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Evaluation and improvement of antioxidant and antibacterial activities of supercritical extracts from clove buds

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ABSTRACT

For the first time, extracts from clove buds obtained by supercritical carbon dioxide extraction were screened for antioxidant and antibacterial activities. Additionally, antioxidant and antibacterial activities of extracts obtained by the supercritical extraction of the clove bud–oregano leaf mixtures were studied. Supercritical extract of pure clove had the highest eugenol (64%) and total phenolic content (530.56 mg GAE/$g_{extract}$). All extracts had antioxidant activity comparable to synthetic antioxidants against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals and formation of peroxides. Presence of 0.6% and 5% of oregano extract in the clove extracts obtained from the clove–oregano plant mixtures improved their antioxidant activity with respect to the extract from pure clove. Clove extract showed moderate antibacterial activities against selected *Staphylococcus* and *Enterococcus* bacterial strains. Presence of 50% of the oregano extract improved antibacterial activity of clove extract against all tested strains and resulted in a synergistic antibacterial activity against Methicillin-resistant *Staphylococcus haemolyticus* strain (MIC $\leqslant$ 1.25 μg/mL). Study demonstrated great potential of supercritical clove extract as natural functional ingredient and the possibility of increasing its antioxidant and antibacterial efficiencies in order to apply lower concentrations and to reduce undesirable flavour notes and toxicological effects in final products.

1. Introduction

Growing interest in functional food ingredients that may impart health-promoting effects other than general nutrition and increasing concern about potentially harmful synthetic additives led to the increasing interest in the use of natural extracts and their components as functional ingredients in foods, drinks, toiletries and health-care products (Sacchetti et al., 2005; Siriamornpun, Kaisoon, & Meeso, 2012; Vulić et al., 2012). Extracts from herbs and spices with a pleasant taste or smell combined with a preservative action against lipid oxidation and spoilage by microorganisms are commonly needed for application within a wide range of the above mentioned products (Dziri et al., 2012; Sacchetti et al., 2005).

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In terms of world trade value, flower buds of clove trees (*Syzygium aromaticum*, syn. *Eugenia caryophyllata*) today represent one of the most important spices used primarily in food industry and though less frequently, in pharmaceutical industry and perfumery. Clove buds comprise around 20% of volatile oil rich in eugenol (85–95%) (Hemaiswarya & Doble, 2009). The United States Food and Drug Administration (FDA) categorizes clove oil and eugenol as generally recognized as safe (GRAS) for use as a food additive or in a dental cement (U.S. Code of Federal Regulations, 21CFR184.1257). Clove bud essential oil obtained by hydrodistillation, extracts isolated from clove buds using organic solvents and their major component eugenol still remain a research priority due to their wide range of pharmacological and biological activities such as antioxidant (Gülçin & Aboul-Enein, 2012; Mastelić et al., 2008; Politeo, Jukic, & Milos, 2010), antibacterial (Hemaiswarya & Doble, 2009; Hoque, Bari, Juneja, & Kawamoto, 2008; Michiels, Missotten, Fremaut, De Smet, & Dierick, 2007), antifungicidal (Omidbeygi, Barzegar, Hamidi, & Naghdibadi, 2007), antiviral, anticarcinogenic and antimutagenic, anesthetic, repellent (Chaieb et al., 2007) and antiprotozoal (Machado et al., 2011) effects.

Nowadays, food and pharmaceutical industries are challenged to detect and eliminate adulterants from foods, food ingredients including functional foods, dietary supplements, drugs, and excipients (Griffiths, Abernethy, Schuber, & Williams, 2009). In this context, supercritical fluid extraction (SFE) with carbon dioxide as environmentally friendly "green" technique has received particular attention in new and improved applications in food and pharmaceuticals processing. Reportedly, the SFE is considered to be the optimal process for isolation of high quality essential oil from clove buds with many important advantages over traditional methods including the absence of traces of organic solvents, higher extraction yields and possibility of concentrating the active principles such as eugenol and eugenol acetate (Della Porta et al., 2007; Ivanovic, Zizovic, Ristic, Stamenic, & Skala, 2011; Wenqiang, Shufen, Ruixiang, Shaokun, & Can, 2007). However, none of the existing studies on SFE from clove buds considered investigation of biological activities of obtained extracts and to the best of our knowledge there are no data in the available literature on the antioxidant and antibacterial activities of clove bud extracts obtained by SFE. Therefore, the primary goal of the present study was to investigate antioxidant and antibacterial activities of supercritical extracts from clove buds for their possible application as functional ingredient in food, drinks and health-care products.

