

Could essential oils of green and black pepper be used as food preservatives?

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Abstract Black and green pepper essential oils were used in this study in order to determine the chemical composition, in vitro antimicrobial activity against food spoilage microorganisms and in situ oils effect on food microorganism, after incorporation in chicken soup, by suggested methodology for calculation of Growth inhibition concentrations (GIC₅₀). Chemical analysis revealed a total of 34 components. The major constituent of black pepper oil was *trans*-caryophyllene (30.33 %), followed by limonene (12.12 %), while β -pinene (24.42 %), δ^3 -carene (19.72 %), limonene (18.73 %) and α -pinene (10.39 %) were dominant compounds in green pepper oil. Antimicrobial activity was determined by microdilution technique and minimal inhibitory (MIC) and minimal bactericidal/fungicidal concentrations (MBC/MFC) were determined. Green pepper oil showed stronger antibacterial and antifungal activity (MIC 0.50–1.87; MBC 0.63–2.5 mg/ml; MIC 0.07–0.16; MFC 0.13–1.25 mg/ml) against black pepper oil (MIC 0.07–3.75; MBC 0.60–10.00 mg/ml; MIC 0.63–5.00; MFC 1.25–10.00 mg/ml). Oils successfully inhibited the growth of *S. aureus* in chicken soup in a dose dependent manner. GIC₅₀ values were calculated after 24, 48 and 72 h and were in range of 0.156–0.689 mg/ml. The 50 % inhibitory concentrations (IC₅₀) of

EOs were 36.84 and 38.77 mg/ml with in 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay respectively.

The obtained results revealed that black and green pepper volatiles are efficient in controlling the growth of known food-spoilage microorganisms.

Keywords *Piper nigrum* L · Black and green pepper · Essential oil · Antimicrobial activity · Novel methodology for GIC₅₀ · Liquid food

Introduction

Despite technological development and food production techniques, food safety remains very important public health issue. According to studies more than 9 million persons each year have a food-borne illness caused by a major pathogen (Scallan et al. 2011). In the United States alone, food recalls and food-borne illnesses bear an annual price tag of approximately 77 billion (including discarded product, revenue, healthcare costs, lost wages and litigation and other expenditures (Scharff 2012). At the same time, Western society appears to be experiencing a trend of ‘green’ consumerism (Chaibi et al. 1997), desiring products with a smaller impact on the environment.

Therefore a need for new methodologies for reducing or eliminating food-borne pathogens, possibly in combination with existing techniques is needed. Many naturally occurring products, such as essential oils from edible and medicinal plants, herbs, spices and isolated compounds have been shown to possess antimicrobial functions and could be used as food additives (Stojković et al. 2011, 2013a, b; Bukvički et al. 2014).

Essential oils (EOs) are plants secondary metabolites. They have been used in medicine, perfumery, cosmetic, and have been added to foods as part of spices or herbs. Particularly, application of essential oils as aroma and flavor ingredients increased recently and became their major employment. Almost 3000 different

Highlights · *Piper nigrum* L. is one of the most used spices in the world.
• Black and green pepper essential oils biological evaluation: in vitro and in situ study.
• Novel tool for determination of GIC₅₀ in liquid foods.
• The oils were efficient in controlling the growth of known food-spoilage microorganisms.
• Food-preservative activity exhibited may be valuable for food industry.

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essential oils are known, and 300 are used commercially in the flavor and fragrances market (Burt and Reinders 2003). The use of essential oils is gaining *momentum* (Reische *et al.* 1998) due to the growing consumers demand for the inclusion of natural products and the reduction and elimination of synthetic additives in the food industry. Many authors, in fact, have reported antimicrobial, antifungal, antioxidant and radical-scavenging properties (Škrinjar and Nemet 2009; De Azeredo *et al.* 2012; Millezi *et al.* 2012) by spices extracts and essential oils. Only in some cases a direct food-related application has been tested (Fisher *et al.* 2009; Emiroğlu *et al.* 2010; Stojković *et al.* 2011; Bukvički *et al.* 2014).

Piper nigrum L. is perennial, evergreen woody vines of the family Piperaceae (Srinivasan 2007). Originating from tropical parts of India and Southeast Asia the plant has pleasant smell and more or less bitter taste. There are several types of pepper, black, green, white, orange, red etc. In fact, they all come from the same plant, and differ in the way of preparation for the market, which gives them a different flavor and degree of spiciness. The plant is known to be rich in essential oil (2–3 %) and a source of numerous biologically active constituents such as monoterpenes, sesquiterpenes, and other volatile compounds (Evans 1996; Jagella and Grosch 1999; Chen *et al.* 2011). *P. nigrum* is one of the most used spices in the world (Meghwal and Goswami 2013). Pepper has many medicinal properties and it is used in treatment of rheumatism, diabetes, chronic indigestion, colon toxins, obesity, fever and diarrhea. It is also reported that pepper enhance the bioavailability of food, drug, anti-carcinogens and phytochemicals. According to literature *P. nigrum* sesquiterpenes enhance the trans-dermal delivery of active substances through skin membrane. Also, increased pancreatic activity and stimulation of gastric acid secretion are recorded (Dorman and Deans 2002; Sashidhar 2002). However, little attention has been given to pepper essential oil research and application. As per our knowledge, there is no report on the preservation of chicken soup with pepper essential oil. In this study, two pepper oils were used. Green pepper oil, which is derived from cooked and dried unripe fruit and black pepper oil obtained from the dried unripe fruit.

