

Original Research

ANTIOXIDANT ACTIVITY OF CLOVE ESSENTIAL OIL

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Abstract

The aim of this study was to determine the antioxidant activity of two different samples of clove essential oils (CEO) and their main bioactive compound, eugenol, in relation to their potential application as natural antioxidants in food products. Radical scavenging activity was evaluated using DPPH and ABTS assays, whereas electron-donating capacity was assessed with the ferric reducing antioxidant power (FRAP) assay. Both analyzed CEO showed a high polyphenol content (TPC), which correlates well with the presence of eugenol as the main bioactive compound in these oils. The high phenolic content was consistent with the results for both radical scavenging activity and reducing power. In all tests, eugenol demonstrated the strongest antioxidant effect, confirming its role as the primary contributor to the antioxidant properties of CEO. Both CEO exhibited strong antioxidant power, with negligible differences likely due to variations in their chemical composition. The results of this study highlights the potential application of CEO and eugenol as natural antioxidants in food production, while further research will focus on their encapsulation to enhance stability and bioavailability.

Keywords: natural antioxidants, clove essential oil, eugenol, radical scavenging, polyphenols.

INTRODUCTION

The primary cause of food degradation is lipid auto-oxidation, particularly in products with a high content of unsaturated fatty acids. This process results in the deterioration of sensory characteristics and nutritional value, as well as changes in chemical composition that may pose a health risk [1,2]. Lipid auto-oxidation occurs when unsaturated fatty acids react with oxygen, producing free radicals, which further initiate oxidation reactions [2–4]. To inhibit this process in food products, antioxidants are used [1]. Antioxidants can act as radical scavengers or through mechanisms that do not involve direct scavenging of free radicals, such as chelating metal ions, inhibiting the decomposition of lipid hydroperoxides, scavenging reactive oxygen species, or regenerating oxidised radical scavengers [2–5]. Synthetic

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antioxidants commonly used in the food industry are phenolic substances, such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), *tert*-butylhydroquinone (TBHQ) and propyl gallate [5]. Due to safety concerns related to the use of synthetic antioxidants and the current demand for cleaner food and natural additives, various natural antioxidants have been investigated [6,7].

Essential oils are complex mixtures of volatile compounds derived from plants and represent a rich source of natural antioxidants [8, 9]. The antioxidant activity of essential oils is dependent on their chemical composition and is associated with the concentration of phenolic compounds. They can neutralize peroxy radicals by donating a hydrogen atom from the phenolic hydroxyl group [9, 10]. Furthermore, the synergistic activity of various bioactive compounds present in essential oils contributes to a stronger antioxidant effect [2].

Clove (*Syzygium aromaticum*) is a significant source of phenolic compounds, including flavonoids, hydroxycinnamic acids, hydroxybenzoic acids, and hydroxyphenyl propenes [11]. Clove essential oil (CEO) is typically obtained from dried buds. The main bioactive compound in CEO is eugenol, with its content often exceeding 70% [11,12]. Both CEO and eugenol have been shown to exhibit strong antioxidant effects, due to the phenolic structure of eugenol and its ability to scavenge free radicals and reactive oxygen species [13,14]. Other bioactive compounds present in CEO, such as eugenol acetate and β -caryophyllene, also contribute to this effect [15]. In addition to antioxidant activity, CEO and eugenol possess strong antibacterial and antifungal properties and are both classified as “generally recognized as safe” by the United States Food and Drug Administration which makes them potential alternatives to conventional food preservatives [16,17].

The aim of this study was to evaluate the radical scavenging activity, ferric reducing antioxidant power (FRAP), and total polyphenol content (TPC) of two different CEO and eugenol, considering their potential application as natural antioxidants in food products.

MATERIALS AND METHODS

Chemicals

Clove essential oil (CEO₁) was obtained from the local market (*Probotanic, Serbia*). Clove oil (CEO₂) and eugenol were purchased from *Sigma-Aldrich*. The other chemicals used in this study were Folin-Ciocalteu reagent (*Carlo Erba*), 96% ethanol (*Betahem*), gallic acid (*Merck*), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (*Sigma-Aldrich*), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (*Thermo Fisher Scientific*), disodium hydrogen phosphate (*Superlab*), potassium dihydrogen phosphate (*Merck*), potassium persulfate (*Riedel de Haën*), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) (*Sigma-Aldrich*), iron(III) chloride hexahydrate (*Moss&Hemoss*). Sodium chloride, potassium chloride, sodium acetate, acetic acid, hydrochloric acid, and iron (II) sulfate heptahydrate were purchased from *Zorka Pharma*.

