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ANTI-MICROBIAL, ANTI-BROWNING AND ANTI-MYCOTOXIGENIC ACTIVITIES OF SOME ESSENTIAL OIL EXTRACTS IN APPLE JUICE

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Five essential oils (EO) extracted from lemon grass (*Cymbopogon citratus*), basil (*Ocimum basilicum*), rosemary (*Rosmariinus officinalis*), sage (*Salvia officinalis*), and clove (*Eugenia aromatica*), were investigated for their inhibitory effect against polyphenoloxidase (PPO) enzymatic browning, microbial activity, as well as effect on food spoilage and mycotoxin producing fungi, *Aspergillus flavus* and *Aspergillus dchraceus*. The TLC technique was used to determine the inhibitory effect of each EO on the radial growth of the fungus, and a dose response of the EO was recorded. Results showed that the EO from lemon grass, clove and rosemary were the most effective and prevented the growth and mycotoxin formation of the two fungi on apple juice when applied at doses of 0.05, 0.2 and 0.3%, respectively. Moderate activity was observed for the EO from basil dose between 0.2% and 0.3%, while the EO from sage was less inhibitory. These effects against food spoilage and mycotoxin producing fungi indicated each essential oil to be a potential food preservative. Also, the results showed that apple juices treated with essential oil (EO) extract from lemon grass, clove and rosemary had a positive effect towards the inhibition of PPO activity and reducing browning as compared to untreated, basil and sage treated juices, at room temperature (25°C) and at refrigerator (4°C), then increased shelf life of apple juice up to 4 weeks. The lowest microbial count for 4 weeks of storage period at 4°C was observed during the pretreatment of apple juice with lemon grass, clove and rosemary extracts. Therefore lemon grass, clove and rosemary extracts used in the study proved to be efficient extractives against food spoilage and mycotoxin producing fungi and in reducing both the enzymatic browning (PPO) and microbial counts during the preservation of apple juice by refrigeration at 4°C, indicating the potential applicability of each essential oil as a food preservative.

INTRODUCTION

Fruits and their products are preserved by different methods to inactivate degradation enzymes and kill spoilage microorganisms. Most recent reviews have concentrated on technology for the improvement of fruit and their products quality without adding chemicals, without affecting their nutritional value and safety of products [Ejechi *et al*., 1998; Eissa *et al*., 2003a, b]. Fresh apple juice is the most unstable fruit juice from both the chemical and microbiological point of view. Consequently, certain types of apple juice that are available on the market largely reflect the preservation techniques that have been used for their production. Pure apple juice is a colorless and virtually odorless liquid. Within seconds of its expression from the fruit, however, it undergoes a sequence of enzymatic changes to produce the color and the aroma which we are familiar with [Spanos & Wrolstad, 1992; El-Assi *et al.,* 1997].

The enzymatic browning reaction is the most severe when the structure of food has been altered or damaged by processing, and is usually an undesirable phenomenon that results in a decreased value and reduced acceptance by consumers. For this reason, extensive research has been done on ways to prevent or control enzymatic browning. Mold growth leads to the production of unacceptable filamentous structures, off-flavors and in certain cases, mycotoxins. The low pH of apple products restricts the growth of a wide variety of microorganisms. Only yeasts, molds and lactic acid bacteria are capable of prolific growth in apple products. Growth of yeast may lead to the production of off-flavors, turbidity, alcohol, and gas in processed apple products.

The raw juice can be protected from microbiological degradation for a few days by storage in a refrigerator, or may be protected indefinitely by pasteurization or by the use of permitted preservatives. Such juice is nearly always turbid, brown in colour and tends to sediment on storage [Lea, 1994]. While application of conventional chemical preservatives such as benzoic and sorbic acids and sulfite to fruit juices is an alternative, the practice is not common as indeed such preserved fruit juices and the chemicals are often imported. For developing countries, preservation should be inexpensive and simple but reliable [Leistner, 1994].

Since ancient times, people have used herbs and spices for preventing food deterioration and food borne diseases. At the end of the last century, antimicrobial and anti-browning activities of the herbs and spices had already been examined and their oils were known to retard microbial spoilage and inhibit enzymatic browning in food products. In recent years,

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there are growing interests in using natural antimicrobial and anti-browning compounds, especially those extracted from plants, for the preservation of food products [Dorantes *et al.*, 2000; Vijaya *et al.*, 2001; Nielsen & Rios, 2000; Panuwat *et al.*, 2003; Eissa *et al.,* 2003a, b; Abdel-Fattah & Abo-Seree, 2004]. On the other hand, the species and herbs give a good flavour and musk the undesirable flavours.