Hence, the aims of this study were to determine: i) chemical composition by means of gas chromatography/mass spectrometry (GC/MS); ii) *in vitro* antimicrobial activity against food poisoning and food spoilage bacteria/fungi; iii) *in vitro* antiradical properties by using DPPH radical and iii) to investigate oils inhibitory effects on food spoilage microorganism *in situ*, after incorporation in soup. The proposed research evaluates the possibility of using pepper essential oils as food preservatives.

Material and methods

Source of the essential oils

Essential oils of *Piper nigrum* L. were commercial samples. Black pepper and green pepper oils were obtained from SENS

IENT ESSENTIAL OILS GMBH, Flughafendamm 11, D-28199 Bremen, Germany.

Essential oils analysis

Procedure used for gas chromatography-flame ionization detector (GC-FID) and GC-MS analyses complies with standards set for Gas Chromatography of essential oils (ISO 7609:1985; ISO 11024-1:1998; ISO 11024-2:1998).

GC-FID analysis was performed on GC Agilent Technologies 7890A apparatus, equipped with the split-splitless injector and automatic liquid sampler (ALS), attached to HP-5 column (30×0.32 mm, film thickness 0.25 µm) and fitted to FID. Operating conditions were as follows: carrier gas was H₂ (1 ml/min/210 °C); temperatures of injector and detector were set at 250 and 280 °C, respectively, while the column temperature was linearly programmed 40–260 °C at 4 °C/min. Solutions of essential oils' samples in ethanol (ca. 1 %) were consecutively injected by ALS (1 µl, split-mode). The percentile presence of components in essential oils' samples were calculated from the peak areas obtained in the area-percent reports (obtained as a result of standard processing of chromatograms) without correction factors, using normalization method.

The GC/MS was performed on HP G 1800C Series II GCD analytical system equipped with HP-5MS column (30 m×0.25 mm, film thickness 0.25 µm). Carrier gas was He (1 ml/min). Other chromatographic conditions were as those for GC-FID. Transfer line was heated at 260 °C. Mass spectra were recorded in EI mode (70 eV), in a range of m/z 40–450. Solutions of essential oil samples in ethanol (ca. 1 %) were consecutively injected by ALS (0.2 µl, split mode).

The identification of essential oils' components was based on matching of their mass spectra peaks with those from Wiley 275 and NIST/NBS libraries. The experimental values for Kovats' retention indices (RI) were determined by using calibrated Automated Mass Spectral Deconvolution and Identification System software (AMDIS (ver. 2.1.)), compared to those from available literature (Adams 2009), and they were used as additional tool to support MS findings.

Microorganisms and culture conditions

The following Gram-positive bacteria were used: *Staphylococcus aureus* (food isolate), *Bacillus cereus* (clinical isolate), *Listeria monocytogenes* (NCTC 7973), and *Micrococcus flavus* (ATCC 10240), and the following Gram-negative bacteria were used: *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 35210), *Salmonella typhimurium* (ATCC 13311), and *Enterobacter cloacae* (ATCC 35030). For the antifungal bioassays, eight fungi were used: *Penicillium aurantiogriseum* (food isolate), *Aspergillus niger* (ATCC 6275), *Aspergillus ochraceus* (ATCC 12066),

Aspergillus versicolor (ATCC 11730), *Aspergillus fumigatus* (human isolate), *Trichoderma viride* (IAM 5061), *Penicillium funiculosum* (ATCC 36839), *Penicillium ochrochloron* (ATCC 9112). The organisms were obtained from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research “Siniša Stankovic”, Belgrade, Serbia.

Antibacterial assay

The MICs and MBCs were determined using 96-well microtitre plates. The bacterial suspension was adjusted with sterile saline to a concentration of 1.0×10^5 CFU/ml. Essential oils were dissolved in 5 % DMSO solution containing 0.1 % Tween 80 (v/v^{-1}) (100 mg/ml) and added in Tryptic Soy broth (TSB) medium (100 μ l) with bacterial inoculum (1.0×10^4 CFU per well) to achieve the wanted concentrations. Microplates were incubated at a rotary shaker (160 rpm) for 24 h at 37 °C. The following day, 30 μ l of 0.2 mg/ml solution of INT (*p*-iodonitrotetrazolium violet) was added to the plates, followed by ½h incubation so as to ensure adequate color reaction. Inhibition of bacterial growth was indicated by a clear solution or a definite decrease in color reaction (Tsukatani et al. 2012). The lowest concentrations without visible growth (at the binocular microscope) were defined as concentrations that completely inhibited bacterial growth (MICs). The MBCs were determined by serial subcultivation of 10 μ l into microtitre plates containing 100 μ l of broth per well and further incubation for 24 h. The lowest concentration with no visible growth was defined as the MBC, indicating 99.5 % killing of the original inoculum. Streptomycin (Sigma P 7794) and Ampicillin (Panfarma, Belgrade, Serbia) were used as positive controls (1 mg/ml in sterile physiological saline). 5 % DMSO solution containing 0.1 % Tween 80 (v/v) was used as a negative control. All experiments were done in triplicate.