Antioxidant activity assays

Determination of total polyphenols

To determine the total polyphenol content (TPC), clove essential oils (CEO) and eugenol were diluted in ethanol at concentrations ranging from 0.0001% to 10%. In a 96-well

microplate, 20 µl of each sample dilution was mixed with 160 µl of distilled water and 10 µl of Folin-Ciocalteu reagent. After 1 minute, 20 µl of sodium carbonate solution (7.5% m/v) was added, and the mixture was incubated in the dark for 1 hour. Absorbance was measured at 750 nm using a microplate reader, against a blank containing 20 µl ethanol instead of the sample.

A calibration curve was prepared by mixing various concentrations of gallic acid (0.05–1.2 mg/ml) with the reagents described above. TPC was expressed as mg gallic acid equivalents (GAE) per 1 ml of undiluted CEO or eugenol.

DPPH radical scavenging activity

To determine DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity, a 200 µM DPPH solution was prepared in 96 % ethanol. In a 96-well microplate, 250 µl of DPPH solution was mixed with 50 µl of essential oil or eugenol samples diluted in ethanol at various concentrations (0.0001–10%). After incubation in the dark for 1 hour, absorbance was measured at 517 nm against a blank (96% ethanol).

Radical scavenging activity (RSA) for each sample concentration was expressed as the percentage reduction of the initial DPPH concentration, calculated using the following equation:

$$\text{RSA, \%} = (A_c - A_s) / A_c \times 100\% \quad (1)$$

where A_c is the absorbance of the control, which contained 50 µl of 96% ethanol and 250 µl of 200 µM DPPH solution, and A_s is the absorbance of the sample. The concentration required to reduce the initial free radical concentration by 50% (IC_{50}) was calculated using logarithmic interpolation:

$$IC_{50\%} = c_1 \times \left(\frac{c_2}{c_1} \right)^{\frac{\log^{50/RSA_1}}{\log^{RSA_2/RSA_1}}} \quad (2)$$

where c_1 and c_2 are the sample concentrations, and RSA_1 and RSA_2 are their radical scavenging activities.

ABTS radical scavenging assay

First, the ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) radical cation was prepared by mixing 5 ml of 7 mM ABTS solution in phosphate-buffered saline (PBS, 5 mM, pH = 7.4) with 0.088 ml of 140 mM potassium persulfate aqueous solution. The mixture was stored in the dark at 4°C for 24 hours prior to use. To prepare the working solution, the ABTS cation was diluted approximately 50-fold with PBS to achieve an absorbance of 0.750 ± 0.050 at 734 nm. In a 96-well microplate, 250 µl of the ABTS working solution was mixed with 30 µl of essential oil or eugenol samples diluted in ethanol at various concentrations (0.0001–10%). Absorbance was measured after 5 minutes at 734 nm against a blank (PBS).

RSA was expressed as the percentage reduction of the initial ABTS concentration, calculated using equation (1), while the IC_{50} value was determined using formula (2). The control sample was prepared by mixing 30 µl of PBS and 250 µl of ABTS working solution.

The ferric reducing antioxidant power (FRAP) assay

The FRAP reagent was prepared by mixing acetate buffer (pH = 3.6), a 10 mM 2,4,6 – tripyridyl-s-triazine (TPTZ) solution in 40 mM HCl, and a 20 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution in distilled water at a volume ratio of 10:1:1. In a 96-well microplate, 10 μl of the sample (diluted in ethanol at concentrations ranging from 0.0001% to 10%) was mixed with 290 μl of the FRAP reagent. After 5 minutes, the absorbance was measured at 593 nm. The blank consisted of ethanol and the FRAP reagent.

A standard curve was prepared using $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ aqueous solutions at concentrations ranging from 0.1 mM to 2.5 mM. For calibration, 10 μl of each standard solution was mixed with 290 μl of the FRAP reagent. Absorbance was measured after 5 minutes at 593 nm against a blank (10 μl of distilled water and 290 μl of the FRAP reagent). Antioxidant capacity was expressed as mmol of Fe^{2+} equivalents per gram of CEO or eugenol.

RESULTS AND DISCUSSION

The antioxidant activity of clove essential oils (CEO) and eugenol was examined using methods based on different mechanisms, including radical scavenging activity (DPPH, ABTS) and reducing antioxidant power (FRAP). Since the antioxidant effect of essential oils is related to phenolic compounds, total polyphenol content (TPC) was evaluated using the Folin-Ciocalteu method.