A recent study (Ivanovic et al., 2011) demonstrated that the extraction rate of the SFE from clove buds can be doubled when small and defined amounts of oregano leaves are added to the clove buds prior to extraction. Thereby, the extraction yield and chemical composition of the obtained clove extracts were negligibly affected. As an extension to the aforementioned study, the second task of the present work was to investigate the influence of adding of oregano leaves to clove buds on antioxidant and antibacterial properties of the supercritical extracts obtained from the clove bud–oregano leaf mixtures.

2. Materials and methods

2.1. Plant material

Dried clove buds (*Eugenia caryophyllata*) originated from Canary Islands (Spain) and leaves of oregano (*Origanum vulgare* L.) grown in Zrenjanin-Čenta (northern Serbia) were purchased from the local markets in Belgrade (Serbia). Air-dried plant material was ground and sieved and fraction with average particle diameter of 0.40 ± 0.10 mm was used for the experiments. The moisture content of the clove buds and oregano leaves determined by Karl Fischer volumetric titration was 8.78% and 9.70%, respectively.

2.2. Chemicals and reagents

The compounds 1,1-diphenyl-2-picrylhydrazyl (DPPH) and gallic acid were acquired from Sigma–Aldrich Chemie GmbH (Taufkirchen, Germany). Folin–Ciocalteu reagent was purchased from Merck and Co., Inc. (New York, NY, USA). All other chemicals and solvents used for analytical and antioxidant activity assays were of the highest commercial grades bought from Lachema Ltd. (Brno, Czech Republic) and Fluka Chemie GmbH (Buchs, Switzerland).

2.3. Supercritical fluid extraction (SFE) procedure

The SFE procedure and laboratory scale extraction unit have recently been described elsewhere (Ivanovic et al., 2011). All extractions were performed under the 10 MPa and 40 °C. Simultaneous SFE of clove buds–oregano leaves mixtures comprising 10%, 50% and 95% of oregano were used to obtain extracts with 0.6%, 5% and 50% of oregano extract ($C_{0.6}$, C_5 and C_{50}, respectively). The calculation was conducted on the basis of experimentally determined extraction yields from pure clove and oregano at the same conditions (Ivanovic et al., 2011). The process of simultaneous SFE was chosen due to the effect of extraction rate enhancement during the extraction from the mixture due to the cosolvent effect of carvacrol on the eugenol extraction rate (Ivanovic et al., 2011). Otherwise, mixing of the calculated quantities of the clove and oregano supercritical extracts would give the extracts of similar chemical compositions. All experiments were performed until the plant material was exhausted and the maximal extraction yields were determined after consumption of 94.3 kg CO_2/$kg_{plant\ material}$ (5.0 ± 0.5 h of extraction). The mass flow rate of the CO_2 was 0.62 ± 0.06 kg/h. All the experiments were carried out in triplicate.

2.4. Determination of total phenolic content (TPC)

The total phenolic content (TPC) in the extracts was determined by a modified version of the method of Folin–Ciocalteu (Singleton & Rossi, 1956). Methanol solutions (100 μL) of the investigated extracts (1 mg oil per 1 mL methanol) were shaken for 1 min on the Vortex mixer with 500 μL of Folin–Ciocalteu reagent and 6 mL of methanol. After the mixture was shaken, 2 mL of 15% Na_2CO_3 were added and the mixture was shaken once again for 0.5 min. Finally, the solution was brought up to 10 mL by adding distilled water. After 2 h, the

absorbance was read on the UV/visible spectrophotometer (Ultrospec 3300 pro, Amersham Bioscience, Uppsala, Sweden) at 750 nm (25 °C) using glass cuvettes against a blank (100 μL of distilled water instead test samples). The TPC was assessed by plotting the gallic acid calibration curve (from 1 to 1500 μg/mL) and expressed as milligrams of gallic acid equivalents (GAE) per gram of extract.