Antifungal activity

In order to investigate the antifungal activity of essential oils, a modified microdilution technique was used. The fungal spores were washed from the surface of agar plates with sterile 0.85 % saline containing 0.1 % Tween 80 (v/v). The spore suspension was adjusted with sterile saline to a concentration of approximately 1.0×10^5 in a final volume of 100 μ l per well. The inocula were stored at 4 °C for further use. Dilutions of the inocula were cultured on solid malt agar to verify the absence of contamination and to check the validity of the inoculum. Minimum inhibitory concentration (MIC) determinations were performed by a serial dilution technique using 96-well microtiter plates. All oils investigated, were dissolved in 5 % DMSO solution containing 0.1 % Tween 80 (v/v) (100 mg/ml) and added in broth Malt medium with inoculum.

The lowest concentrations without visible growth (at the binocular microscope) were defined as MICs. The fungicidal concentrations (MFCs) were determined by serial subcultivation of a 10 μ l of tested compounds dissolved in medium and inoculated for 72 h, into microtiter plates containing 100 μ l of broth per well and further incubation 72 h at 28 °C. The lowest concentration with no visible growth was defined as MFC indicating 99.5 % killing of the original inoculum (Espinel-Ingroff 2001). 5 % dimethyl sulfoxide (DMSO) solution containing 0.1 % Tween 80 (v/v) was used as a negative control, commercial fungicides, bifonazole (Srbolek, Belgrade, Serbia) and ketoconazole (Zorkapharma, Šabac, Serbia), were used as positive controls.

Free radical scavenging activity

The antioxidant activity of black and green pepper oils was determined using the stable radical DPPH method (Blois 1958). Briefly, 500 μ l of various concentrations of EO in methanol (20, 40, 60, 80 mg/ml) were mixed with 500 μ l of 20 μ M methanol solution of DPPH. Absorbance at 517 nm was measured after 30 min in dark. Methanol plus sample solution was used as a blank. DPPH solution and methanol were used as controls. The inhibition percentage was calculated using following formula:

$$\% \text{ inhibition} = \left[\frac{AC-AS}{AC} \right] \times 100$$

where AC is the absorbance of the negative control and As is absorbance of the samples. The results were compared with butylated hydroxyanisole (BHA).

In situ preservation of chicken soup

Isolation and identification of chicken soup contaminant bacterium

Chicken soup used in this study was purchased from the local market. The packaging stated no artificial preservatives. Chicken soup was left open at 25 °C for 3 days. After that period, the experiment with dilutions was repeated to check for contaminants. Mueller–Hinton (MH) plates were inoculated with different dilutions of chicken soup in sterilized water and kept at 37 °C in order to investigate possible bacterial contaminants. Bacterial culture reinoculate in Casein soya bean digest broth, homogenize and incubate at 35 °C to 37 °C for 18 to 48 h. Subculture on a plate of Bair–Parker agar and incubate at 35 °C to 37 °C for 18 to 72 h. Black colonies of gram positive cocci, surrounded by a clear zone indicate the presence of *S. aureus*. After confirmation by suitable biochemical tests such as the coagulase test and the deoxyribonuclease test, the culture was identified by M. Sci.

Tatjana Stević (Institute for Medicinal Plant Research “Dr Josif Pančić”), as *S. aureus* and deposited to Bacteria Collection Unit of the Department for Plant Physiology, Institute for Biological Research “Siniša Stanković”, Belgrade, Serbia, with the number Sa-DS-12.

In situ antibacterial assay in chicken soup

Chicken soup was prepared in sterile conditions and according to the manufacturer instructions. Different concentrations of the oils were added to the chicken soup to achieve final concentrations in range of 0.01–105 mg/ml respectively. The controls contained chicken soup but not essential oil. The flasks were homogenized for 30s to ensure mixing of the oils with chicken soup. The soup mixture was inoculated with $\sim 10^6$ cells of foodborne *S. aureus* that had been prepared by growing overnight bacterium at 37 °C in TSB medium. Cell suspension was adjusted with sterile saline to approximately 1×10^6 cells/ml of chicken soup. The inoculum

