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Antioxidant Activity, Free Radical Scavenging Potential and Chemical Composition of *Litsea cubeba* Essential Oil

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Abstract: The antioxidant activity and free radical scavenging potential of *Litsea cubeba* essential oil (LCEO) were evaluated from different points of view and compared with citral, a major component in LCEO, ascorbic acid (Vc) and synthetic antioxidants including butylated hydroxytoluene (BHT) and propyl gallate (PG). The results revealed that LCEO has notable antioxidant activity. The order of hydroxyl radical scavenging activity was LCEO >BHT > citral > PG >Vc. Their half maximal inhibitory concentration (IC_{50}) values of hydroxyl radical scavenging activity were 0.19, 0.28, 0.50, 0.79 and 1.07 mg/mL, respectively, and IC_{50} values of scavenging superoxide were 0.45, 0.64, 0.67, 1.08 and 0.51 mg/mL, respectively. Furthermore, the peroxidation of linoleic acid was significantly suppressed by the addition of LCEO. The order of activity was BHT >Vc >LCEO >citral >PG. Chemical composition of LCEO was identified by GC and GC-MS, and major components in the oil were limonene (26.25 %), followed by α-citral (25.97 %), β-citral (21.90 %), and β-pinene (6.20 %). Citral plays an important role in antioxidant activity and free radical scavenging.

Key words: Antioxidant activity, free radical scavenging, *Litsea cubeba* essential oil, citral, GC-MS.

Introduction

Reactive oxygen species (ROS) include free radicals such as superoxide (O_2-·), hydroxyl radical (HO·), peroxyl radical (RO_2·) as well as nonradical species such as hydrogen peroxide (H_2O_2) [1]. Some ROS play a positive role in living organism such as energy production, phagocytosis, regulation of cell growth and intracellular signaling [2]. On the other hand, ROS are also capable of damaging a wide range of essential biomolecules such as proteins, DNA and lipids [3]. ROS are not only strongly associated with lipid peroxidation resulting in deterioration of food materials, but also are involved in development of a variety of diseases including aging, carcinogenesis, coronary heart disease, diabetes and neurodegeneration [1,4]. Over production of ROS in human beings, by endogenous or external sources, e.g. tobacco smoke, certain pollutants, organic solvents or pesticides [5], leads to oxidative stress. Oxidative stress is defined in general as excess formation and/or incomplete removal of highly reactive molecules such as ROS [1]. It is well-known to cause many diseases e.g. cancer, arteriosclerosis and other cardiovascular problems, diabetes and even ageing are promoted [6-9].

In order to reduce the damage of free radicals and to prevent foods from the oxidation, ascorbic acid (Vc) and synthetic antioxidants such as butylated hydroxytoluene (BHT) and propyl gallate

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(PG) are often used in foods. However, synthetic antioxidants have been suspected to cause or promote negative health effects [10,11]. Thus, it is essential to develop and utilize effective and natural antioxidants so that they can protect the human body from free radicals and retard the progress of many chronic diseases [12]. Therefore, the use of spices and herbs as antioxidants in processed foods is a promising alternative to the use of synthetic antioxidants. Numerous reports of antioxidant activity of spices have appeared, strongly inspired by an increasing consumer interest in "natural" food additives. Both antioxidant and antimicrobial properties are very important in increasing the shelf life of the particular food material.

Litsea cubeba is a family *Lauraceae* plant and occurs mainly in mountainous regions in China, India, Indonesia and some other parts of Southeast Asia. It is a traditional spice and herbal drug with a long history in above-mentioned countries. The small and pepper-like fruits of the plant contain essential oil with an intensely lemon-like, fresh, sweet odour and the oil is rich in citral (as high as 70 percent). Its major use both in international markets and in China is both as a flavor enhancer in foods, cosmetics, cigarettes [13] and medicine because of its antimicrobial and antibacterial action [14], and as a raw material source for the isolation of natural citral. Citral is a key starting material for various fine chemical products, in particular for the synthesis of several types of pseudoionones that are an intermediate in the commercial production of ionones, which possess a violet-like fragrance, and vitamins [15]. In addition, the methanol and butanol extracts of *L. cubeba* were found to have good free radical scavenging potential [16]. The objectives of this study were to analyze the chemical composition of *L. cubeba* essential oil (LCEO) from China by GC and GC-MS techniques, and to investigate the antioxidant activities and free radical scavenging potential of LCEO.