2.5. Analytical procedures

2.5.1. Gas chromatography (GC/FID)

Gas chromatographic analyses of the extracts were carried out on the HP-5890 Series II GC apparatus [Hewlett–Packard, Waldbronn (Germany)], equipped with split–splitless injector and automatic liquid sampler, attached to HP-5 column (25 m × 0.32 mm, 0.52 μm film thickness) and fitted to flame ionization detector (FID). Carrier gas flow rate (H_2) was 1 mL/min, split ratio 1:30, injector temperature was 250 °C, detector temperature 300 °C, while column temperature was linearly programmed from 40 to 260 °C (at rate of 4 °C/min), and then kept isothermally at 260 °C for 10 min. Solutions of extracts' samples in chloroform were consecutively injected in amount of 1 μL. Area percent reports, obtained as a result of standard processing of chromatograms, were used as base for the quantification analysis.

2.5.2. Gas chromatography/mass spectrometry (GC/MS)

The same analytical conditions as those mentioned for GC/FID were employed for GC/MS analysis, along with column HP-5MS (30 m × 0.25 mm, 0.25 μm film thickness), using HP G 1800C Series II GCD system (Hewlett–Packard, Palo Alto, CA, USA). Helium was used as the carrier gas. Transfer line was heated at 260 °C. Mass spectra were acquired in EI mode (70 eV); in *m/z* range 40–450. The amount of 0.2 μL of sample solution in chloroform was injected. The components of the extracts were identified by comparison of their mass spectra to those from the available Wiley 275 and NIST/NBS libraries, using different search engines. The experimental values for Kovats retention indices were determined by the use of calibrated Automated Mass Spectral Deconvolution and Identification System Software (AMDIS ver. 2.1), compared to a previously available literature (Adams, 2007) and used as additional tool to approve MS findings.

2.6. Determination of DPPH radical scavenging capacity

The free radical scavenging capacities of the supercritical extracts were determined using the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical according to the previously described method (Cuendet, Hostettmann, & Potterat, 1997). The aqueous solutions of the extracts (50 μL) with different concentrations (1, 2, 5, 10 and 20 mg/mL) were further dissolved in methanol to obtain the following concentrations: 10, 20, 50, 100 and 200 μg/mL. After that 1 mL of 0.2 mM DPPH in methanol was added (0.0078 g of pure DPPH (M_W = 394.32 g/mol) in 10 mL of methanol). The solutions so obtained were vigorously shaken and left at room temperature in the dark. After 30 min of incubation in the dark at room temperature, the absorbance was measured against a blank (methanol) at 517 nm using the UV/visible spectrophotometer (Ultrospec 3300 pro, Amersham Bioscience, Uppsala, Sweden). Inhibition of DPPH radical was calculated as a percentage (%) using Eq. (1):

$$\text{Percentage inhibition}\ (\%) = [(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \cdot 100 \quad (1)$$

where, A_{control} is the absorbance of the control reaction (containing all reagents except tested extract), and A_{sample} is the absorbance of the tested extract. IC_{50} (concentration of extract required to scavenge 50% of free radicals) was calculated from the regression equation, prepared from the concentration of samples and percentage inhibition of free radical formation (percentage inhibition of the DPPH was assayed). L-Ascorbic acid was used as a positive control and all tests were carried out in triplicates.

2.7. Determination of the ferric reducing antioxidant power (FRAP)

Method for determination of the ferric reducing antioxidant power (FRAP) of the clove supercritical extracts described previously (Szöllösi & Szöllösi Varga, 2002) was used to assess overall antioxidant activity. To prepare the FRAP reagent, a mixture of 300 mmol/L acetate buffer pH 3.6 (containing 6.4 mL 2 M sodium acetate solution and 93.6 mL 2 M acetic acid solution diluted in a volumetric flash (1 L)), 10 mmol/L of 2, 4, 6-tripyridyl-S-triazine (TPTZ) in 40 mmol/L HCl and 20 mmol/L ferric chloride (10:1:1, v/v/v) was made. Methanol solution of plant extracts (150 μL) were mixed with 4.5 mL of FRAP reagent. In the FRAP method, the yellow Fe^{3+}–TPTZ complex is reduced to the blue Fe^{2+}–TPTZ complex by electron-donating substances under acidic conditions. Any electron donating substance with a half reaction of lower redox potential than Fe^{3+}/Fe^{2+}–TPTZ will drive the reaction and the formation of the blue complex forward. The absorbance readings started after 5 min at 593 nm using a spectrophotometer (Ultrospec 3300 pro, Amersham Bioscience, Sweden). The blank probe consisted of FRAP reagent. The final absorbance of each sample was compared with those obtained from the standard curve made from ferric sulphate ($FeSO_4 \times 7H_2O$) (200–1000 μmol/L). The results were expressed in μmol Fe^{2+}/g of extract.