Therefore, this work was aimed at investigating apple juice with satisfactory properties obtained by the addition of volatile or essential oil extracts of lemon grass, clove, rosemary, basil and sage. At the same time the antifungal activity of these additives and their effect on PPO activity, enzymatic browning and microbiological properties of the resultant apple juice during storage at room temperature and at 4 °C were studied as compared to untreated apple juice (control). Also, the study focused on developing a technology to produce high quality and long shelf life apple juice with controlled enzymatic browning and delayed spoilage.

MATERIALS AND METHODS

Mycotoxins standard

Aflatoxins B_1, B_2 and G_1, G_2 and ochratoxin A were obtained from Sigma Chemical Co. St. Louis MO, USA.

Organisms and spore suspension

Aspergillus flavus (aflatoxigenic local strain) and *A. ochraceus NRRL 3174* were obtained as a lyophilized preparation from the Mycotoxin Lab., National Research Center, Dokki, Giza, Egypt. These culture strains were grown on (PDA) slants for 10 days at 25°C until sporulation. The spores were washed from the slants with a sterile 0.01% solution of Tween 80 as a spore dispersing agent. The final spore preparations were resuspended in the appropriate volume of sterile saline to yield a direct microscopic count of approximately 105--106 spores/mL, of each tested fungus.

Plant materials and essential oil extract preparations

Powder from lemon grass (*Cymbopogon citratus*), basil (*Ocimum basilicum*), rosemary (*Rosmariinus officinalis*), sage (*Salvia officinalis*), and clove (*Eugenia aromatica*) was obtained from a local market and the essential oils (EO) tested were extracted by the hydrodistillation method using a Clevenger's apparatus [Lamaty *et al.*, 1987]. The recovered oils were dried over anhydrous sodium sulphate and stored in darkness at 4°C. The yield of the essential oils as percent of plant material weight was as follows: 0.57%, 6.2%, 0.42%, 0.36% and 0.50% for the EO from *C. citratus, M. myristica, O. gratissimum, T. vulgaris* and *Z. officinale*, respectively. For each, the filtrate was used as the test extract.

Preparation of juice samples

Apple *(Red delicious)* samples representing common cultivars were obtained from local food stores during the fall and winter of 2006 and stored briefly at 4°C until needed. One hour prior to use, the fruits were taken out of the refrigerator and equilibrated to room temperature. Apple fruits were rinsed with water and sectioned to longitudinal slices. Apple juice samples were prepared from individual apples with a juice extractor. Juice was collected in a beaker containing 5 mg ascorbic acid/100 mL juice with stirring. The amount of ascorbic acid used was not enough to prevent browning for more than 1 h. However, the ascorbic acid was used to prevent instantaneous browning, thereby providing a short lag time to allow test materials (volatile or essential oil extracts) to be added and mixed [Sapers & Douglas, 1987].

Antifungal activity testing

Apple juice was used as a basal medium in this study. Fifty millimeter of apple juice was dispensed into each of a series of 250-mL Erlenmeyer flasks. The medium was pasteurized at 80°C for 15 min and the cooled. Next, 0.0, 0.05, 0.20 or 0.30% of each plant extract was added. Then, the medium was inoculated with 1 mL of the appropriate spore suspension and incubated for 45 days at 25±2°C. Each assay was performed by triplication.

Mycotoxin analysis

Quantitative determination of aflatoxins B_1 and G_1 was performed on silica gel D.G-plates according to the A.O.A.C. methods [1995]. Aflatoxin B_1, B_2, G_1 and G_2 were qualitatively identified on 20 x 20 cm thin layer chromatographic plates (0.5 mm thickness) by comparing the R_f values with those of standards (10 μg/mL of each) dissolved in benzene:acetonitrile (98:2, v/v). Crude aflatoxin(s) (0.2 mL) were applied in a line approximately 6 cm wide across a chromatoplate, 3 cm from the bottom, and developed in a trichloroethylene – chloroform – methanol (8+1+1) mixture. This solvent system cleanly separates aflatoxin(s) B_1 and B_2 as one band (R_f=0.5) and aflatoxin(s) G_1 and G_2 as another band (R_f =0.4). Aflatoxin(s) were located by viewing under UV light (365 nm) and aflatoxin concentration in each band was determined by means of fluorodensitometry.

