Algerian Journal of Natural Products 4:2 **(2016)** 316-322

OPEN ACCESS

Online ISSN: 2353-0391

Algerian Journal of Natural Products
www.univ-bejaia.dz/ajnp

Type of the Paper (Article)

Evaluation of the chemical composition and antioxidant activity of *Citrus limon* essential oil and its application in margarine preservation

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Received: 05/06/2016 /Accepted: 07/08/2016

Abstract: Essential oils are aromatic oil liquids obtained from various plant parts. The growing interest in the substitution of synthetic antioxidant agents by natural ones has fostered research on vegetable sources and the screening of plant materials in order to identify new compounds. The study aims to evaluate the use of essential oil extract by cold pression of *Citrus limon* (Lisbon variety) as a substitute of Tocoblend (mixture of α, β, γ and δ-tocopherol) used as antioxidant in margarine preservation. The major constituents of the essential oil extract Identified were Limonene (66.75%) followed by β-Pinene (13.92%) and γ-Terpinene (3.10%). In both DPPH scavenging and bleachability of β-carotene in linoleic acid system assays, the essential oil extract exhibited the highest activity compared to the Tocoblend. Tests conducted at pilot scale showed that the margarine elaborated with essential oil extract was more resistant to oxidation than the margarine reference with Tocoblend. In addition, the physicochemical properties were not modified.
Keywords*: Citrus limon, essential oil, antioxidant activity, chemical composition, margarine.*

I. Introduction

Nowadays, the interest in naturally occurring antioxidants has considerably increased for use in food, cosmetic and pharmaceutical products to replace synthetic antioxidants which are being restricted due to their carcinogenicity [1, 2]. *Citrus* is the most abundant crop in the world, with about 64 million tons of orange and 13 millon tons of lemon products produced during 2004 [3]. The amount of residue obtained from *Citrus* fruit account for 50% of the original amount of whole fruit [4]. *Citrus* peel of fruit processing which provides a great potential for further commercial use. During the process of juice extraction oil sacs break and release volatile oils which are in pockets localized in the external part of the mesocarpe of fruit (flavedo). These oils are used in food and pharmaceutical industries, but can also provide flavouring ingredients to drinks, ice creams and other food products [5]. Essential oils are complex mixers comprising many single compounds. Chemically they are derived from terpenes and their oxygenated compounds. Each of these constituents contributes to the beneficial or adverse effects [6]. The main goal of the present study is to examine the chemical composition and antioxidant properties of the essential oil of *Citrus limon* (Lisbon variety) extracted by cold pression oil and his incorporation at margarine of table.

II. **Experimental Section**

II.1. Essential oil

II.1.1. Process of extraction

The essential oil of peels *Citrus limon* is manually extracted by cold pression. The epidermis and oil glands were lacerated. The oil is carried down to a decantation vessel in a stream of water, the emulsion being collected and then separated by centrifugation.
The obtained essential oil is dried over anhydrous sodium sulfate and stored in sealed brown vials at 4°C.

II.1.2. Gas chromatography-mass spectrometry

The essential oils were analyzed by gas chromatography coupled to mass spectrometry (GC-MS) (Hewlett-Packard computerized system comprising a 6800 gas chromatograph coupled to a MSD 5973 mass spectrometer) using two fused-silica-capillary columns with different stationary phases. The non-polar column was HP5MS (30 m×0.25mm, 0.25 µm film thickness) and the polar one was a Stabilwax consisting of Carbowax-PEG (60 m× 0.2 mm, 0.25 mm film thickness). GC-MS spectra were obtained using the following conditions: carrier gas He ; flow rate 0.5 $mL \cdot min^{-1}$; split-less mode ; injection volume 0.2 µL ; injection temperature 250 °C ; the oven temperature program was 60 °C for 8 min increased at $2°C \cdot min^{-1}$ to 250 °C and held at 250 °C for 10 min ; the ionization mode used was electronic impact at 70 eV. The relative percentage of the components was calculated from gas chromatography with flame ionization detection (GC-FID). Most constituents were tentatively identified by comparison of their GC Kovats retention indices (I), determined with reference to an homologous series of C_5-C_{28} n-alkanes and with those of authentic standards available in the authors laboratory. Identification was confirmed when possible by comparison of their mass spectral fragmentation patterns with those stored in the MS database (National Institute of Standards and Technology and Wiley libraries) and with mass spectra literature data [7-10].