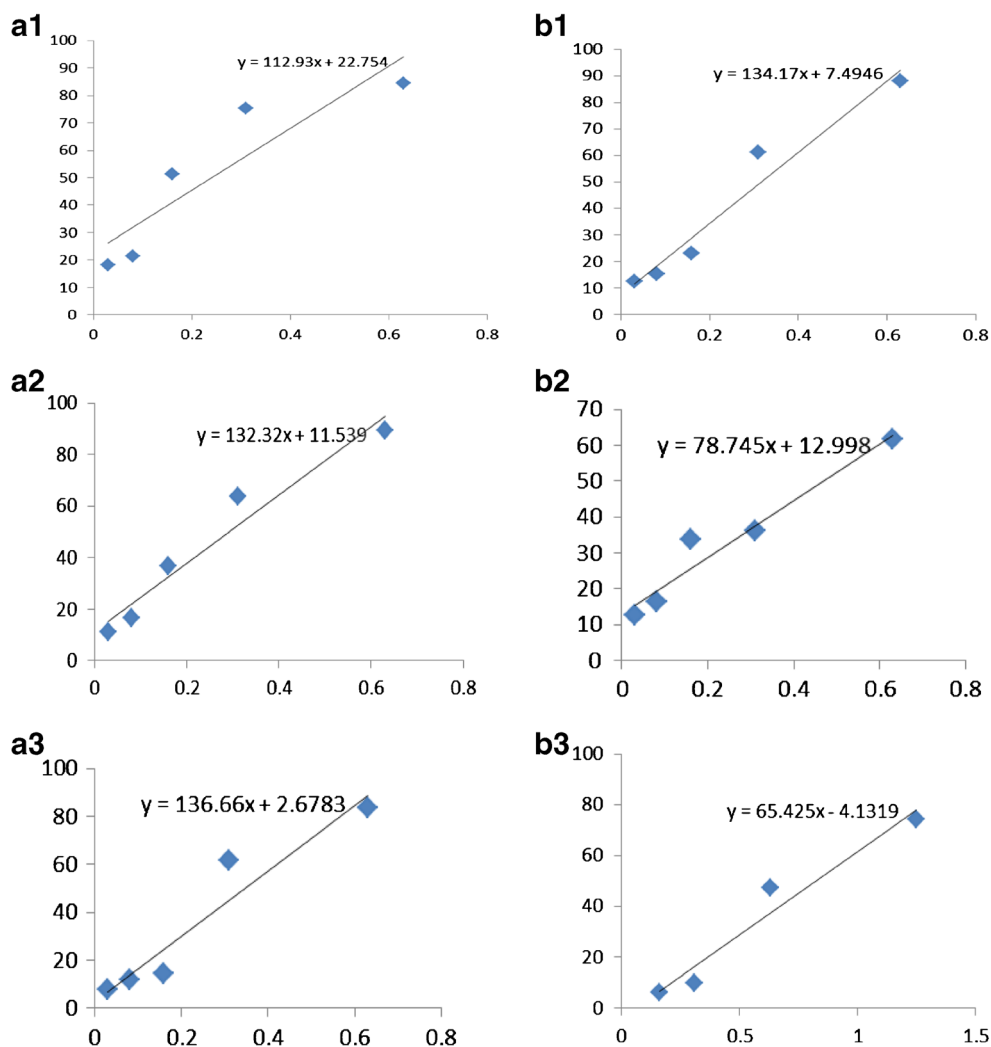
was mixed thoroughly with the soup, by hands. Experimental microplates were kept at 25 °C. The inhibition percentage at 25 °C was calculated by optical density measured by ELISA plate reader (Tecan Austria, GmbH-Austria, Ependorf-AG, Germany) using the following equations:

$$\% \text{ inhibition} = \left(\frac{OD_{\text{sample}} - OD_{\text{sample}}^0}{OD_{\text{growth}} - OD_{\text{growth}}^0} - OD_{\text{blank}} \right) \times 100$$

where OD^0_{sample} and OD_{sample} corresponded to the absorbance at 612 nm of the strain growth in the presence of the extract before and after incubation, respectively; OD^0_{blank} and OD_{blank} corresponded to the broth medium with dissolved compound before and after incubation, respectively; and OD^0_{growth} and OD_{growth} to the strain growth in the absence of the oils before and after incubation at 25 °C, respectively (Stojković et al. 2013a, b; Bukvički et al. 2014).

The results were further presented graphically and calculated, using equations presented on Fig. 1, as growth inhibition concentrations (GIC) that cause 50 % retardation of

Fig. 1 Inhibition values (50 %) for green pepper (PG) and black pepper (PN) after 24, 48 and 72 h. x axes is presented in mg/ml, y axis presents percentages of inhibition (%). Figures: A1, A2, A3 present GIC_{50} values determination for green pepper oil, after 24 h (0.241 mg/ml), 48 h (0.290 mg/ml), 72 h (0.346 mg/ml) of incubation, respectively. Figures: B1, B2, B3 present GIC_{50} values determination for black pepper oil, after 24 h (0.317 mg/ml), 48 h (0.470 mg/ml), 72 h (0.827 mg/ml) of incubation, respectively



bacterial growth. The concentrations were calculated after 24, 48 and 72 h following the incubation period.