Experimental

Plant Materials

The dry fruits of *L. cubeba* were collected from Jiangxi Province, China. LCEO was obtained by hydrodistillation. Standard citral (the mixture of α- and β-citral) was purchased from Sinopharm Chemical Reagent Ltd. Co. (Shanghai, China). Ferrous sulfate, 1,10-phenanthroline, BHT, Vc, PG, pyrogallol, and thiobarbituric acid (TBA) were purchased from Tianjin Chemical Co. (Tianjin, China). Hydrogen peroxide and anhydrous sodium sulfate were obtained from Tianjin Kaitong Chemical Reagent Ltd. Co. (Tianjin, China). Linoleic acid and trichloroacetic acid (TCA) were obtained from Shanghai Laize Chemical Institute (Shanghai, China). Standard *n*-alkanes reference mixture of C_8-C_{40} was obtained from AccuStandard Inc. (CT, USA). All chemicals used were of analytical reagent grade. Distilled water was used throughout.

Instrumentation

A Unico UV-2102 spectrophotometer (Shanghai Unico Co. Ltd., Shanghai, China) matched with 10-mm quartz cells was used to measure the absorbance. GC and GC-MS analyses of the essential oil were performed on a Finnigan Trace DSQ GC-MS, equipped with an AL 3000 autoinjector (Thermo Fisher Scientific Inc., MA, USA) and a capillary column (Thermo TR-5MS, crosslined 5 % phenyl polysilphenylene-siloxane, 30 m x 0.25 mm i. d., 0.25 μm film thickness).

GC-MS

The column was coupled directly to the MS, and the flow rate for the helium carrier gas was 1.0 mL/min. The injector temperature was 250°C. A 0.4 μL sample was injected in the split mode with a split ratio of 40:1. The temperature was program from 50 to 230°C at a rate of 10°C/min, then from 230 to 280°C at a rate of 20°C/min and subsequently held isothermal for 10 min. Ionization voltage (EI) was 70 eV. Ion source and interface temperature was 250°C. Scan mass range was 50-450 m/z. Solvent delay time was 1 min. Alkenes were used as reference points in the calculation of retention indices (RI).

The components of essential oil were identified by comparison of their mass spectra with those of Mainlib Library and Replib Library Database, and confirmed by comparison of their retention indices.

Determination of total antioxidant capacity

The antioxidant activities of LCEO and citral were evaluated by the formation of phospho-molybdenum complex method [17,18]. Briefly, an aliquot of 0.4 mL of sample solution at different concentrations was added to a vial containing 4 mL of each of reagent solutions (0.6 mmol/L, sulfuric acid, 28.0 mmol/L sodium phosphate and 4.0 mmol/L ammonium molybdate). The vial was capped and incubated in a hot water bath at 95°C for 90 min. After the sample was cooled at room temperature, the absorbance of the mixture was measured at 695 nm. The antioxidant activity was expressed relatively to those of BHT, PG and Vc. All determinations were done in triplicate.