II.1.3. Evaluation of the antioxidant and antiradical activities

II.1.3.1. Scavenger effect on DPPH

The electron donation ability of the samples (essential oil and Tocoblend) was measured by bleaching of the purple-colored solution of 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) according to the methode of Archana et al. (2005). DPPH radicals have an absorption maximum at 517 nm, which disappears with reduction by an antioxidant compound. A $DPPH^{\cdot}$ solution in absolute methanol (60 µM) was prepared, and 3 mL of this solution were mixed with 100µl of the samples at different concentrations. These solution mixtures were kept in the dark for 30 min and optical density was measured at 517 nm using spectrophotometer against methanol. The antioxidant capacity was expressed as percentage of inhibition of DPPH radical (%DPPH inhibition) calculated according to the following equation.

$$\%DPPH\ inhibition = (Abs_{control} - Abs_{sample}/Abs_{control}) \times 100.$$

Where $Abs_{control}$ is the absorbance of DPPH radical + methanol ; Abs_{sample} is the absorbance of DPPH radical + sample extract. From data elaboration (% inhibition plotted versus samples concentration), the concentration of samples required to reach 50% radical inhibition (IC_{50}) was calculated.

II.1.3.2. Bleachability of β-carotene in linoleic acid system

The antioxidant activity of the examined essential oils was evaluated using a β-carotene-linoleate model system following the method described by [11]. A solution of β-carotene was prepared by dissolving 2.0 mg of β-carotene in 10 ml of chloroform. One millilitre of this solution was pipetted into a round-bottom flask and 20ml of purified linoleic acid and 200 mg of tween 40 emulsifier were added. After the chloroform was rotary evaporated at 40 °C under vacuum, 50 ml of oxygenated water were added to the flask under vigorous shaking. Aliquots (5 ml) of this emulsion were transferred into a series of tubes containing 500 ml of essential oil (2 mg/ml) or 2 mg/ml of Tocoblend for comparative

purposes. As soon as the emulsion was added to each tube, the zero time absorbance was read at 470 nm. Subsequent absorbance readings were recorded at 120 min by keeping the sample in a water bath at 50 °C until the visual color of β-carotene in the control sample disappeared. Antioxidant activities (Inhibition %) of the samples were calculated using the following equation:

$$\textbf{Inhibition \%} = (A_{\beta\text{-carotene after 2h assay}} / A_{\text{initial } \beta\text{-carotene}}) \times 100$$

Where $A_{\beta\text{-carotene after 2h assay}}$ is the absorbance of β-carotene after 2h assay remaining in the samples and $A_{\text{initial } \beta\text{-carotene}}$ is the absorbance of β-carotene at the beginning of the experiments.