Statistical analysis

The results are expressed as mean values and standard deviation (SD), and analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha=0.05$. This analysis was carried out using SPSS v. 18.0 program.

Results

The results obtained by GC-FID and GC-MS chemical analysis of black and green pepper essential oils are presented in the Table 1. Results showed that sesquiterpene hydrocarbons presented the major portion of black pepper while monoterpene hydrocarbons were major compounds in green pepper oil. Thirty compounds were identified in black pepper oil, which accounts for 100 % of the total oil. The major constituent of the oil was *trans*-caryophyllene (30.33 %) followed by limonene (12.12 %). Analysis of green pepper oil showed 12 compounds representing 98.44 % of the total oil. The main constituent β -pinene (24.42 %) was followed by δ^3 -carene (19.72 %), limonene (18.73 %) and α -pinene (10.39 %).

Antimicrobial activity results, of the essential oils investigated by microdilution method, are summarized in Tables 2 and 3. Oils inhibited the growth of all bacterial and fungal strains tested. Green pepper oil showed slightly stronger antibacterial and antifungal activity (MIC 0.50–1.87; MBC 0.63–2.50 mg/ml; MIC 0.07–0.16; MFC 0.13–1.25 mg/ml) compared to black pepper oil (MIC 0.07–3.75; MBC 0.60–10.0 mg/ml; MIC 0.63–5.00; MFC 1.25–10.00 mg/ml). The most resistant bacterial species was found to be *L. monocytogenes* while the most susceptible to oils was *B. cereus*. Fungi appear to be more sensitive compared to bacteria. The most sensitive fungi was *A. versicolor* while *A. niger* was the most resistant species. Comparing the results of EOs with that of standard drugs, Ampicillin (MIC 0.05–0.30; MBC 0.10–0.50 mg/ml) and Streptomycin (MIC 0.30–0.80; MBC 0.40–1.25 mg/ml), it was concluded that the oils possess less potent antibacterial activity. However, bifonazole (MIC 0.10–0.20; MFC 0.20–0.30 mg/ml) and ketoconazole (MIC 0.15–1.50; MFC 0.50–2.00 mg/ml) showed lower activity compared to green pepper oil and higher activity in comparison to black pepper oil.

In the DPPH assay, the radical scavenging capacity of the tested EOs increased in a dose dependent manner. The values for 50 % scavenging activity (IC_{50}) are presented in Table 4. The black pepper oil showed slightly better antiradical scavenging activity (36.84 mg/ml) than green pepper oil (38.77 mg/ml). Both oils exhibited weaker antiradical activity compared to control BHA (0.42 mg/ml). However,

Table 1 Chemical composition of black and green pepper oils

No	Constituents	RI	Yield percentage	
			Black papper oil	Green papper oil
1	α -Thujene	919	0.51	nd
2	α -Pinene	924	2.88	10.39
3	Camphene	939	nd	4.68
4	Sabinene	964	7.52	nd
5	β -Pinene	967	7.42	24.42
6	Myrcene	985	0.69	nd
7	α -Phellandrene	997	0.33	nd
8	δ^3 -Carene	1002	5.28	19.72
9	p-Cymene	1017	0.70	0.32
10	Limonene	1021	12.12	18.73
11	<i>trans</i> -Pinocarveol	1131	nd	0.31
12	<i>cis</i> -Sabinene hydrate	1062	0.31	nd
13	p-Mentha-2,4(8)-diene	1082	0.73	nd
14	Terpinen-4-ol	1171	0.28	nd
15	Myrtenol	1191	nd	2.81
16	Geraniol	1256	2.06	nd
17	δ -Elemene	1328	0.60	4.13
18	α -Cubebene	1340	0.99	nd
19	α -Copaene	1365	0.56	3.15
20	β -Elemene	1382	1.74	nd
21	<i>cis</i> -Caryophyllene	1408	nd	0.58
22	<i>trans</i> -Caryophyllene	1409	30.33	nd
23	α - <i>trans</i> -Bergamotene	1426	3.40	nd
24	α -Guaiene	1428	0.36	nd
25	β -Selinene	1475	3.26	nd
26	α -Salinene	1484	5.32	nd
27	α -Zingiberene	1486	0.74	nd
28	Bicyclogermacrene	1493	0.31	nd
29	β -Bisabolene	1499	4.78	nd
30	γ -Cadinene	1513	0.65	nd
31	γ - <i>trans</i> -Bisabolene	1522	1.39	nd
32	Hedycaryol	1541	0.37	nd
33	Germacrene B	1545	0.22	nd
34	Caryophyllene oxide	1571	4.16	9.20
	monoterpene hydrocarbons	38.17	78.26	
	oxygenated monoterpenes	2.65	3.12	
	sesquiterpene hydrocarbons	53.26	7.86	
	oxygenated sesquiterpenes	5.92	9.20	
	Total identified	100.00	98.44	

nd, not detected

comparison with the results should be careful, because BHA is an individual compound and not a mixture. The antiradical activity of essential oils might be attributed to the presence of phenolic and related compounds (Sultana et al. 2007).