Superoxide radical scavenging activity

The measurement of superoxide anion scavenging activities of LCEO and citral was based on the autoxidation of pyrogallol method described by Liu and coworkers [19] with a slight modification. The detailed process was as follows: 4.5 mL of 50 mmol/L Tris-HCl buffer solution (pH 8.2) and 1 mL of 2.0 mmol/L EDTA were added into the tube containing 0.1 mL of the essential oil solution at different concentrations. After the tube was incubated in water bath at 25°C for 20 min, 0.4 mL of 25 mmol/L pyrogallol solution was added. The reaction was stopped by adding 50 µl of 12 mol/L hydrochloride acid after 4 min. The absorbance was measured at 299 nm. The control group was prepared as above-mentioned process, but ethanol in the tube instead of the essential oil solution. Further, the essential oil sample was replaced by an equal volume of BHT, PG or Vc solutions as the reference standards. The percentage inhibition of superoxide anion generation was calculated by the following equation:

Superoxide radical scavenging activity (%) = $(A_o - A_1)/A_o \times 100$

where A_o is the absorbance of the control group, and A_1 is the absorbance of the test sample.

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was determined according to the previous literature [20] with some modifications based on the autoxidation of 1,10-phenanthroline- Fe^{2+}. The HO· was usually created by Fenton reaction. The detailed process was as follows: The tube was filled with an aliquot of 5 mL of reagent mixture composed of PBS 150 mmol/L, 1,10-phenanthroline 0.75 mmol/L and $FeSO_4$ 0.75 mmol/L (pH 7. 4), with or without 0.01 % H_2O_2 (the former is used as an injury tube). The reagent mixture above-mentioned in the presence of 0.01 % H_2O_2 were mixed with LCEO or citral or synthetic antioxidants at different concentrations. In comparison with, the essential oil or synthetic antioxidants samples were the same concentrations. After the test tube was incubated in water bath at 37°C for 1 h, the absorbance was determined at 536 nm. All experiments were carried out in triplicate. The hydroxyl radical scavenging ability was calculated by the following equation:

Hydroxyl radical scavenging activity (%) = $A_{sample} - A_{injury}/(A_{non\text{-}injury} - A_{injury}) \times 100\%$

where A_{sample} is the absorbance of LCEO, citral and other synthetic antioxidants, A_{injury} and $A_{non\text{-}injury}$ are the absorbance of the injury and non-injury tube.

Determination of inhibiting lipid oxidation activity

The inhibiting lipid oxidation activity of LCEO was determined according to ferric thiocyanate method (FTC)[21-23] and thiobarbituric acid method (TBA) with slight modifications. The detailed process of FTC were: To a screw-cap vial, in which contains 4.1 mL of 2.5 % linoleic acid in 99.5 % ethanol, 8.0 mL of 0.02 mol/L phosphate buffer (pH 7.0, the mixture solution of potassium dihydrogen phosphate and disodium hydrogen phosphate according to a molar ratio of 1:1) and 3.9 mL of water, an aliquot of 4 mL of 1 mg/mL essential oil in 99.5 % ethanol was added and well mixed. The vial was placed in an oven at 40°C in the dark. In addition, 4 mL of 1 mg/mL essential oil in 99.5 % ethanol was replaced, respectively by each of an equal volume of 1 mg/mL BHT, PG or Vc as reference. At a 24 h interval, 0.1 mL of

this mixture was transferred to a test tube. Then 9.7 mL of 75 % (v/v) ethanol, 0.1 mL of 30 % ammonium thiocyanate and 0.1 mL of 0.02 mol/L ferrous chloride in 3.5 % hydrochloric acid were added in succession. Three minutes later, the absorbance was measured at 500 nm. This step was repeated until the control reached its maximum absorbance value. The detailed process of TBA[21, 22, 24] were: To a 10 mL centrifuge tube, 2 mL of the sample solution prepared as FTC method, 1 mL of 20 % aqueous trichloroacetic acid and 2 mL of 0.3 % aqueous thiobarbituric acid were added. The mixture was placed in a boiling water bath for 10 min. After atmosphere naturally cooled, it was centrifuged at 3000 rpm for 20 min. Absorbance of the supernatant was measured at 532 nm, which is represented for the quantity of MDA in the lipid. Antioxidant activity was based on the absorbance of the final day of TBA assay.