The Folin-Ciocalteu method is based on the transfer of electrons from phenolic compounds and other reducing species to molybdenum, forming blue complexes that are measurable spectrophotometrically at 750 nm [18]. The TPC for each sample was calculated using a gallic acid standard curve ($R^2=0.9988$). Eugenol, as a pure phenolic compound, exhibited the highest TPC (585.11 ± 4.47 mg GAE/g). Both CEO showed notable polyphenol content, with CEO₁ displaying higher TPC (472.97 ± 2.90 mg GAE/g) than CEO₂ (442.77 ± 2.80 mg GAE/g). The examined essential oils displayed a greater TPC than that reported by Nada *et al.* (9.32 ± 0.35 mg GAE/g) [19]. Lower TPC values for CEO were also reported by Sarrami *et al.* (345.95 ± 7.85 mg GAE/g) [20], while Zengin *et al.* reported a higher polyphenol content (635.327 ± 11.71 mg GAE/ml) [21]. The variation in the phenol content of essential oils arises from differences in their chemical composition, which depends on the method and conditions of extraction, the biological properties and geographical origin of the plant, as well as the soil quality and climatic conditions where the plant was cultivated [20].

The DPPH radical scavenging activity assay is a colorimetric method that measures the ability of an antioxidant substance to reduce the free radical diphenyl-picrylhydrazyl to its non-radical form. During this reaction, the colour of the free radical changes from purple to yellow and is measured spectrophotometrically at 517 nm [8]. The free radical scavenging activity at various concentrations of the examined samples was calculated using equation (1). Results were expressed as IC_{50} , the concentration of the substance required to decrease the initial free radical concentration by 50%. IC_{50} values were calculated using equation (2). IC_{50} values decrease as antioxidant activity increases; therefore lower values indicate stronger antioxidant activity.

Table 1 Antioxidant activity of clove essential oils and eugenol

	IC _{50%} , µg/ml		FRAP, mmol Fe ²⁺ /g	TPC, mg GAE/g
	DPPH	ABTS		
CEO ₁	75.78 ± 2.34	17.25 ± 0.02	12.85 ± 0.09	472.97 ± 2.90
CEO ₂	96.80 ± 2.86	28.89 ± 0.87	10.71 ± 0.19	442.77 ± 2.80
Eugenol	66.24 ± 1.83	17.30 ± 0.75	16.69 ± 0.29	585.11 ± 4.47

The results of the DPPH assay are presented in Table 1. Eugenol exhibited stronger DPPH radical scavenging activity than both CEO, with the lowest IC₅₀ value (66.24 ± 1.83 µg/ml). CEO₁ (75.78 ± 2.34 µg/ml) had a higher IC₅₀ than eugenol, but displayed a stronger radical scavenging effect than CEO₂ (96.80 ± 2.86 µg/ml). The DPPH results were consistent with the TPC results. The correlation between the DPPH radical scavenging activity and the TPC is due to the ability of antioxidants to neutralize free radicals, such as DPPH, by donating a hydrogen atom from the phenolic hydroxyl group [9,10].

The ABTS radical scavenging activity assay measures the ability of antioxidant compounds to neutralize the intensely blue-coloured ABTS radical cation. The decrease in colour intensity depends on the antioxidant effect and can be monitored spectrophotometrically at 734 nm. Because the ABTS cation is soluble in both water and organic solvents, this method enables the determination of both lipophilic and hydrophilic compounds [22]. Radical scavenging activity was determined using equation (1) and used to calculate IC₅₀ with formula (2). Results are shown in Table 1. CEO₂ displayed the lowest antioxidant activity, with the highest IC₅₀ value (28.89 ± 0.87 µg/ml). Unlike the DPPH assay results, the IC₅₀ values for eugenol (17.30 ± 0.75 µg/ml) and CEO₁ (17.25 ± 0.02 µg/ml) were very similar. This inconsistency between methods is due to the fact that ABTS radicals are more reactive than DPPH radicals. Reactions with ABTS radicals involve both hydrogen atom and electron transfer, whereas reactions with DPPH radicals involve only hydrogen atom transfer [5,17]. Therefore, complex mixtures such as essential oils are more sensitive to the ABTS assay than to DPPH. In addition, the ability of CEO to scavenge free radicals may be stronger than that of eugenol due to the synergistic effect of bioactive compounds, such as eugenol acetate and β-caryophyllene [2,15].