2.8. Peroxide value (PV)

In order to measure peroxide value (PV) 50 μL of extracts' samples were added to 50 g of unrefined sunflower oil placed in 150 mL beakers. The commercial antioxidant butylated hydroxytoluene (BHT) (1 mg) was used as a positive control and pure unrefined sunflower oil was used as a negative control. The beakers were covered with pitted foil and placed at 60 °C in incubators with artificial light. Measuring of PV was performed every day during 7 days period. Measurement of the PV of the oils was performed according to the AOAC (1989) Official Method Cd 8b-53 except that 0.01 instead of 0.1 mol/L of $Na_2S_2O_3$ standard solution was used as the titrant (Karakaya & Şimşe, 2011).

2.9. Statistical analyses

All measurements for determination of TPC and antioxidant activity of tested samples were performed in triplicate. The

mean value and standard deviation were calculated using the Microsoft Excel software (Microsoft Corporation, Redmond, WA, USA). A one-way ANOVA (analysis of variance) method followed by post hoc Tukey's test was used to evaluate the significant difference among various treatments with the criterion of $P < 0.01$.

2.10. Determination of antibacterial activity

Enterococcus and *Staphylococcus* species isolated from clinical and food specimens as well as one reference strain from the American Type Culture Collection (ATCC) were used for investigation of antibacterial activity of the obtained extracts. For the purpose of isolation and identification of bacteria, conventional microbiological methods were applied with usage of commercial kits for species identification-BBL Crystal Gram-positive ID kit (Becton Dickinson). For the detection of Methicilin-resistant staphylococci strains, cefoxitin discs were used (Becton Dickinson) in routine disc diffusion investigations, and confirmation was achieved by detecting *mec*A gen using PCR according to previously described protocols (Isenberg, 2004). For investigation of antibacterial activity of extracts, broth microdilution method was applied for determining MIC (minimal inhibitory concentration) values and in accordance with the CLSI recommendations (2003). Cation adjusted Mueller Hinton II broth was used (CAMHB, Becton Dickinson) and antibacterial activity of the extracts were investigated were investigated at concentrations of 1280, 640, 320, 160, 80, 40, 20, 10, 5, 2.5, 1.25 and 0.625 μg/mL. The desired inoculum's density of 5×10^5 CFU/mL was achieved by preparing the suspension of bacteria of approximately 1–2×10^8 CFU/mL, which was the density equal to McFarland standard 0.5 (Becton Dickinson). The prepared suspension was diluted 10 times, to obtain final inoculum density of approximately 1–2×10^7 CFU/mL. Fifty microliters of this suspension was applied to CAMHB, to obtain the number of bacteria in the media of approximately 5×10^5 /mL. The media were incubated on 37 °C for 18 h. For MIC values the broth with the lowest concentration of extract, with no visible bacterial growth, was used.

3. Results and discussion

3.1. Total phenolic content

Antioxidant properties of plant extracts are commonly associated with the presence of phenolic compounds possessing the ability to donate hydrogen to the radical. The total phenolic content and antioxidant activities of the extracts isolated from pure clove and clove–oregano mixtures are given in Table 1. Total phenolic content (TPC) in the obtained supercritical extracts determined by the Folin–Ciocalteu method was in the range of 161.98–530.56 mg GAE/g of extract (Table 1). The highest TPC was reported for the pure supercritical extract of clove buds (530.56 mg GAE/g).

As can be seen from Table 1, the TPC of the extracts decreased with the increase of the fraction of oregano extract (Table 1). The presence of 50% of oregano extract in the total extract resulted in even three times lower TPC compared to the pure clove extract. This was expected due to the reported lower TPC of oregano oil compared to clove oil (Viuda-Martos et al., 2011). Presence of the smaller amount of the supercritical oregano extract (0.6%) reduced the TPC for 5.56% compared to the pure clove extract.