Results and discussion

Chemical composition of the essential oil was analyzed using GC and GC-MS. Thirty-eight components were identified representing 98.95 % of total essential oil. The major component was limonene (26.25 %), followed by α-citral (25.97 %), β-citral (21.90 %), and β-pinene (6.20 %). The results are similar to previous literature [25]. In addition, some other components in minor quantities were first found in LCEO from China including eucalyptol, limonene oxide, caryophyllene oxide, verbenol, α-copaene, β-elemene, β-farnesene, α-selinene, cadinene, farnesol, nerol acetate, ethyl geranate, and widdrol. Relative percentages and retention indices of the characterized compounds calculated from GC are shown in Table 1. The contents of whole aldehydes and alcohols were 48.34 % and 6.69 % respectively. It was well known that aldehydes are highly resistant to oxidative deterioration. Relative high contents of citral (47.87 % of total oil) existed in the essential oil as potentially major antioxidant components. In addition, because of the presence of other minor antioxidant components in the oil, antioxidant activity of LCEO was perhaps a synergistic interaction among the antioxidant components in the essential oil.

Table 1. Chemical composition of *Litsea cubeba* essential oil

Compound	RI	Content (%)
α-Thujene	936	0.15
α-Pinene	947	3.8
Camphene	966	0.82
β-Phellandrene	985	4.51
β-Pinene	992	6.2
Dehydrocineole	1001	0.09
α-Phellandrene	1019	0.05
Limonene	1044	26.25
Eucalyptol	1049	1.61
γ-terpinene	1071	0.14
cis-β-Terpineol	1085	0.07
α-Terpinolene	1097	0.14
Linalool	1107	1.35
cis-Limonene oxide	1150	0.11
trans-Limonene oxide	1155	0.17
α-Citronellal	1164	0.47
cis-Verbenol	1172	0.46
Verbenol	1190	0.97
Terpinen-4-ol	1198	0.52

table 1. (continued).

Compound	RI	Content (%)
Terpineol	1214	0.49
(Z)-Carveol	1228	0.05
cis-Geraniol	1237	0.46
β-Citral	1256	21.90
trans-Geraniol	1262	0.61
α-Citral	1284	25.97
Terpinyl acetate	1362	0.09
Nerol acetate	1385	0.04
α-Copaene	1395	0.11
β-Elemene	1405	0.06
β-Farnesene	1460	0.05
Caryophyllene	1481	0.79
α-Selinene	1521	0.04
Cadinene	1538	0.04
Caryophyllene oxide	1614	0.19
Limonen-6-ol pivalate	2058	0.03
Farnesol	2090	0.06
Ethyl geranate	2169	0.05
Widdrol	2177	0.04
Total		98.95

Due to the complexity of antioxidant activity in foods, the evaluation of the activities of essential oil and citral was carried out under the same experimental conditions. The antioxidant activities of LCEO and citral were expressed as the values of absorbance at 695 nm, which was based on the reduction of Mo (VI) to Mo (V) by the antioxidant and the formation of a green molybdenum (V) phosphate with a maximal absorption at 695 nm. The results are compared with those known antioxidants including BHT, PG and Vc (Figure 1). The antioxidant activity of LCEO increased with the increasing of the essential oil concentrations. The activities of the essential oil and citral seem to be higher than BHT, PG, and Vc.

Figure 2 shows the superoxide radical scavenging activities of LCEO and citral in the comparison with conventional antioxidants, BHT, PG and Vc. The scavenging activities of the essential oil and antioxidants were obvious and related to their concentrations. As shown in Figure 2, for the essential oil and citral at the concentration below 0.45 mg/mL, the scavenging activity was higher than BHT, PG and Vc. To the contrary of concentration over 0.8 mg/mL, the scavenging activities of LCEO and citral were lower than those of Vc and BHT. The IC_{50} values of scavenging superoxide of essential oil, citral, BHT, PG and Vc were 0.45, 0.67, 0.64, 1.08 and 0.51 mg/mL, respectively. These results clearly suggest that the antioxidant activities of all samples were related to their abilities to scavenge superoxides.

