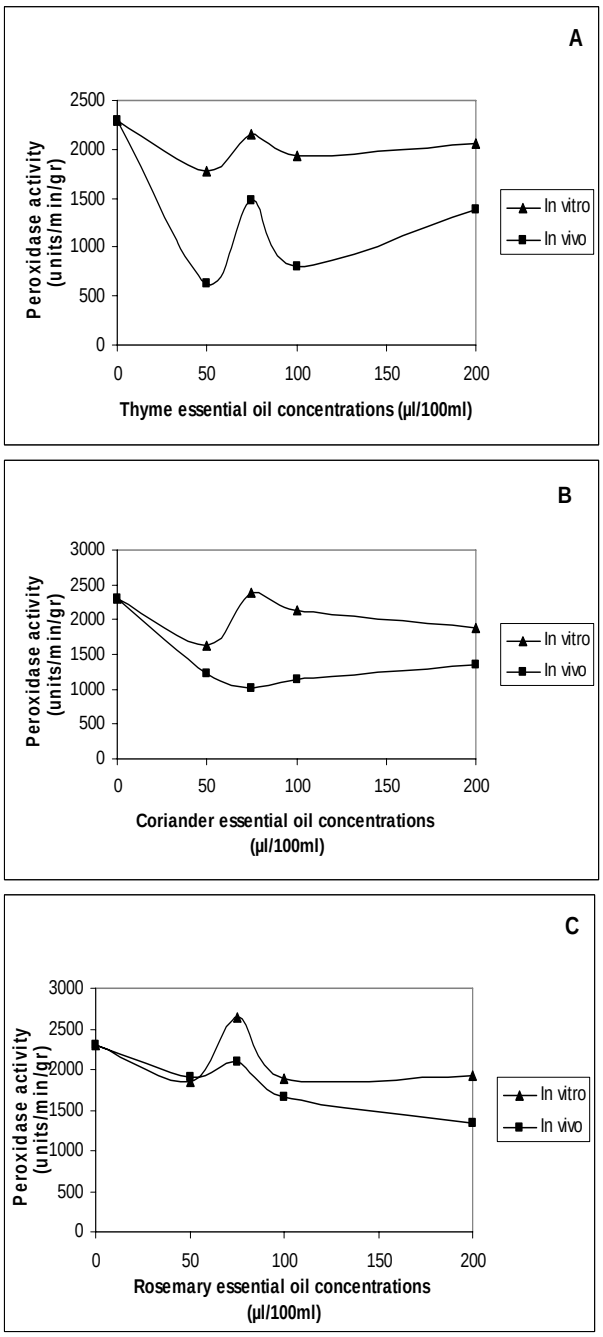

Fig 3. Change of peroxidase activity in presence of thyme (A), coriander (B) and rosemary (C) *in vitro* and *in vivo* application of essential oils in the lettuce ($LSD_{0.05}$ = 309.3).

oils assayed (Ponce et al., 2004). *In vivo* using of essential oils applied to lettuce and spinach improved the antioxidant activity. So, *in vivo* treatments were more effective than *in vitro* treatments on the lettuce and spinach (Fig. 2 and 3). Ponce et al. (2008) reported no antioxidant effects on *in vitro* studies over the peroxidase. However, in *in vivo* studies the antioxidant properties enhanced over peroxidase enzyme.

Ascorbic acid

The antioxidant activity of essential oils was assessed in presence of ascorbic acid. The results revealed that *in vitro* and *in vivo* application of ascorbic acid decreased the peroxidase activity. *In vivo* and *in vitro* ascorbic acid exerted the highest reduction of peroxidase activity on the celery, in which the antioxidant activity was reached to 84% and 88%, respectively. *In vivo* and *in vitro* peroxidase activity was affected by ascorbic acid (reductions in activity 75% and 57%, respectively) when the enzyme was obtained from spinach. The peroxidase activity in the *in vivo* and *in vitro* romaine lettuce was minimized with application of ascorbic acid, in which the reduction of peroxidase was obtained 63% and 44%, respectively (Table 2). Ponce et al., (2004) concluded that the reductions of peroxidase activity in vegetable extracts was 87% in spinach, 74% in butter lettuce, 56% in romaine lettuce and 35% in chard and cabbage. Talano et al. (2008) found that peroxidase activity was inhibited by ascorbic acid. Ascorbic acid plays an essential

role in capturing hydrogen peroxide and protects thiol groups of enzymes and proteins from oxidation (Vina and Chaves, 2006). Zhang et al., (2005) illustrated a significant decrease in vitamin C content of fresh-cut celery during the storage for 9 days at 4°C. So, addition of ascorbic acid decreased the rate of browning development on cut surfaces, and was effective in extending the refrigerated shelf life of celery (Johnson et al., 1974). Wang et al. (2004) reported that in a solution containing 0.05 % (w/w) ascorbic acid, fresh-cut asparagus lettuce could retain color for 5 days at 4°C. This point is noticeable that unlike ascorbic acid, the essential oils affect as powerful antioxidants with some antimicrobial properties as well. Lanciotti et al. (2004) stated that application of natural essential oils could inhibit the microbial activity in fresh fruits crops.

Conclusion

The use of essential oils applied to celery, spinach and lettuce improved the antioxidant protection of the leafy vegetables, offering a great advantage in the prevention of peroxidase activity. Because of the peroxidase content of celery (375 units/min/g), spinach (4552.5 units/min/g) and lettuce (2300 units/min/g), essential oils and ascorbic acid have excellent ability to minimize the peroxidase activity. Comparison of *in vitro* and *in vivo* application of essential oils showed different behaviors in antioxidant properties. In spinach and lettuce, *in vivo* application exerts essential oils over peroxidase. In the celery, thyme and rosemary when applied *in vitro*, and coriander, when applied *in vivo*, provided effective antioxidant activity. However, *in vivo* application of thyme, coriander and rosemary essential oils as coating or direct apply to spinach and lettuce, and also *in vivo* oil of coriander to celery, enhanced the antioxidant properties over peroxidase. This result indicates that as respects to antioxidants properties of essential oils, this natural compound, especially as a coating, could be potentially used to minimize the peroxidase activity and enzymatic browning in the celery, spinach and lettuce.

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