Ochratoxin A was estimated qualitatively as above by thin layer chromatography (TLC). A plate was developed with solvent: toluene: ethyl acetate: formic acid (8:2:0.5) and confirmed with ammonia foams. After plate development, it was examined under long wave UV light to confirm the presence or absence of ochratoxin A, once mycotoxin was present, it was marked with a dot at this position, then standards were scanned in a usual manner; sample spot position was noted in relation to marking, and scanned flourodesitometry was conducted according to the A.O.A.C. methods [1995].

Evaluation of capacity of microbiological growth in juice

Various concentrations of volatile or essential oil spice extracts (0, 0.05, 0.2, 0.3%, v/v) were added to fresh apple juice in glass bottles. The treated and untreated samples were incubated for 4 weeks at 30°C and analysed for changes in yeast or bacterial populations by plating every two weeks on malt extract agar and plate count agar, respectively. The changes were expressed as density of growth for six replicates, based on the initial population of organisms [Ejechi *et al.,* 1998].

Effect of essential oil extracts on microbiological activity in refrigerated juice

The untreated and 1% of volatile or essential oil extracts treated juice was stored for 4 weeks at 4°C and subjected

to microbiological analyses every week. The populations of total bacteria, yeast and molds were determined by the method of Sadler *et al.* [1992]. Untreated and treated juice samples were serially diluted with 0.1% peptone (DIFCO Labs. Detroit, MI) and pour-plated in duplicate. Total plate counts (TPC) were determined as follows: one mL of aliquot of each dilution was plated using a total plate count agar medium (Merck KGaA, Darmstadt, Germany), and incubated at 35-37°C for 48 h to counting. Also, yeast and mold counts (Y and M) were obtained using malt extract agar (Merck KGaA, Darmstadt, Germany) and incubated at 25°C for 3 days prior to counting. The number of colonies (TPC or Y and M) that appeared on the plates was counted and expressed as Colony Forming Unit per mL of juice (CFU/mL juice).

Evaluation of capacity of browning in juice and colour assessment

Portions of volatile or essential oil extracts treated juices and untreated control (25 mL of juice in 50-mL beakers containing magnetic stirrer bars, covered to prevent evaporation) were magnetically stirred at 4000 rpm for as long as 24 h at room temperature on a stirrer to accelerate enzymatic browning if it were to occur. Juices were held at room temperature with stirring during which time tristimulus reflectance L*-, a*- and b*-values for controls and treated juices were periodically measured during 24 hs, using a spectrocolorimeter (Tristimulus Colour Machine) with the CIE lab colour scale (Hunter, Lab Scan XE – Reston VA, USA) in the reflection mode. The instrument was standardized each time with White Tile of Hunter Lab Color Standard (LX No.16379): X= 72.26, Y= 81.94 and Z= 88.14(L*= 92.46; a*= -0.86; b*= -0.16) [Sapers & Douglas, 1987].

Assay of polyphenoloxidase (PPO) activity

Extraction of polyphenoloxidase (PPO) was carried out using the method of Galeazi *et al.* [1981] and its activity was determined spectrophotometrically according to the methods of Sun & Song [2003] and Oktay *et al.* [1995] using 2 mL of catechol 0.1 mol/L as a substrate. The reaction was initiated by adding 1 mL of the polyphenoloxidase extract at 25 °C. Absorbance was measured at 420 nm in 30 s intervals for 3 min using a 4054 UV/ visible spectrophotometer (LKB--Biochrom Comp., London, England). One unit of PPO activity was defined as an increase in the absorbance at 420 nm per minute [Sun & Song, 2003]. Polyphenoloxidase (PPO) activity was reported in arbitrary units (unit per mL).

Statistical analysis

Significant differences between treatments and strains sensitivity were analysed using MSTAT-C statistical software, version 1.3 [MSTAT-C, 1990]. The Least Significant Difference (LSD) was used for mean separation tests at 5% significance level.

RESULTS AND DISCUSSION

Antifungal activity of the essential oil extract from lemon grass, basil, sage, clove and rosemary on mycotoxin production by *A. flavus* and *A. ochraceus*

In regard to results in Table 1, the essential oils under investigation, except sage oil, showed considerable antifungal activity against growth and aflatoxin production by *A. flavus* (Table 1). No aflatoxin was detected even at a low level of lemon grass, basil, clove and rosemary oils (0.05%). On the other hand, sage oil showed a weak inhibition level against growth and aflatoxin production. Results in Table 2 revealed that sage and basil oils were comparatively different in their action against *A. ochraceus*. The inhibitory action by these oils against growth and ochratoxin A formation was found at concentration of 0.05% and increased as the concentration in the medium increased to reach the maximum at 0.3%. But lemon grass, clove and rosemary oils completely inhibited

TABLE 1. Effect of the different essential oil extracts on aflatoxin production by *A. flavus* in apple juice.