Figure 3 shows the hydroxyl radical scavenging activities of LCEO and citral in the comparison with that of BHT, PG and Vc. The results show that the plots of scavenging abilities vs the initial concentrations of the samples were linear in a given concentration range of 0.1-3.0 mg/mL. Among these samples tested, BHT exhibited the strongest scavenging activity against hydroxyl radical, and Vc was the weakest. Moreover, LCEO and citral showed higher scavenging activity against hydroxyl radical than PG and Vc. At a concentration of 0.1-3.0 mg/mL, the scavenging activities were 19.54-89.4 % for citral,

23.45-92.1 % for LCEO, 23.2-90.9 % for BHT, 15.43-85.7 % for PG, and 10.21-81.3 % for Vc.

The IC_{50} values, which are of LCEO, BHT, citral, PG and Vc were 0.19, 0.28, 0.50, 0.79 and 1.07 mg/mL, respectively. Therefore, the order of hydroxyl radical scavenging activity of the samples tested is LCEO >BHT > citral > PG >Vc. This is because that many components in LCEO have the hydroxyl and superoxide radical scavenging activities, such as some monoterpenes, sesquiterpenes and their oxygenated derivatives which can generate the relative radicals and result powerfully in quenching of hydroxyl and other radicals. Therefore, the hydroxyl and superoxide radical scavenging activities of LECO are better than 100 % of citral. These results clearly demonstrated that LCEO can quench hydroxyl radicals and are available for human health.