Table 2 Antibacterial activity of green and black pepper essential oils against food-spoilage bacteria (mg/ml)

Oils		<i>B.c.</i>	<i>M.f.</i>	<i>S.a.</i>	<i>L.m.</i>	<i>E.c.</i>	<i>En.cl.</i>	<i>P.a.</i>	<i>S.t.</i>
Green pepper	MIC	0.50±0.07 ^c	1.25±0.14 ^b	0.63±0.22 ^c	1.87±0.26 ^b	0.63±0.07 ^b	1.00±0.00 ^d	1.00±0.21 ^c	1.00±0.14 ^c
	MBC	0.63±0.22 ^c	2.5±0.28 ^b	1.25±0.25 ^c	2.50±0.28 ^b	1.25±0.14 ^b	1.25±0.14 ^d	1.25±0.14 ^b	1.25±0.14 ^a
Black pepper	MIC	0.31±0.09 ^b	3.75±0.38 ^c	0.31±0.03 ^b	2.50±0.28 ^c	2.50±0.28 ^c	0.07±0.04 ^a	1.25±0.14 ^d	0.63±0.21 ^b
	MBC	0.60±0.00 ^{bc}	5.0±0.88 ^c	0.60±0.22 ^b	10.00±1.44 ^c	5.00±0.28 ^c	0.31±0.03 ^a	5.00±1.44 ^c	1.25±1.01 ^a
Ampicillin	MIC	0.1±0.03 ^a	0.2±0.01 ^a	0.05±0.03 ^a	0.2±0.02 ^a	0.2±0.03 ^a	0.3±0.02 ^b	0.2±0.02 ^a	0.2±0.00 ^a
	MBC	0.2±0.01 ^a	0.3±0.02 ^a	0.1±0.03 ^a	0.3±0.09 ^a	0.3±0.006 ^a	0.5±0.02 ^b	0.3±0.02 ^b	0.3±0.02 ^a
Streptomycin	MIC	0.3±0.00 ^b	0.3±0.01 ^a	0.3±0.003 ^b	0.4±0.006 ^a	0.3±0.006 ^a	0.4±0.02 ^c	0.8±0.01 ^b	0.3±0.01 ^a
	MBC	0.4±0.01 ^{ab}	0.4±0.02 ^a	0.4±0.006 ^{ab}	0.5±0.01 ^a	0.5±0.006 ^{ab}	0.8±0.01 ^c	1.25±0.02 ^c	0.5±0.02 ^a

B.c., Bacillus cereus; *M.f.*, Micrococcus flavus; *S.a.*, Staphylococcus aureus; *L.m.*, Listeria monocytogenes; *E.c.*, Escherichia coli; *En.cl.*, Enterobacter cloacae; *P.a.*, Pseudomonas aeruginosa; *S.t.*, Salmonella typhimurium

In each column, and for each concentration, different letters mean significant differences between samples ($p < 0.05$)

The results from the antimicrobial activity tested in situ are summarized in Table 5. It is evident that green pepper essential oil had better results against *S. aureus* in chicken soup after 24, 48 and 72 h, respectively. Inhibition percentage was dose dependent and inhibition percentage decreased with lower tested doses of oils. The best effect was noticed for the highest concentration used (5 mg/ml) at 25 ° C. Furthermore, inhibition decreased during the days of storage. It could be suggested that *S. aureus* was able to slightly overcome the effect of active compounds from oils. This could be correlated to the concentration of the oils used.

GIC₅₀ values for the oils were calculated after 24, 48 and 72 h from the equations presented on Fig. 1. Inhibitory effect on *S. aureus* was better for green pepper oil after 24 h from the beginning of incubation and GIC₅₀ value was 0.241 mg/ml, while for black pepper oil GIC₅₀ was higher - 0.317 mg/ml. The trend of increasing GIC₅₀ values was continued throughout the experiment and days of storage. GIC₅₀ values showed that green pepper better preserved the chicken soup during days of storage at lower concentrations than black pepper oil. GIC₅₀ had small increase in concentration value for green

pepper oil, while the value had larger increase with time for black pepper oil. GIC₅₀ was quiet constant with tendency of negligible small increase in concentration value for green pepper oil, while GIC₅₀ value was almost higher from 0.3 mg/ml after each investigated period for black pepper oil.