Extract level (%)	Aflatoxin production (μg/50 mL apple juice)				
	Lemon grass	Basil	Sage	Clove	Rosemary
Control, 0.0%	$880^{Aa} \pm 14.7$	$880^{Aa} \pm 14.7$	$880^{Aa} \pm 14.7$	$880^{Aa} \pm 14.7$	$880^{Aa} \pm 14.7$
0.05	$0.00^{Ba} \pm 0.00$	$0.00^{Ba} \pm 0.00$	$1050^{Bb} \pm 18.6$	$0.00^{Ba} \pm 0.0$	$0.00^{Ba} \pm 0.00$
0.2	$0.00^{Ba} \pm 0.00$	$0.00^{Ba} \pm 0.00$	$905^{Cb} \pm 23.5$	$0.00^{Ba} \pm 0.0$	$0.00^{Ba} \pm 0.00$
0.3	$0.00^{Ba} \pm 0.00$	$0.00^{Ba} \pm 0.00$	$915^{Cb} \pm 17.0$	$0.00^{Ba} \pm 0.0$	$0.00^{Ba} \pm 0.00$
LSD (5%)			47.9		

A,B – different capital litters in the column denote significant differences (p≤5%) between means in the same extract and *vice versa*; a,b – the same small litters in rows denote no significant differences (p≤5%) between extracts and *vice versa*.

TABLE 2. Effect of the different essential oil extracts on ochratoxin A production by *A. ochraceus* in apple juice.

Extract level (%)	Ochratoxin A- production (mg / 50 mL apple juice)				
	Lemon grass	Basil	Sage	Clove	Rosemary
Control, 0.0%	$2090^{Aa} \pm 33.4$	$2090^{Aa} \pm 33.4$	$2090^{Aa} \pm 33.4$	$2090^{Aa} \pm 33.4$	$2090^{Aa} \pm 33.4$
0.05	$0.00^{Ba} \pm 0.00$	$2040^{Ab} \pm 38.5$	$3110^{Bc} \pm 45.1$	$0.00^{Ba} \pm 0.00$	$0.00^{Ba} \pm 0.00$
0.2	$0.00^{Ba} \pm 0.00$	$965^{Cb} \pm 18.6$	$3125^{Bc} \pm 72.0$	$0.00^{Ba} \pm 0.00$	$0.00^{Ba} \pm 0.00$
0.3	$0.00^{Ca} \pm 0.00$	$910^{Cb} \pm 23.4$	$3095^{Bc} \pm 29.5$	$0.00^{Ba} \pm 0.00$	$0.00^{Ba} \pm 0.00$
LSD (5%)			103		

A,B – different capital litters in column denote significant differences (p≤5%) between means in the same extract and *vice versa*; a,b – the same small litters in rows denote no significant differences (p≤5%) between extracts and *vice versa*.

the growth and ochratoxin formation. Our results are contrary with those observed on sage oil, by Basilico & Basilico [1999] and by Sokovic *et al.* [2002]. Fluctuation in the results may be attributed to differences in the extract used depending on the preparation method employed [Lienert *et al.,* 1998; Vilegs *et al.,* 1997].

The antifungal activity of the tested essential oils could be due to phenolic, alcoholic and aldehydic contents which were reported as antimicrobial substances [Variyar *et al.,* 1998; Sacchetti *et al.,*2005]. As known literatures about antifungal properties of sage, rosemary and ginger were still rare, whereas their inhibitory action observed might be associated with the phenolic constituents, which are namely phenolic di-terpenes.

In this respect, Ho *et al.* [2000] reported that alcohol extracts of rosemary and sage contain active antioxidative factors such as phenolic di-terpenes, flavonoids and phenolic acids. On the other hand, Campo *et al.* [2000] found that antimicrobial activity of rosemary extract was linked to the compounds extracted with hexane, which are presumably phenolic di-terpenes.