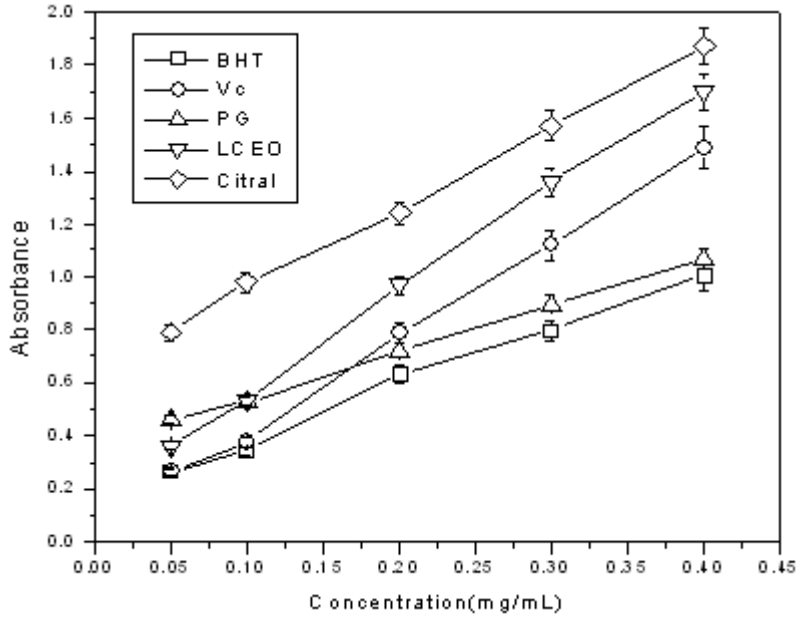

Figure 1. Total antioxidant activity of the samples tested

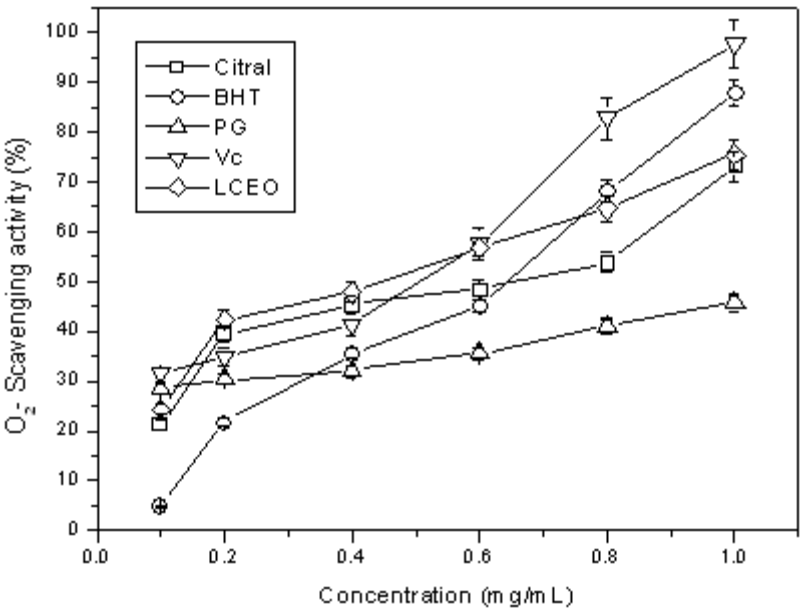

Figure 2. Superoxide radical scavenging activity of the samples tested

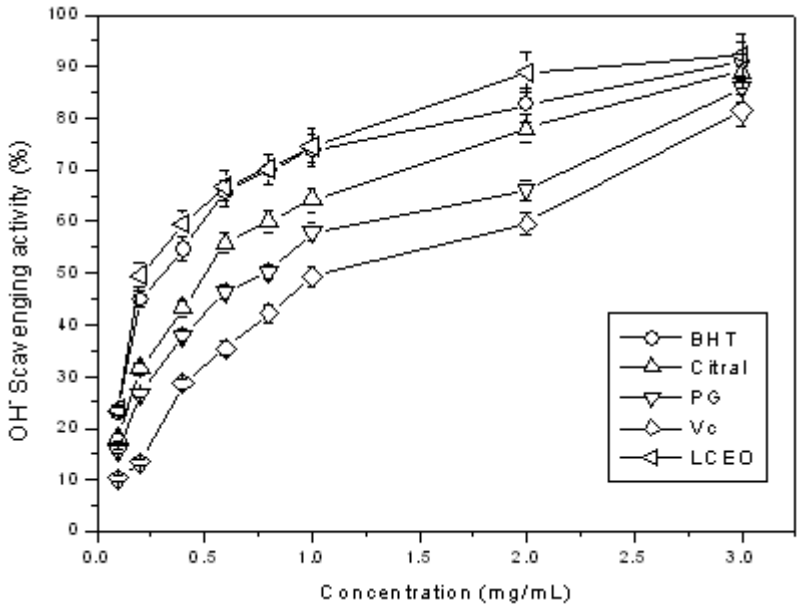

Figure 3. Hydroxyl radical scavenging activity of the samples tested

The FTC method measures the amount of peroxide in the initial stages of linoleic acid peroxidation. Low absorbance values in the FTC method indicate high level of antioxidant activity. Figure 4 shows the absorbance value of LCEO and other conventional antioxidants compared to the control. The antioxidant activities of LCEO and citral in this method were lower than that of PG, BHT, and Vc. During the oxidation process, peroxide was gradually decomposed to lower molecular compounds and the relative concentrations were measured using TBA method.

Figure 5 shows the absorbance values of the essential oils and the standards measured at 532 nm. The absorbance was measured on the final day of FTC assay. The results were consistent with the results of the assay using FTC method.