II.2. Margarine

II.2.1. Pilot production of margarines

Margarines were produced at pilot scale. Fats (82%) and liquid (18%) phases were prepared. The lipid phase contained palm oil, sunflower oil, coprah oil hydrogenated, palm oil hydrogenated and Tocoblend or essential oil (100ppm) and the liquid phase contained water (16%), β-carotene (7ppm), aroma (diacetyl: 0.8g/kg), salt (0.3%), lactic acid (0.5 g/kg) and potassium sorbate (1.3g/kg).
Fats were first melted at 70°C in a mixing tank with agitator. Emulsifier was dissolved in a small amount of melted fat, then added to the fat phase in the mixing tank. The aqueous phase and its ingredients were prepared in a separate tank before being added to the fat phase.
Agitation was then set for at least 10 min in order to allow the formation of a good pre-emulsion.
Margarine was processed in a simplified perfector pilot plant made of one cooler tube followed by a resting tube (2.3 l). the modified parameters were the water bath temperature running in the tube exchanger of the second small tank and in the double envelope tube (45°C or 55°C), the pump rate of the emulsion (400 or 500 rpm), the blades rotational speed (400 or 500 rpm), the scraped surface heat exchanger temperature (15°C or 20°C) and the temperature of the water bath running in the double envelop of the resting tube (15°C or 20°C).
The produced margarines were collected in plastic containers (250g) at the end of the processing line after stabilization of the temperatures. Samples were then stored at 4°C before analysis.

II.2.2. Determination of physicochemical properties of the margarines developed

II.2.2.1. Moisture

The moisture content was determined on 3g of margarine brought to 100°C until constant weight [12].

II.2.2.2. pH

Margarine pH was determined directly on the aqueous phase.

II.2.2.3. Determination of the peroxide.

The peroxide index determination involves mixing 5g of melted margarine with 12 ml of chloroform, 18 ml of acetic acid and 0.5 ml of potassium iodide solution. After 1 min, 75 ml of distilled water and a few drops of starch (colour indicator) were added. The mixture was then titrated with sodium thiosulfate solution (0.01 N) until the colour changes to pale yellow. A blank was prepared in the same conditions [13].
The peroxide index is expressed in meq O_2/kg calculated using
The following equation:

$$\textbf{PI} = (V/M) \times 1000$$

Where V is the volume (ml) of sodium thiosulfate and M the sample mass (g).

II.2.2.4. Determination of melting point

The capillary tubes were placed in cold water then heated (0.5°C/min) until the level of the fat matter rises in the capillary tube. The melting temperature was noted [14].

II.2.2.5. Determination of solid content (solid fat content)

To determine the level of solids in the margarine, each sample was melted in an oven at 100°C and then filtered. The filtrate obtained was then poured into three tubes up to 2 cm.
The tubes were incubated separately at three different temperatures : 20°C/20 min, 30°C/20 min and 40°C/20min. Values were read using a nuclear magnetic resonance (NMR) apparatus (type minispec mq 20, Germany), processed and the final results were given in percentage of solids [15].

II.2.3. Determination of the oxidative stability of margarines (Rancimat test)

The Rancimat test is an accelerated technique most commonly used for assessment of the oxidative stability of edible fats, oils and fat-containing foods [16].
To determine the oxidative stability of the prepared margarines, 3g of sample were put in accelerated oxidation conditions : temperature set at 120°C and air flow to 10 l/h. As a result, volatile compounds are formed and trapped in the tube containing distilled water (60 ml) that induces the increase of its conductivity. The induction period and the oxidative stability of the samples are given in hours. It is determined from the inflection point of the curve of conductivity [17].

III. Results and Discussion

III.1. Essential oil

III.1.1. Gas chromatography-mass spectrometry (GC-MS)

The average yield in essential oil was 0.81± 0.092%. The output of the Eureka variety is 1.02% ± 0.04% [18]. At 0.05, the two outputs present a significant difference, which implies that the variety has an impact on the output. Thus point of considering quantitative, it is interesting to carry out the lemon essential oil extraction of the Eureka variety.
The chemical composition of essential oil of *Citrus limon* (Lisbon variety) was characterized by 29 constituents which accounted for 99.82% of the total oil (table 1).