The control samples in microliter plates (chicken soup with *S. aureus*) could grow up to 10⁸ CFU/100 μL of medium, as we have checked by serial dilutions and cultivation of cells on laboratory agar broths (it is widely accepted that bacteria grow up to 10⁹ CFU/mL). From this observation if we assume that bacterium concentration was 10⁸ in control sample (100 % growth) and if we have 95 % inhibition in our experimental well (5 % growth), it means that there are still bacteria in experimental well with concentration of 5 × 10⁸. This value could be read spectrophotometrically, since spectrophotometric measurement is capable of determining cell density of 10⁶ CFU/mL or more. The method was developed to check inhibitory influence of the oil and further to calculate GIC₅₀ values. Though it is not surprising that the results slightly different from microdilution method. Of course 100 % inhibition does not obligatory mean that bacterium was killed. The

Table 3 Antifungal activity of green and black pepper essential oils against food spoilage fungi (mg/ml)

Oils		<i>A.fum.</i>	<i>A.v.</i>	<i>A.o.</i>	<i>A.n.</i>	<i>T.v.</i>	<i>P.f.</i>	<i>P.o.</i>	<i>P.a.</i>
Green pepper	MIC	0.07±0.04 ^a	0.07±0.05 ^a	0.01±0.07 ^a	0.12±0.01 ^a	0.20±0.16 ^a	0.16±0.09 ^a	0.16±0.04 ^a	0.63±0.21 ^b
	MFC	0.16±0.09 ^a	0.16±0.09 ^a	0.13±0.02 ^a	0.16±0.09 ^a	0.31±0.18 ^a	0.31±0.13 ^a	0.31±0.00 ^a	1.25±0.00 ^{ab}
Black pepper	MIC	1.25±0.14 ^a	0.63±0.21 ^b	2.50±0.28 ^b	5.00±0.00 ^c	2.50±0.28 ^c	2.50±0.28 ^b	2.50±0.38 ^c	2.50±0.32 ^d
	MFC	2.50±0.28 ^b	1.25±0.14 ^c	5.00±0.28 ^b	10.0±1.44 ^d	5.00±0.43 ^c	5.00±0.00 ^c	5.00±0.00 ^c	5.00±1.08 ^c
Ketoconazole	MIC	0.20±0.01 ^a	0.20±0.06 ^a	0.15±0.00 ^a	0.20±0.03 ^b	1.00±0.00 ^b	0.20±0.03 ^a	1.00±0.30 ^b	1.50±0.00 ^c
	MFC	0.50±0.06 ^a	0.50±0.06 ^b	0.20±0.30 ^a	0.50±0.03 ^a	1.50±0.30 ^b	0.50±0.00 ^b	1.50±0.10 ^b	2.00±0.00 ^b
Bifonazole	MIC	0.15±0.03 ^b	0.10±0.06 ^a	0.15±0.03 ^a	0.15±0.03 ^a	0.15±0.03 ^a	0.20±0.06 ^a	0.20±0.06 ^a	0.20±0.00 ^a
	MFC	0.20±0.06 ^a	0.20±0.06 ^a	0.20±0.00 ^a	0.2±0.06 ^a	0.20±0.00 ^a	0.25±0.03 ^a	0.25±0.03 ^a	0.30±0.00 ^a

A.fum., Aspergillus fumigatus; *A.v.*, Aspergillus versicolor; *A.o.*, Aspergillus ochraceus; *A.n.*, Aspergillus niger; *T.v.*, Trichoderma viride; *P.f.*, Penicillium funiculosum; *P.o.*, Penicillium ochrochloron; *P.a.*, Penicillium aurantiogriseum

In each column, and for each concentration, different letters mean significant differences between samples ($p < 0.05$)

Table 4 Free radical scavenging activity of green and black pepper essential oils (mg/ml)

Oils	Green pepper	Black pepper	BHA
IC ₅₀	36.84±2.04 ^b	38.77±11.13 ^b	0.42±0.01 ^a

In each column, and for each concentration, different letters mean significant differences between samples (*p*<0.05)

oil had microbicidal activity at high concentrations, which was checked by subcultivation of bacterium. Thus, the method was developed for validating inhibitory activities of natural preservatives in liquid foods and for the determination of GIC₅₀ value.

Discussion

Previous studies of chemical composition of the *P. nigrum* essential oil showed that α-pinene, β-pinene, myrcene, α-phellandrene, limonene, linalool, methylpropanal, 2- and 3-methylbutanal, butyric acid, and 3-methylbutyric acid were the main odorants present in the oil (Jagella and Grosch 1999). In the study of Liu et al. (2007) thirty compounds were identified from fruits of *P. nigrum*. The most dominant were: β-caryophyllene (23.49 %), δ³-carene (22.20 %), D-limonene (18.68 %), β-pinene (8.92 %) and α-pinene (4.03 %)

respectively. Our results on chemical profiling of black and green pepper oils are in agreement with studies mentioned above. However, several factors, namely climatic, geographic conditions and growth stage of collected plants may severely affect essential oil yield, their composition and their biological properties, so certain differences in different pepper oil chemical composition are quite common (Zouari et al. 2012).

Overall, both oils, especially green pepper oil showed promising antimicrobial activity. According to literature, few studies have examined the antimicrobial activity of pepper essential oils. In the study of Sasidharan and Nirmala (2010) pepper essential oil showed strong in vitro activity against *B. subtilis*, *P. aeruginosa*, *A. niger*, *Penicillium spp*, *C. albicans*, *S. cerevisiae* and *Trichoderma spp*.