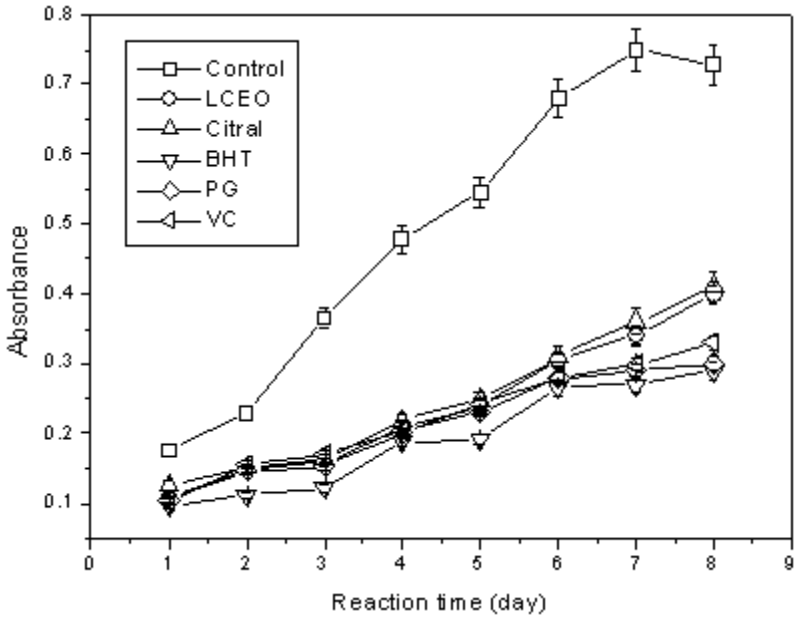

Figure 4. Antioxidant activity of the samples tested using FTC method

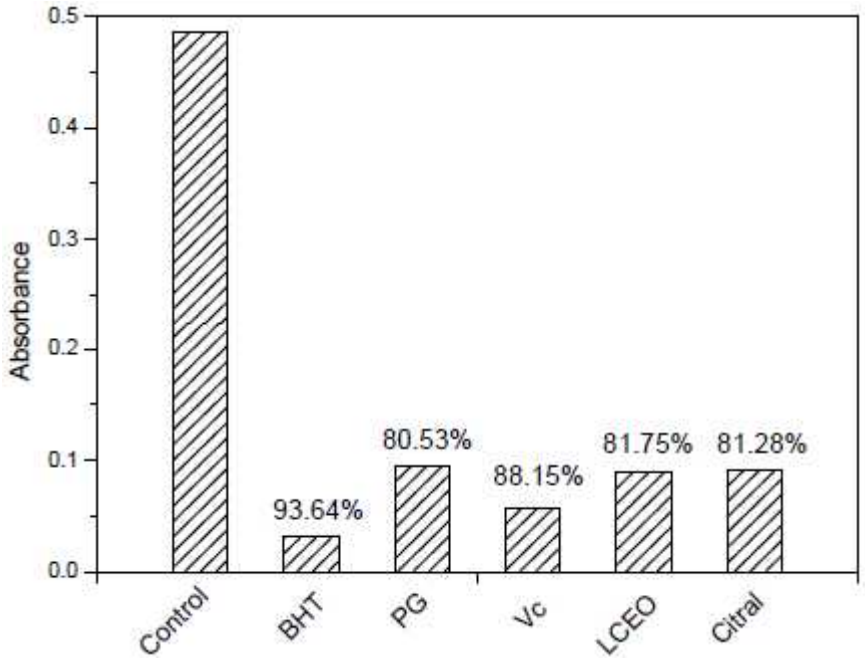

Figure 5. Antioxidant activity of the samples tested using TBA method. The inserted data are the antioxidant activity

Conclusions

Chemical composition of LCEO was identified by GC and GC-MS. Further, the antioxidant and free radical scavenging activity of LCEO were evaluated. In some aspects, the antioxidant activity of LCEO was higher than that of citral. This may be due to the essential oil is a mixture of many kinds of terpenoids and alkenes. The results indicate that the essential oil could be used as easily accessible source of natural antioxidant for food industry so that it is interesting to examine its application as natural antioxidant additive in some food products.

In addition, the present study shows that no single testing method is sufficient to estimate the antioxidant activity of a studied sample, which is identical with the Koleva's result [26]. The combination of several methods, applied in this study, was a good choice to evaluate the antioxidant activity of essential oils.

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