Phenolic compounds are considered to be responsible for health promoting effects due to a broad range of pharmacological and biological activities. Because of their versatile chemical structures, phytochemicals present in the clove and oregano extracts can possess different pharmacological and biological activities (Bakkali, Averbeck, Averbeck, & Idaomar, 2008; Mastelić et al., 2008; Michiels et al., 2007; Santoyo et al., 2006). Therefore, particular phenolic compounds present in the supercritical extracts of clove and clove–oregano mixtures were further identified and quantified.

3.2. Identification and quantification of active compounds

Major compounds in the supercritical extracts were presented in Table 2. Supercritical extract of clove comprised high content of phenolic monoterpene alcohol eugenol (64%) and its derivative eugenyl acetate (20%). Phenolic monoterpene alcohol carvacrol was found to be the main compound of the supercritical extract isolated from oregano (57%). Chemical compositions of the clove extracts containing 0.6% and 5% of oregano extract ($C_{0.6}$ and C_5, respectively) were similar to the composition of the extract from pure clove (C) (Table 2). Contents of eugenol and eugenyl acetate in the $C_{0.6}$ were decreased for 1.7% and 6.1%, respectively, with respect to the extract from pure clove. In the C_5, eugenol and eugenyl acetate contents were reduced for 1.3% and 10%, respectively, in comparison to the extract isolated from pure clove buds.

3.3. DPPH radical scavenging capacity

The antioxidant activities of six different concentrations (5, 10, 20, 50, 100 and 200 μg/mL) of the supercritical extracts

Table 1 – Total phenolic content (TPC) and antioxidant activities of the supercritical extracts of clove.

Sample	Fraction of oregano extract (%)	Eugenol (%)	Carvacrol (%)	TPC (mg GAE/$g_{extract}$)	IC_{50} (μg/mL)	FRAP (μmol Fe^{2+}/$g_{extract}$)
C	0	64.1	–	530.56 ± 0.09[a]	20.64 ± 0.15[a]	1040.73 ± 2.00[a]
$C_{0.6}$	0.6	63.0	0.3	500.41 ± 0.24[b]	14.92 ± 0.30[b]	1244.80 ± 1.53[b]
C_5	5	60.1	2.8	317.44 ± 0.20[c]	11.17 ± 0.35[c]	613.00 ± 0.53[c]
C_{50}	50	44.2	17.5	161.64 ± 0.25[d]	42.29 ± 0.36[d]	277.67 ± 0.87[d]
L-Ascorbic acid		–	–	–	6.29 ± 0.26[e]	–

Values marked with different letters (a, b, c, d and e.) within column are significantly different ($P = 0.01$).

Table 2 – Content of the main active principles in obtained supercritical extracts.

Plant material		Clove	Clove–oregano mixture			Oregano
Oregano fraction in feed[a] (%)		0	10	50	95	100
Fraction of oregano extract in final extract[b] (%)		0	0.6	5	50	100
Sample		C	$C_{0.6}$	C_5	C_{50}	O
Components	RI	Content (%, w/w)				
Thymoquinone	1248	–	0.2	0.2	1.4	13.3
Thymol	1289	–	0.1	0.3	0.5	6.0
Carvacrol	1298	–	0.3	2.8	17.5	57.3
Eugenol	1356	64.1	63.0	60.1	44.2	–
trans-Caryophyllene	1417	13.0	12.7	11.4	8.2	1.0
Eugenyl acetate	1521	19.9	19.7	17.9	13.6	0.1
Waxes		Traces	0.3	0.7	4.5	4.9
Total identified (%)		96.9	96.2	93.5	89.7	82.6

[a] Mass percent of the oregano leaves in the extracted clove buds/oregano leaves mixture.
[b] Mass fraction of the supercritical extract of oregano in the final supercritical extract of clove buds was calculated on the basis of experimentally determined extraction yields of pure clove and oregano supercritical extracts (18.2% and 1%, respectively).

were measured in terms of hydrogen donating or radical scavenging ability, using stable DPPH radical. This method is considered to be a valid and easy assay to evaluate scavenging activity of antioxidants, since the radical compound is stable and does not have to be generated as in other radical scavenging assays. The radical scavenging effects of the tested supercritical extracts as well as L-ascorbic acid on the DPPH radical were concentration dependent (Fig. 1). All samples with the highest concentration of extracts (200 μg/mL) had strong scavenging effect comparable to the L-ascorbic acid, showing DPPH radical inhibition ability ranged from 83.3% to 94.5%.

