



Antibacterial and Antioxidant Potential of Essential Oils of Five Spices

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HIGHLIGHTS

- Clove, black pepper, and cinnamon Essential Oils (EOs) showed considerable antibacterial and antioxidant properties.
- Eugenol, cinnamaldehyde, and β -caryophyllene were the main constituents of clove, cinnamon, and black pepper EOs, respectively.
- Clove, black pepper, and cinnamon EOs may serve as effective natural preservatives in the food industry.

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Acronyms and abbreviations

EO=Essential Oil
BHT=Butylated Hydroxy-toluene
EDTA=Ethylenediamine-tetraacetic Acid
DPPH=1,1-diphenyl-2-picrylhydrazyl
DMSO=Dimethylsulfoxide
IZD=Inhibition Zone Diameter
MIC=Minimum Inhibitory Concentration
CFU=Colony Forming Unit
GC-MS=Gas Chromatography-Mass Spectrometry

ABSTRACT

Background: Essential Oils (EOs) of spices may serve as a potential source of antibacterial and antioxidant agents due to the presence of a diverse group of phytochemicals. In the present investigation, an attempt has been made to seek EOs from five commonly used spices that have both strong antibacterial and antioxidant potential to shed some light on these important aspects.

Methods: *In vitro* antibacterial efficacy of black cumin, black pepper, cinnamon, clove, and nutmeg EOs were evaluated against some food-borne bacteria using agar well diffusion, microbroth dilution, and time-kill assay methods. Antioxidant potential was evaluated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, β -carotene linoleic acid bleaching and Fe^{2+} ion chelating methods. Chemical characterization of EO components was performed by Gas Chromatography-Mass Spectrometry (GC-MS). Statistical analysis of data was performed using SPSS software, version 18.0.

Results: Black pepper, cinnamon, and clove EOs had significantly ($p < 0.05$) higher antibacterial properties comparing to the black cumin oil and nutmeg EOs. Clove and cinnamon EOs showed the highest and lowest antioxidant potential, respectively. GC-MS analysis revealed that eugenol, cinnamaldehyde, and β -caryophyllene were the main constituents of clove, cinnamon, and black pepper oils, respectively.

Conclusion: The results provide evidence that EOs of clove, black pepper, and cinnamon may serve as effective natural preservatives in the food industry. Further studies are needed for their plausible applications in the food industry.

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Introduction

Food-borne disease is an increasingly major public health problem all over the world (Newell et al., 2010). Microbial contamination and oxidation of food compo-

nents especially lipids in foods are considered to be mainly responsible for food spoilage and contamination. A range of synthetic antimicrobial and antioxidant preservatives are currently being used to extend the shelf-

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life of food items but they have accumulated evidence that they could be toxic and carcinogenic. Besides, another great concern is the emergence of antimicrobial resistance in food-borne pathogens (Mathew et al., 2007). Natural antimicrobials and antioxidants seem to be the most promising answer to many of the increasing concerns and could yield better results than synthetic food preservatives. Therefore, novel types of effective as well as healthy antimicrobial and antioxidant agents that could protect food against spoilage and also microbial contamination are highly demanded (Lucera et al., 2012).

In this context, Essential Oils (EOs) may serve as a potential source of such compounds due to the presence of diverse groups of phytochemicals and may play a significant role in the development of eco-friendly plant-based food preservatives (Prakash and Kiran, 2016; Raut and Karuppayil, 2014). EOs are effective against a number of microbes due to their hydrophobic nature which allows them to penetrate microbial cells and cause alterations in its structure and function (Burt, 2004). In addition to their antimicrobial properties, EOs may have some antioxidant potential. Therefore, application of EOs as an antimicrobial and also antioxidant agent is a recent growing trend reflecting the interest towards “green consumerism” (Loizzo et al., 2015).