Effect of essential oil extracts on microbial content in incubated juice (30°C)

The microbial stability of apple juice supplemented with volatile oil extracts of lemon grass, clove, rosemary, basil and sage during storage at an incubator (30°C) for 4 weeks was investigated. The ability of various volatile oil extracts with different concentrations to control the endogenous micro flora in apple juice can be seen in Tables 3 and 4.

The results showed that lemon grass, clove and rosemary extracts were effective at 0.3% having prevented growth in the yeast and bacteria populations whilst basil and sage extracts had weaker activity in inhibiting the microbial population. Results obtained showed a decreased activity of volatile oil extracts of lower concentration, these results were in agreement with findings of Aureli *et al.* [1992] and Eissa *et al.* [2003a]. Supplementing apple juice with an aqueous extract at a concentration of 0.3% inhibited the growth of microorganisms, but 0.2 and 0.3% extracts yielded a product with un unacceptable taste; while the concentration of 0.05% essential oil extracts produced an apple juice with acceptable taste and caused a marked reduction in yeast and bacteria population. For the purpose of maintaining an acceptable degree of freshness or sensory value and reduction in microbial population 0.05% of volatile oil extracts were used. Ejechi *et al.* [1998] demonstrated that the concentration of volatile oil extracts alone needed to inhibit microbial growth was high. Such high concentrations may alter the sensory value or freshness of fruit juices. The results of Nakatani [1994] showed that ginger contains pungent components such as zinger one, gingerroot, and shogaol and nutmeg contains myristicin and sabinen that possess antimicrobial activity.

Effect of essential oil extracts on enzymatic browning in juice stored at room temperature and refrigeration

Raw apple juice might represent a more useful system than the cut surface of plugs for the comparison of multilevel treatments to inhibit browning since it would be homogeneous and more easily manipulated. However, preliminary experiments with five kinds of volatile oil extracts (lemon grass, clove, rosemary, basil and sage) on apple juice (*Red Delicious*) indicated that browning in the freshly prepared juice

TABLE 3. Effect of essential oil extracts on the growth of yeast and molds in apple juice during storage at incubator (30°C) for 4 weeks.

Essential oils	Addition of essential oils extract in respective storage weeks							
	0.00%		0.05%		0.2%		0.3%	
	2weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks
Lemon grass	+5	+7	+4	+5	+1	+1	-	-
Clove	+5	+7	+4	+5	+1	+1	-	-
Basil	+5	+7	+7	+6	+3	+4	+2	+3
Rosemary	+5	+7	+4	+5	+1	+1	-	-
Sage	+5	+7	+4	+7	+3	+5	+2	+3

(+7)= very high population, (+1)= very low population, (-)= no microorganisms detected.

TABLE 4. Effect of essential oil extracts on the growth of bacteria in apple juice during storage at incubator (30°C) for 4 weeks.

Essential oils	Addition of essential oils extract in respective storage weeks							
	0.00%		0.05%		0.2%		0.3%	
	2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks
Lemon grass	+5	+7	+2	+4	+1	+1	-	-
Clove	+5	+7	+2	+4	+1	+1	-	-
Basil	+5	+7	+5	+6	+3	+4	+2	+3
Rosemary	+5	+7	+3	+4	+1	+1	-	-
Sage	+5	+7	+5	+7	+3	+4	+3	+4

(+7)= very high population, (+1)= very low population, (-)= no microorganisms detected.

occurred too rapidly to permit sample treatment and evaluation. Reflectance measurements and a*-values were increased in the browning juices (Figure 1). Browning of the apple juices was measured by a* (green-red). An increase in a*-value is indicative of browning [Monsalve-Gonzales *et al.,* 1993]. No heat treatment was given to the apple juices; thus enzymatic activity of polyphenoloxidase was assumed. The results that show the effect of treating apple juices with the different volatile oil extracts as anti-browning agents and storage at room temperature (25°C) for 24 h and for 4 weeks at 4°C on inhibiting the browning reactions are graphically represented in Figures 1 and 2. These figures illustrate the changes in the colour of apple juices in terms of a*-values over 24 h after adding lemon grass, clove, rosemary, basil and sage volatile oil extracts at a concentration of 0.05% for each one. Also, the colour a*-values were recorded for the untreated apple juice over 24 h directly after preparing.

It can be observed that the apple juices treated with lemon grass volatile oil extract have no browning or the lowest a*-value (<3.98) followed by those treated with clove extract (<3.99) then with rosemary (<6.76) after 24-h storage at room temperature (25°C); while browning or a*-value of basil, sage and untreated apple juices was too high (>7.13) after 24-h storage at room temperature (25°C). On other hand, both lemon grass and clove extracts are considered to be anti-browning agents than other volatile or essential oil extract, in controlling enzymatic browning reactions, as seen in Figure 1.