As can be seen from Fig. 1, supercritical extracts containing 0.6% and 5% of oregano extract ($C_{0.6}$ and C_5) had superior scavenging effect than the one isolated from pure clove (C). Lower antioxidant activity of C_{50} is expected due to the previously reported superior DPPH radical-scavenging activity of clove oil compared to oregano oil (Viuda-Martos et al., 2011). Improved DPPH scavenging effect of clove extracts with 0.6% and 5% of oregano extract was observed for all tested sample concentrations (5–200 μg/mL). Moreover, the C_5 extract effectively scavenged free radicals (80%) even at the lower concentration (20 μg/mL) (Fig. 1). C_5 and $C_{0.6}$ extracts showed superior DPPH scavenging activity despite lower total phenolic and eugenol content compared to the pure supercritical clove extract (Tables 1 and 2) and there was no correlation between TPC and DPPH radical scavenging activity. There are several possible causes of the ambiguous relationship between the antioxidant activity and TPC. The applied method for TPC determination does not include all compounds with antioxidant activity. The antioxidant activity is not only dependent on the concentration but also on the structure and interactions among antioxidant compounds. Finally, carvacrol and thymol (mainly present in oregano) can act as pro-oxidants at high concentrations (Bakkali et al., 2008). This could be the reason for the decrease of radical scavenging activities of clove extracts with higher percentage of oregano extract.

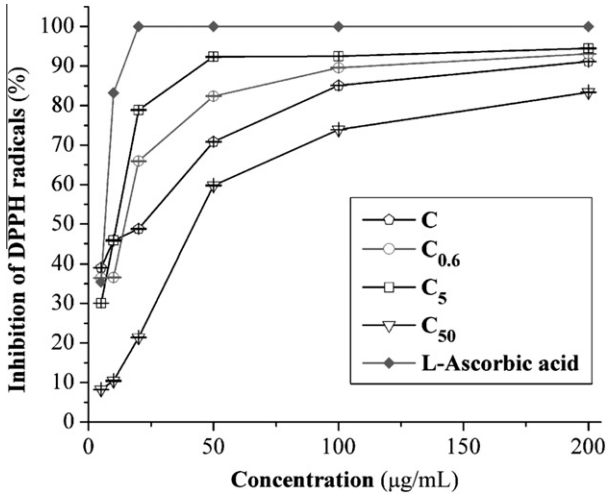

Fig. 1 – Scavanging activity against DPPH radicals.

In accordance with the DPPH scavenging ability of the tested extracts, IC_{50} values increased in the following order: L-ascorbic acid < C_5 < $C_{0.6}$ < C < C_{50} (Table 1). Value of IC_{50} (20.64 μg/mL) for supercritical extract of pure clove was similar to that previously reported for clove volatile oil obtained by hydrodistillation (21.50 μg/mL) (Gülçin & Aboul-Enein, 2012). This could be due to the fact that clove supercritical extract contained mostly essential oil components (97%) (Table 2). In same study, IC_{50} for clove oil was lower than that for BHT, α-tocopherol, butylated hydroxyanisole (BHA) and trolox.

3.4. *FRAP method*

Results of the measurement of the total antioxidant capacity of the supercritical extracts applying the FRAP method are presented in Table 1. This method is based on the ability of antioxidants to reduce Fe^{3+} to Fe^{2+} in the presence of the TPTZ forming an intensely blue coloured Fe^{2+}–TPTZ complex with an absorbance maximum at 593 nm. The reducing power of the extracts demonstrates their ability for donating electron and neutralizing free radicals by forming stable products.

As can be seen from Table 1, the ferric reducing antioxidant ability increased in the following order of $C_{50} < C_5 << C < C_{0.6}$. The presence of small amounts of oregano extract (0.6%) in the supercritical extract resulted in improved antioxidant power in comparison with the extract of pure clove. On the other hand, supercritical extract with the highest amount of oregano extract (C_{50}) showed the lowest ferric reducing antioxidant ability. This could be due to the previously demonstrated lower ferric reducing capacity of oregano oil compared to clove oil (Gülçin & Aboul-Enein, 2012; Politeo et al., 2010; Viuda-Martos et al., 2011).