Table 1: *Chemical composition of the essential oil of Citrus limon of the Lisbon variety*

N°	RT min	Compound	Area %	I
1	10.250	α-Thujene	0.33	931.078
2	10.660	α- Pinene	2.15	938.46
3	13.503	β-Pinene	13.92	978.974
4	14.428	β-Myrcene	1.41	997.846
5	17.696	Limonène	66.75	1013.859
6	19.282	γ-Terpinene	3.10	1066.170
7	21.238	α-Terpinolene	0.15	1070.709
8	22.617	Linalool	0.25	1108.870
9	24.082	Trans -Limonene oxide	0.14	1131.203
10	24.554	Cis-Citronellol	0.50	1138.001
11	24.930	Trans-Citronellol	0.86	1143.324
12	25.171	Camphor	0.28	1146.693
13	25.436	Citronellal	0.19	1150.361
14	29.118	Myrtenol	0.21	1197.710
15	29.248	α-Terpineol	0.36	1199.271
16	31.350	Trans-Carveol	0.26	1231.182
17	32.212	Cis-Carveol	0.09	1244.105
18	32.506	Cis-Citral	1.27	1248.347

19	34.665	Geranial	2.76	1278.377
20	39.962	Citronellyl Acetate	0.12	1356.164
21	40.396	Piperitenone oxide	1.32	1363.477
22	40.704	Neryl Acetate	0.90	1367.996
23	41.967	β-Elemene	0.74	1386.175
24	44.015	Trans-Caryophyllen	0.16	1417.241
25	44.208	α-Humulene	0.17	1455321
26	45.037	Trans -α- Bergamotene	0.17	1435.464
27	46.565	Germacrene	0.19	1460.447
28	49.663	γ-Cadinene	0.23	1510
29	54.126	Caryophyllene oxide	0.84	1584.81

The oil was dominated by Limonene (66.75%) followed by β-Pinene (13.92%), γ-Terpinene (3.10%), Geranial (2.76%) and α-Pinene (2.15%).
The essential oil of *Citrus limon* (Lisbon variety) is strongly dominated by monoterpene hydrocarbons (89.17%). The oxygenated monoterpene and sesquiterpene compounds are scarcely represented in this oil (5.81% and 2.33% respectively).

III.1.2. Antioxidant and antiradical activities

As shown in Table 2, the essential oil has antioxidant ability for preventing the linoleic acid oxidation and to reduce DPPH radicals.

Table 2. *Antioxidant capacity of essential oil of Citrus limon and Tocoblend*

	Essential oil	**Tocoblend**
IC 50 (µg/ml)	0.120 ± 0.049	0.837 ± 0.143
Inhibition in linoleicacid system (%)	88.47±0.026	39.75±0.015

Values are means ± standard deviation of three separate experiments.

The DPPH assay has been widely used to evaluate the free radical scavenging effectiveness of various antioxidant substances. Lower IC_{50} value indicated higher antioxidant activity. The essential oil of *Citrus limon* (0.120 ± 0.049 µg/ml) showed higher scavenging ability on DPPH radicals when compared to Tocoblend (0837 ± 0143 µg/ml).
The antioxidant activity of Tocoblend and essential oil of *Citrus limon* was also evaluated by the β-carotene-linoleate bleaching method. As for antiradical scavenging activity the essential oil extract showed higher ability to prevent the bleaching of β-carotene than that of Tocoblend.
The antioxidant activity of essential oil is mainly contributed by the active compounds of fraction present in them. Wei and Shibamoto (2007) showed the presence of a significant antioxidant potential of essential oils rich in hydrocarbon monoterpenes (82.82%) (limonene α- and β-pinene) and hydrocarbon sesquiterpenes (2.33%) (trans-caryophyllene, β-Elemene, α-Humulene, Caryophyllene oxide, γ-Cadinene and Germacrene) were responsible of the DPPH neutralization which is the case of *Citrus limon* [19].

III.2. Margarine
III.2.1. Physicochemical properties of margarines developed

The pH values of the aqueous phase and the moisture of the two margarines (margarine with Tocoblend and margarine with essential oil) were however within the standard range (table 3).