Furthermore, for long-term storage at 4°C, the results showed that the apple juices treated with lemon grass extract have no browning or the lowest a*-value (<4.3) followed by those treated with clove and rosemary extracts (<5.28) then with basil and sage extracts (>8.18) after 4 weeks of storage at refrigeration (4°C); while the a*-value of untreated apple juices was too high (2.6-10.77) after 4 weeks of storage at refrigeration (4°C), as seen in Figure 2. The results showed that application of a browning inhibitor solution containing lemon grass, clove and rosemary extracts could control the enzymatic browning of apple juices. The use of these treatments, especially lemon grass, clove and rosemary and refrigeration at 4°C for 4 weeks constitutes an effective method of quality improvement and shelf life extension. However, refrigerating and 0.05% volatile or essential oil extracts treatments caused inhibition of enzymatic browning up to 4 weeks at 4°C and preservation of the quality of apple juice. These results are consistent with the results of Eissa *et al.* [2003a] and Ejechi

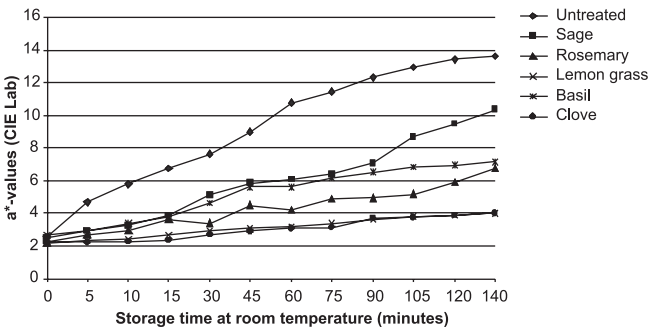

FIGURE 1. Effect of some essential oil extracts on enzymatic browning (a*-value) in apple juice during storage at room temperature (25°C).

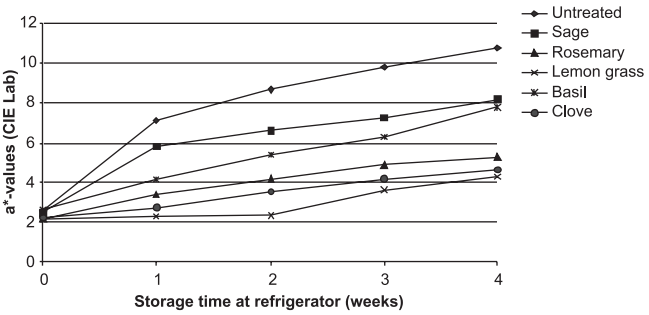

FIGURE 2. Effects of some essential oil extracts on enzymatic browning (a*-value) in apple juice during storage at refrigeration (4°C).

et al. [1998] who revealed that the tropical spices might prove useful in preservation of fruit juices by hurdle technology.

The percentage inhibition of polyphenoloxidase (PPO) activity in apple juice treated with lemon grass, clove, rosemary, basil and sage after storage at refrigeration (4°C) for 4 weeks were 92.65, 91.05, 78.86, 56.48 and 22.81%, respectively (Figure 3). These results indicate that addition of volatile or essential oil extracts to apple juice inhibited polyphenoloxidase (PPO) activity, therefore, the volatile oil of all treatments were higher than those of the control apple juice throughout storage periods at 4 °C.

The most effective volatile or essential oil extracts pretreatments on enzymatic browning (a*-value) were lemon grass and clove in apple juices during storage at room temperature (25°C) for 24 h, as well as lemon grass, clove and rosemary in apple juices during storage at 4°C for 4 weeks, as seen in Figures 1 and 2. These results indicted that the volatile or essential oil extracts treatment inhibited browning of refrigerated apple juices as compared with that of the untreated samples. These results nearly[consistent with results given by Eissa *et al.* [2003a, b], Vijay-Sethi [1991] and Monsalve-Gonzales *et al.* [1993] who proved the effectiveness of some spices and 4-hexylresorcinol as anti-browning agents in tomato juice and apple slices, respectively. Results from Figure 2 showed that refrigeration temperature of 4°C could enhance the inhibitory effect of lemon grass, clove and rosemary but not of basil and sage.