3.5. *Peroxide value*

Edible oils, fats and fatty foods, in the presence of oxygen from the air, undergo autoxidation processes over time due to external factors such as heat, light, enzyme and traces of metals. Consequently, the breakdown products such as peroxides, aldehydes and ketones are formed causing unpleasant taste and smell due to oil rancidity and turning products unacceptable for consumption. Peroxide value (PV) is widely used for measuring the concentration of peroxides and hydroperoxides formed in the initial stages of lipid oxidation. The PV of the supercritical extracts measured by titration with iodide ion are expressed as the amount of peroxide oxygen in mmol per 1 kg of sunflower oil and given in Fig. 2.

During seven days of storage at 60 °C tested extracts showed significant effectiveness against lipid peroxide formation in the initial stage of autoxidation of sunflower oil comparable to BHT (Fig. 2). After the 7th day of storage, samples containing 0.6% and 5% of oregano extract had lower peroxide values compared to the pure clove supercritical extract. Moreover, supercritical extract with 0.6% of oregano extract was more effective than BHT.

The results of comparative analysis of antioxidant potential for all the applied methods are summarized in Table 3.

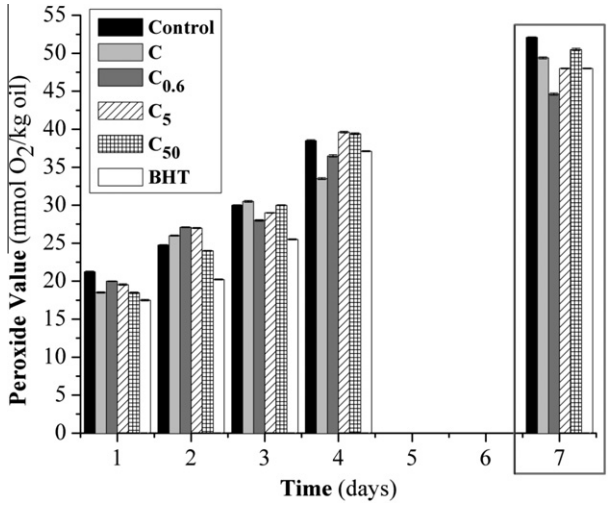

Fig. 2 – The effect of various fraction of oregano extract in the supercritical clove extracts on the PV of sunflower oil at 60 °C.

All the antioxidant assays demonstrated that the presence of 0.6% of oregano supercritical extract in the total extract enhanced its antioxidant activity. The DPPH and PV assays showed that the presence of 5% of the oregano extract in the total extract also improved its antioxidant activity. Therefore, it can be concluded that the presence of small quantity of oregano extract (0.6%) in supercritical clove extract is favourable since it had little influence on its TPC and composition (Tables 1 and 2) and enhanced its antioxidant activity from 10% to 27.7% depending on applied method for determination of antioxidant properties (Table 3).

3.6. *Antibacterial activity*

Antibacterial activity of the supercritical extracts against selected staphylococci and enterococci has been investigated. Values of MIC for the representative samples are presented in Table 4.

As can be seen, the pure clove supercritical extract showed moderate antibacterial activity against tested *Staphylococcus* and *Enterococcus* species including MRSA strains with MIC values in the range of 320–1280 μg/mL. Supercritical extract obtained from the pure oregano showed strong antibacterial effect against staphylococci (MIC = 10–20 μg/mL), including MRSA strain (MIC = 10 μg/mL). The tested enterococci were also highly susceptible to the supercritical oregano extract (MIC = 40–80 μg/mL). Stronger antibacterial potential of the oregano oil compared to the clove extract might be due to previously suggested stronger antibacterial activity of carvacrol compared to eugenol (Michiels et al., 2007). Still, the values of MIC for the supercritical clove extract in this study (320–640 μg/mL) were significantly lower than previously reported for the ethanol and aqueous extracts of clove for growth inhibition of four tested *Staphylococcus aureus* strains (1500–2500 μg/mL) (Hoque et al., 2008). This is partially due to the use of higher final inoculum density (approximately 10^6 CFU/mL) in the mentioned work (Hoque et al., 2008) compared to this study where the final bacterial count was 5×10^5 CFU/mL.