Rabadia et al. (2011) tested black pepper essential oil and potential synergistic effect against pathogenic fungi *C. albicans*, *C. tropicalis* and *Trichophyton mentagrophytes* by disk diffusion method. All fungal strains were inhibited and Pepper oil exhibited significant inhibition against *T. mentagrophytes*.

Singh et al. (2005) found that black pepper oil showed complete reduction in number of colonies against *B. cereus*, and inhibited *S. aureus*, *B. cereus*, *L. monocytogenes*, *E. coli*, *S. typhimurium* and *P. aeruginosa* in concentration dependent manner.

A correlation of the antimicrobial activity of the EOs from our study and their chemical composition suggests that the

Table 5 Percentage of inhibition of *S. aureus* in chicken soup by essential oils of green and black pepper after 24, 48 and 72 h

Concentration (mg/ml)	EO	Percentage of inhibition of <i>S. aureus</i> in chicken soup		
		24 h	48 h	72 h
5.00	green pepper	93.15±0.39 ^d	91.84±1.02 ^e	89.86±0.95 ^e
	black pepper	92.87±2.18 ^e	93.01±2.21 ^h	84.85±1.97
2.50	green pepper	90.66±1.47 ^d	85.17±2.00 ^e	83.68±1.46 ^d
	black pepper	87.24±2.01 ^c	84.92±1.73 ^e	77.44±4.12 ^e
1.25	green pepper	90.49±2.91 ^d	86.96±2.06 ^e	89.49±1.40 ^e
	black pepper	92.01±2.83 ^{de}	81.38±1.07 ^f	74.08±2.21 ^d
0.63	green pepper	84.33±1.73 ^c	89.17±1.08 ^e	83.37±3.69 ^d
	black pepper	87.98±1.22 ^{cd}	61.79±1.20 ^e	47.43±5.02 ^d
0.31	green pepper	75.29±4.30 ^b	63.83±1.40 ^d	61.62±1.79 ^c
	black pepper	61.07±1.18 ^c	35.98±0.89 ^d	9.67±1.49 ^c
0.16	green pepper	51.25±2.13 ^b	36.97±3.18 ^c	14.52±1.65 ^b
	black pepper	22.96±2.83 ^b	33.64±2.91 ^d	6.04±0.81 ^b
0.08	green pepper	21.40±3.09 ^a	16.68±2.34 ^b	11.69±1.38 ^b
	black pepper	15.24±2.82 ^a	16.19±2.54 ^c	7.41±4.34 ^{ab}
0.03	green pepper	18.15±1.72 ^a	11.15±1.44 ^{ab}	7.55±1.70 ^a
	black pepper	12.57±2.68 ^a	12.67±2.74 ^b	5.17±1.02 ^{ab}
0.01	green pepper	22.34±6.07 ^a	5.36±3.86 ^a	2.68±2.89 ^a
	black pepper	15.31±3.34 ^a	6.35±1.25 ^a	3.27±1.88 ^a

In each column, and for each concentration, different letters mean significant differences between samples (*p*<0.05)

activity of the oils could be attributed to the presence of the major constituents. Although the major oil constituents are probably not the only responsible for good antimicrobial activity achieved, the involvement of less abundant constituents should also be considered.

In our study none of the oils showed better results than positive control (BHA). In addition, antiradical activities observed in volatile oil could be the synergistic effect of more than one or two compounds that may be present in the system.

Kapoor et al. (2009) concluded that pepper oil showed strong antioxidant activity measured by different techniques and pointed that scavenging power of oil was better than those of BHA and BHT. According to Singh et al. (2004) pepper oil and its extract had almost the same antioxidant activity throughout the experiment as compared with BHA and BHT. However, the results from various studies are difficult for comparison, presumably due to different test methods applied, as well as the different sources of antioxidant and samples used. There are literature reports (Nikolić et al. 2014; Xi et al. 2014) regarding antioxidant activity of various herbs and spice extracts, but, to our knowledge, there is a scarce literature about antiradical activity of pepper oils.

To avoid undesirable sensory changes, toxicity and other harmful and undesirable characteristics, application of low amounts of naturally occurring antimicrobial agents as ingredients should be also explored, in order to control spoilage. Research findings from this work provide a basis for developing effective naturally occurring antimicrobial agents to extend shelf life of soup and perhaps other types of traditional foods. The present study provides strong evidence that pepper essential oils could be used in the control of *S. aureus* in soup.

Conclusion

Medicinal plants are good alternatives to chemically synthesized antibiotics. Presented results from the above showed that green and black pepper oils significantly inhibited the growth of food spoilage microorganisms and they could serve as natural free radical scavengers. The importance of conducting the study on essential oils lies not only in the chemical characterization but also in the possibility of linking the chemical contents with particular functional properties such as preservation of food *in vitro* and *in situ*. Inhibition of *S. aureus* in chicken soup correlates with the functional properties potentially useful in the fight against foodborne pathogens and contaminants. If the active substances are to be added to foods in greater concentrations than is currently practiced for flavorings, further safety studies might be necessary.

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