Table 3. *Physico-chemical characteristics of elaborate margarines*

Parameters	Margarine with Tocoblend	Margarine with essential oil
Moisture (%)	15.76 ± 0.003	15.50 ± 0.090
pH	4.0 ± 0.162	4.03 ± 0.132
Peroxide index (meq/kg)	1.97 ± 0.019	1.89 ± 1.123
Point melting (°C)	36 ± 0.011	35.43 ± 0.078

The two margarines exhibited a similar peroxide index (1.97 ± 0.019Meq O_2/kg for margarine with Tocoblend and 1.89 ± 1.123Meq O_2/kg for margarine with essential oil) far below the maximum values allowed by the international standards (5 Meq O_2/kg). The peroxide index is one of the most widely used tests for oxidative rancidity in oils fats. It is the amount of peroxide oxygen per 1 kg of product. It is a very useful test and a satisfactory sensitivity to appreciate the early stages of oxidative damage [20].

In addition to the physicochemical properties, two indicators of the organoleptic properties of the two margarines were measured i.e. the melting point and the Solid Fat Content (SFC).

The melting point gives an indication of the temperature at which the margarine should be smooth in the mouth. The melting point international standard range of margarine are between 28 and 34°C which implies that margarine can melt rapidly in the mouth and be firm at room temperature to resist to mechanical work during its spreadability. The melting points of the margarines developed were not significantly different and within this the international range since margarine with Tocoblend and margarine with essential oil fused at 36 ± 0.011°C and 35.43 ± 0.078°C respectively.

The SFC is the percentage of solidified triglycerides in oil at a given temperature. SFC is an important indicator of several characteristics of a product, including its appearance and organoleptic properties. It can be used as a measure of the degree of crystallisation of fats during treatment [21].

The SFC rates of the two samples varied with the temperature. Indeed, it decreases with increasing temperature. The values of SFC obtained for the margarine with Tocoblend and the margarine with essential oil are respectively 34.8 ± 0.04% and 34.3 ± 0.01% at 10°C, they are 4.4 ± 0.17% and 4.3 ± 0.02% at 35°C and they are 2 ± 0.01% and 2.2 ± 0.14% at 40°C.

These results were in agreement with those obtained by Karleskind [20]. At 37°C SFC must be less than 6%. In the present study, margarines had already a SFC lower than 6% at 35°C which indicates that these margarines melts easily in the mouth.

III.2.2. Oxidative stability of the margarine (Test of Rancimat)

The purpose of the test is to predict the oxidative stability of the fat matter. The Rancimat results are represented as a curve (conductivity as a function of time). The induction period is determined from the inflection point of the conductivity curve [22].

The induction time obtained for the margarines, were 5.34 ± 0.028h (margarine with Tocoblend) and 9.02 ± 0.014h (margarine with essential oil). It appears that the oxidation is decreased significantly ($P< 0.05$) in the margarines containing the essential oil by comparison to the one containing Tocoblend.

Compared to the margarine with Tocoblend, margarines developed with essential oil of *Citrus limon* showed clearly a longer induction time and therefore better resistance to oxidation.

IV. Conclusion

The present study was designed to replace the commonly used Tocoblend as an antioxidant in margarine preservation, by essential oil of *Citrus limon* (Lisbon variety). Essential oil extract presented significant percentage of Limonene compound (66.75%) that play an important role against oxidation as shown by scavenger effect on DPPH and bleachability of β-carotene in linoleic acid system.

The same concentration of essential oil extract, incorporated in margarine, showed higher performance than Tocoblend which usually used at 100 ppm.

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Please cite this Article as:

HIMED Louiza, MERNIZ Salah and BARKAT Malika, Evaluation of the chemical composition and antioxidant activity of *Citrus limon* essential oil and its application in margarine preservation, ***Algerian J. Nat. Products,* 4:2 (2016) 316-322.**

www.univ-bejaia.dz/ajnp
Online ISSN: 2353-0391
Editor in chief: Prof. Kamel BELHAMEL