Hence, the diversified use of EOs with broad-spectrum antimicrobial and antioxidant efficacy could play a major role in food preservation both by retarding microbial growth and oxidative deterioration, with enhanced flavour properties. But, knowledge is lacking about EOs that have both strong broad-spectrum antibacterial activity against food-borne bacteria as well as strong antioxidant potential. These evidences are particularly important to facilitate the development of safe and effective natural antimicrobial and antioxidant food preservatives. Although a number of antimicrobial and antioxidant activity study of EOs have been reported by several researchers (Amorati et al., 2013; Mith et al., 2014; Smith-Palmer et al., 1998), but most of the studies were performed using one or two *in vitro* test models. Evaluation of the antimicrobial as well as antioxidant performance of EOs is, however, a crucial issue, because each of the test models varies in different respects. The lack of standardized methods makes direct comparison of results between studies impossible which subsequently may mislead future research (Amorati et al., 2013; Jennie et al., 2003). Keeping these important points in mind, in the present study, a comprehensive investigation using standard methods has been made to screen safe and effective EOs that have both broad-spectrum antibacterial activity against food-borne bacteria as well as antioxidant potential.

Materials and methods

Chemicals

p-iodonitrotetrazolium violet (INT), β -carotene, linoleic acid, Butylated Hydroxytoluene (BHT), ferrozine, ferrous chloride, EDTA, and gallic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Brain Heart Infusion Broth (BHIB), Brain Heart Infusion Agar (BHIA), Tryptic Soy Broth (TSB), Tryptic Soy Agar (TSA), Mueller-Hinton Broth (MHB), Mueller-Hinton Agar (MHA), Ciprofloxacin and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Hi-Media, Mumbai, India. All other chemicals were purchased from Merck, Germany.

Collection, extraction, and formulation of plant materials

Ethnobotanical description of selected five spices (black cumin, black pepper, cinnamon, clove, and nutmeg) is given in Table 1. These spices were purchased from the local market (Baranagar Bazar Market, Kolkata, India), identified by a botanist and voucher specimens (BC1701, BP1702, CN1703, CL1704, and NT1705) were deposited in Herbarium of Agricultural and Ecological Research Unit, Indian Statistical Institute, Kolkata. The test spices were washed thoroughly in distilled water, dried at 40 °C and powdered. EOs of powdered test spices were extracted by hydro-distillation in a Clevenger's type apparatus. Yield of test EOs are shown in Table 1. Working solution of test EOs (1000 µg/ml) was prepared by reconstituting them in 0.5% dimethylsulfoxide (DMSO) with Tween 20 (0.02% v/v) as emulsifier. All working solutions were prepared freshly prior to use.

Microorganisms

The bacterial strains [*Bacillus cereus* (n=6), *Listeria monocytogenes* (n=8), *Micrococcus luteus* (n=7), and *Salmonella* Typhimurium (n=7)] used in the present study were isolated and identified from a total of 50 spoiled and contaminated foods collected from local markets in and around Baranagar, Kolkata, India using standard methods (Kumar et al., 2011). Standard strains [*B. cereus* (MTCC 1272) and *S. Typhimurium* (MTCC 3224)] were procured from the Institute of Microbial Technology, Chandigarh, India. Bacterial cultures were maintained on selective agar slants (*L. monocytogenes* in BHIB; *S. Typhimurium* in TSB; *B. cereus* and *M. luteus* in MHB) following standard guidelines (CLSI, 2005).

Standardization of inoculum size

The inoculum size of test bacterial strains was standardized according to the Clinical and Laboratory

Standards Institute guidelines (CLSI, 2005). Briefly, the bacterial strains were incubated for 3-6 h at respective temperature (30 °C/37 °C) until the culture attained a turbidity of 0.5 McFarland Unit. The final inoculum size was adjusted to 5×10^5 CFU (Colony Forming Unit)/ml.