The use of volatile or essential oil extracts as a preservative may be due to the fact that they contain aldehydes and

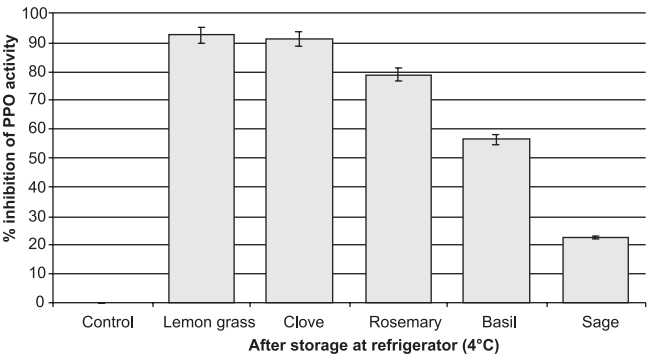

FIGURE 3. Effects of some essential oil extracts on% inhibition of polyphenoloxidase (PPO) in apple juice after storage at refrigeration (4°C).

volatile compounds that have efficient on the inhibition of enzymatic browning and growth microorganisms. These results were confirmed by the findings of Nakatani [1994] who found that the essential oil of spices like eugenol and isoeugenol in clove and other phenolic volatile components exhibited appreciable effect but they have too strong characteristic odors to be used as food additives.

Effect of essential oil extracts on microbial content in refrigerated juice

The microbial stability of apple juice supplemented with extracts of lemon grass, clove, rosemary, basil and sage during storage at refrigeration (4°C) for 4 weeks was investigated. Tropical spices may prove useful in preservation of fruit juices by hurdle technology [Ejechi *et al.*, 1998]. Total microbial count of different apple juices treatments with 0.05% of lemon grass, clove, rosemary, basil and sage volatile oil extracts, and of untreated apple juices were followed up through 4 weeks at 4°C. The effects of treating apple juices with the studied various volatile or essential oil extracts and storage at 4°C for 4 weeks on inhibiting the microbial counts are seen in Tables 5 and 6. It can be observed that the apple juices treated with lemon grass, clove and rosemary extracts have the highest inhibition of yeast and molds (Y and M) and bacteria (B) followed by those treated with basil and sage extracts after 4 weeks at 4°C. The microbial count of untreated apple juices was 10.19 log (CFU / mL) of B and 11.0 log (CFU / mL) in case of Y and M. Whereas, the microbial count of apple juices pre-treated with sage extract was 10.4 log (CFU / mL) of B and 10.0 log (CFU / mL) of Y and M.

The results from Tables 5 and 6 showed that the apple juices treated with lemon grass, clove and rosemary have also the highest reduction of Y and M and B followed by basil and sage, but untreated samples were characterised by the lowest reduction of Y and M and B for 4 weeks of storage at 4°C. These results are partially confirmed by those of Kanako *et al.* [1998]. They found that lemon grass and clove exhibited strong anti-fungal activity for 30 days. Whereas no colonies were seen for 30 days and fungal growth was inhibited for more than 30 days. On the other hand, Sebti & Tantaoui-Elaraki [1994] showed that cinnamon powder although very efficient at inhibiting the fungi, imported a dark colour to the papers and therefore is not recommended. While, cinnamon water extract did not inhibit fungal growth up to concentration of 80 g/kg (8%). Also, results from Tables 5 and 6 showed that refrigeration temperature of 4°C of apple juice could enhance the inhibitory effect of volatile or essential oil extracts. These results are nearly consistent with results given by Eissa *et al.* [2003a, b] and Ting & Deibel [1992] who demonstrated that refrigeration temperature (4°C) could enhance the inhibitory effect of sage but not of cloves or oregano. When 0.5 or 1.0% of cloves were tested, the organism died more rapidly in tryptic soy broth at 24°C than at 4°C. Whereas, >5 log reduction in CFU was observed after 7 days of incubation at 4°C and after 3-h incubation at 24°C in tryptic soy broth.