The results obtained for the ABTS and DPPH assays were consistent with previous reports on the radical scavenging activity of CEO. Depending on the extraction procedure and resulting chemical composition, reported IC₅₀ values for DPPH ranged from 0.2 to 170.5 µg/ml for CEO and from 4.4 to 130.5 µg/ml for eugenol [23, 24]. Higher IC₅₀ values for CEO in both assays were reported by Olszowy *et al.* (IC₅₀ = 0.32 ± 0.02 mg/ml for DPPH and IC₅₀ = 0.16 ± 0.01 mg/ml for ABTS) [25]. Thongmee *et al.* reported stronger radical scavenging activity for CEO in both assays (IC₅₀ = 3.54 ± 0.2 µg/ml for ABTS and IC₅₀ = 8.70 ± 2.26 µg/ml for DPPH) [26]. These differences can be attributed to varying reaction conditions and the chemical composition of the essential oils.

The FRAP assay relies on the ability of antioxidants to reduce the yellow Fe³⁺-TPTZ complex to the blue Fe²⁺-TPTZ complex through electron transfer. The reducing capacity of a compound is an important indicator of its antioxidant activity, as electron-donating properties enable the neutralization of free radicals [17]. The intensity of the blue-coloured complex was measured spectrophotometrically at 593 nm, and the antioxidant capacity was calculated

using a calibration curve ($R^2 = 0.9994$). The results, expressed as mmol Fe^{2+} equivalents per gram of undiluted sample, are presented in Table 1.

Eugenol showed the strongest reducing power (16.69 ± 0.29 mmol Fe^{2+}/g), followed by CEO₁ (12.85 ± 0.09 mmol Fe^{2+}/g), while CEO₂ (10.71 ± 0.19 mmol Fe^{2+}/g) displayed the lowest activity. These results indicate strong reducing capacity and are consistent with the DPPH radical scavenging activity and TPC. Costa *et al.* reported similar findings (at a sample concentration of 25 $\mu\text{g}/\text{ml}$, 285.62 μM Fe^{2+} for eugenol and 270.79 μM Fe^{2+} for CEO) [27]. However, the values in the present study are significantly higher than those reported by Olszowy *et al.* ($62,273 \pm 3,000$ μmol $\text{Fe}^{2+}/100$ g essential oil) [25]. Olszowy *et al.* also documented higher IC_{50} values for both radical scavenging activity assays, indicating lower overall antioxidant activity. Similarly, Sarrami *et al.* reported lower FRAP values (112.37 ± 8.81 mM Fe^{2+}), higher IC_{50} values for the DPPH assay (0.83 mg/ml), and lower total polyphenol content (345.95 ± 7.85 mg GAE/g) [20]. The high variability in the results may be due to differences in the chemical composition of the examined essential oils and variations in reaction conditions, such as incubation time and sample-reagent volume ratio. Additionally, the use of an aqueous FRAP reagent presents a limitation for analysing hydrophobic essential oils, in contrast to the DPPH reagent, which is diluted in an organic solvent. Furthermore, overestimated FRAP values have been reported for different substances, indicating that there is no relationship between the Fe^{2+} equivalent value and the number of electrons an antioxidant can donate [18]. This may be caused by the formation of oxidation products that absorb at 593 nm, increasing the measured absorbance [28].

In all assays, except for ABTS, eugenol exhibited the strongest antioxidant activity, indicating its role as the primary contributor to the antioxidant activity of CEO. Both CEO demonstrated strong antioxidant activity, suggesting a high eugenol content. However, CEO₁ showed slightly higher antioxidant power across all assays. This is because the antioxidant activity of essential oils depends on their chemical composition, which is influenced by various factors, such as extraction methods, genetics, planting area, and the phenological stage of the clove tree [18,29].

CONCLUSION

The results of this study show that clove essential oil (CEO) and its major constituent, eugenol, have a high polyphenol content and the ability to scavenge free radicals and reduce ferric ions. Eugenol displayed the strongest DPPH radical scavenging activity and electron-donating properties, indicating its role as the primary contributor to the antioxidant activity of CEO. Both essential oils demonstrated strong ferric reducing and radical scavenging activities, suggesting a high eugenol content. The observed differences between the two CEO in this study confirm that the antioxidant activity of essential oils derived from the same plant species may vary, most likely as a result of differences in their chemical composition. For a more precise characterization of essential oils, gas chromatography - mass spectrometry (GC-MS) analysis should be employed. The results highlight the potential application of CEO and eugenol as natural antioxidants in food products. Due to their strong aroma and high volatility, further research should focus on encapsulation strategies.

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