The presence of small amounts of oregano extract (0.6% or 5%) did not affect the antibacterial activity of the total extract. In contrast, presence of 50% of oregano extract in total extract (C_{50}) resulted in improved antibacterial activity against all tested strains when compared to pure clove extract (Table 4). Moreover, synergistic antibacterial effect was observed for C_{50} extract against one Methicillin-resistant *Staphylococcus haemolyticus* strain, with MIC $\leqslant$1.25 μg/mL significantly lower compared to MICs for supercritical extract from pure clove (320 μg/mL) and oregano (40 μg/mL) (Table 4). The synergistic effect might be due to the fact that carvacrol disintegrates the outer membrane, thus making it easier for eugenol to enter the cytoplasm and combine with proteins as previously suggested by Pei, Zhou, Ji, and Xu (2009).

Practical implications of improved antioxidant and synergistic antibacterial activities of the clove extract include application of the lower concentration needed to yield a similar antioxidant and/or antibacterial activity and to reduce flavour notes and/or toxicological effects in the given products. The other advantage of the obtained clove extracts with the improved antioxidant and antibacterial activities is possibility

Table 3 – Comparative results on antioxidant potential of the extracts.

Method	Units	Effectiveness	Improvement of antioxidant activity[a] (%)
TPC	mg GAE/g dry extract	$C > C_{0.6} > C_5 > C_{50}$	–
DPPH	IC_{50} (μg/mL)	L-Ascorbic acid > $C_5 > C_{0.6} > C > C_{50}$	C_5 (45.9) $C_{0.6}$ (27.7)
FRAP	μmol Fe^{2+}/g	$C_{0.6} > C > C_5 > C_{50}$	$C_{0.6}$ (20.0)
PV	mmol O_2/kg[b]	$C_{0.6} > C_5$ = BHT > $C > C_{50}$	$C_{0.6}$ (10.0) C_5 (3.0)

[a] Improvement of antioxidant activity expressed in corresponding units compared to the pure clove extract.
[b] Per kg of non refined sunflower oil.

Table 4 – MIC values for the chosen bacterial strains.

	Bacterial strain	MIC (μg/mL)		
	Samples	C, $C_{0.6}$ and C_5	C_{50}	O
1	*Staphylococcus aureus*	640	40	20
2	Methicillin-resistant *Staphylococcus aureus* (MRSA)	320	40	10
3	Methicillin-resistant *Staphylococcus haemolyticus*	320	⩽1.25	40
4	*Staphylococcus aureus* ATCC 11632	640	160	20
5	*Enterococcus* sp.	1280	160	40
6	*Enterococcus* sp.	1280	160	80
7	*Enterococcus* sp.	640	160	40
8	*Enterococcus* sp.	1280	160	80

of their easy and fast isolation by simultaneous supercritical fluid extraction from the mixtures of clove buds–oregano leaves with defined mass fractions of particular plant components.

Further studies could consider beneficial effects of the presence of the oregano extracts in the clove extract on other biological activities of the clove extract and toxicological tests. For the ultimate application of the clove extracts in the particular functional food and pharmaceutical products it would be necessary to investigate the influence of particular parameters of a given medium (pH, fat, protein, water content, incubation time/temperature, packaging procedure, physical structure, etc.) to antioxidant and antibacterial activities of the clove extract.

4. Conclusions

The present study is the first report on the *in vitro* evaluation of the antioxidant and antibacterial activities of supercritical extracts of clove buds. This study has demonstrated great potential of supercritical clove extract as a natural antioxidant with comparable activity to synthetic antioxidants. Additionally, it was proven that the antioxidant activity of the supercritical clove extract can be improved with a small amount of supercritical oregano extract (0.6% and 5%) using simultaneous SFE from the clove–oregano plant mixture. Clove extracts showed moderate antibacterial activities against selected *Staphylococcus* and *Enterococcus* bacterial strains. The presence of small quantities of oregano extract did not affect the overall antibacterial activity, while the presence of 50% of oregano extract improved antibacterial activity against all tested strains. Moreover, it resulted in a synergistic antibacterial activity against Methicillin-resistant *Staphylococcus haemolyticus* strain.

In order to promote practical use of plant extracts as natural antioxidant and/or antibacterial agents, food industry is challenged to develop effective low dose combinations of plant extracts for maintaining product safety and shelf-life with minimal influence on undesirable sensory changes and toxicological effects associated with the addition of high concentrations of plant extracts. In this context, improvement of antioxidant and antibacterial activities of supercritical clove extract suggested in this study could be important for reducing its usage concentrations when it is incorporated as a functional ingredient in foods, drinks and pharmaceuticals.

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