In general, the refrigeration of apple juices increased the inhibition of bacteria, yeast and mold counts. Also, the results showed no browning and the lowest microbial count (Y and M, and B) in apple juices pre-treated with lemon grass and clove extract during storage at 4°C for 4 weeks. Nakatani [1994] proposed that the inhibitory mechanism for the anti-fungal action of the aldehydes was due to the ability to form charge transfer complexes with electron donors and reactivity with SH group in cystein or glutathione moieties. Shelef [1983] studied the inhibition of gram positive and gram negative food borne bacteria, yeast and mold by garlic, onion, cinnamon, cloves and other spices in pickles, rice and meat products. Eugenol, carvacrol and thymol had been identified as the major anti-microbial compounds in cloves and cinnamon. Ejechi *et al.* [1998] investigated the microbial stability of mango juice supplemented with extracts of ginger and nutmeg during 3 months of ambient-temperature storage. Supplementing mango juice with an aqueous extract of 15% ginger and 20% nutmeg inhibited the growth of challenge microorganisms, but produced a product with unacceptable taste. Heating the mango juice at 55°C for 15 min and supplementing with 4% nutmeg and 4% ginger markedly inhibited microbial growth and produced a product with acceptable taste. Zaika, [1988] has given an excellent summary of the antimicrobial effectiveness of spices. However, the addition of spices can be expected to aid in preserving foods held at refrigeration temperatures, at which the multiplication of microorganisms is slow. A partial listing of this summary is confirmed with our results as follows: (1) Microorganisms differ in their resistance to a given spice; (2) A given microorganism differs in its resistance to various spices; (3) Bacteria are more resistant than fungi; (4) The effect of a spice may be inhibitory or germicidal; and (5) Active components of spices

TABLE 5. Effect of essential oil extracts on the log (CFU / mL) of growth of yeast and molds (Y&M) in apple juice during storage at refrigeration (4°C) for 4 weeks.

Treatments	Zero time	First week	Three weeks	Four weeks
Control	2.12±0.05	5.00±0.19	9.70±0.09	11.00±0.46
Lemon grass	2.05±0.07	2.40±0.09	3.90±0.41	5.70±0.27
Clove	2.18±0.14	3.40±0.47	4.30±0.68	5.90±0.38
Rosemary	2.09±0.34	3.30±0.18	5.70±0.28	7.20±0.62
Basil	2.11±0.47	3.87±0.54	8.47±0.37	9.60±0.81
Sage	2.22±0.08	4.91±0.37	8.69±0.07	10.00±0.08

* SD = Standard Deviation (σn-1) of the log values of yeast and molds.

TABLE 6. Effect of essential oil extracts on the log (CFU / mL) of growth of bacteria (B) in apple juice during storage at refrigeration (4°C) for 4 weeks.

Treatments	Zero time	First week	Three Weeks	Four Weeks
Control	1.8±0.19	3.68±0.74	7.48±0.78	10.19±0.37
Lemon grass	1.72±0.37	1.84±0.08	3.00±0.68	4.20±0.84
Clove	1.69±0.61	1.95±0.29	4.00±0.19	5.00±0.69
Rosemary	1.37±0.66	3.87±0.52	4.50±0.44	5.80±0.59
Basil	1.51±0.49	4.64±0.91	8.40±0.34	9.74±0.81
Sage	1.66±0.71	4.88±0.68	9.40±0.64	10.40±0.55

* SD = Standard Deviation (σn-1) of the log values of bacteria or total plate counts.

at low concentrations may interact synergistically with other factors (NaCl, acids and preservatives) to increase preservative effect.

Thus, Zaika [1988] reported that food product safety and shelf life depend in some part on the type, quantity, and character of volatile oil spices extracts added to the products. Then, our results showed that refrigerating at 4°C and 0.05% volatile or essential oil extracts treatments caused a marked reduction in yeast and bacteria populations with acceptable taste and extended shelf life of apple juice up to 4 weeks.

CONCLUSIONS

It is concluded that no browning (inhibition of PPO) and the lowest microbial count (Y and M, and B) were observed in apple juices pre-treated with lemon grass and clove volatile oil extracts during storage at 4°C for 4 weeks. In addition, lemon grass, clove and rosemary volatile oil extracts treatment were the most preferred in all the studied characteristics after 4 weeks of storage at 4 °C. The use of these treatments (volatile or essential oil extracts) constitutes an effective method of quality improvement and shelf life extension of apple juices stored at 4 °C. In general, the results showed that lemon grass, clove and rosemary volatile oil extracts with refrigeration at 4 °C are considered to be anti-browning and anti-microbial agents than other volatile oil extracts, in controlling both enzymatic browning reactions and microorganisms, with potential antifungal properties. It can be concluded from the results of this work that 3 volatile oil extracts (lemon grass, clove and rosemary) treatments with refrigeration at 4 °C may serve as an alternative to conventional chemical preservatives in the preservation of fruit juices by hurdle technology. A practical application of spices in food as natural inhibitors against browning and microorganisms should be further studied.

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