Evaluation of antibacterial potential

-Determination of Inhibition Zone Diameter (IZD)

IZD of tested EOs against the studied bacteria was determined by a modified agar well diffusion method (Okeke et al., 2001). Briefly, one ml of inoculum (5×10^5 CFU/ml) was spread evenly with a glass rod spreader on selective agar plates (*L. monocytogenes* in Brain BHIA; *S. Typhimurium* in TSA; *B. cereus* and *M. luteus* in MHA) and six mm diameter wells were created on the surface of agar plates. EO (100 µl) from reconstituted working solution (1000 µg/ml) was pipetted into the wells. Plates were then kept for 2 h at room temperature to allow diffusion of EOs and then incubated at respective temperature (30 °C/37 °C) for 24 h. IZD was measured in mm. Ciprofloxacin (10 µg/ml) and 0.5% DMSO were used as experimental positive and negative control, respectively. The threshold limit values for antimicrobial susceptibility testing based on IZD data were classified according to Bauer et al. (1966) as follow: (IZD \geq 11 mm: sensitive; 8 mm \leq IZD<11 mm: intermediate activity; IZD<8 mm: resistant).

-Determination of Minimum Inhibitory Concentration (MIC)

MICs of tested EOs against the studied bacteria were determined according to the guidelines of CLSI (2005) with a slight modification. Briefly, test EOs were diluted two-fold serially at varying concentrations (6.25-100 µg/ml) with selective broth from which 100 µl solution was given in each well of 96-well microtiter plates containing 90 µl of selective broth. Ten µl of working inoculum suspension (5×10^5 CFU/ml) was added to each well. A few wells were kept for control of sterility (without giving inoculum), inoculum viability (without giving sample solution) and DMSO inhibitory effect. The plates were then incubated for 24 h at respective temperature (30 °C/37 °C). Then 40 µl of 0.4 mg/ml of INT solution was added to each well and incubated for 6 h. The microtiter plates were examined to determine a colour change. A change in colour from faint yellow to red-purple occurs when viable microorganisms in wells interact with INT solution. The lowest dilution with no colour change was considered as the MIC for that individual oil. The threshold limit values for antimicrobial susceptibility testing based on MIC data were classified according to Chlipala et al. (2010) as follow: (MIC<100 µg/ml: active; MIC \geq 100 µg/ml: inactive). On the basis of the threshold limit values of antimicrobial susceptibility

testing based on IZD and MIC data, EOs that were found active (IZD \geq 11 mm; MIC<100 µg/ml) against both Gram-positive and Gram-negative bacteria were screened and subjected to further studies.

-Time-kill kinetics assay

Kill-kinetics study of test active EOs was performed following the method of Levinson (2004). Briefly, 90 µl of respective broth, 10 µl of bacterial suspension (5×10^5 CFU/ml) and 100 µl of test active EOs at different concentrations (0.5 \times MIC, 1 \times MIC, 2 \times MIC and 4 \times MIC) were added in microtiter plate wells and mixed thoroughly. The plates were then incubated at respective temperature (30 °C/37 °C) for 24 h. Ten µl sample was removed from wells at 0, 3, 6, and 24 h of incubation, and diluted serially with respective broth. Viable counts were determined by plating 100 µl of diluted aliquots on respective agar plates and incubated for 24 h at respective temperature (30 °C/37 °C). Agar plates with 30 to 300 colonies were used for CFU counting. Log₁₀ CFU/ml was plotted against time for construction of time-kill kinetics curves. Each experiment was repeated thrice. The antibacterial effects of EOs were considered bactericidal when the reduction in colony count was ≥ 3 log₁₀ CFU/ml and bacteriostatic when the reduction was <3 log₁₀ CFU/ml at 24 h with respect to control values (French, 2006).

Evaluation of antioxidant potential

-DPPH free radical scavenging assay

Radical scavenging activity of test active EOs was determined using DPPH radical scavenging assay method (Wang et al., 1998). For this, test active EOs were diluted two-fold serially (6.25-100 µg/ml) and 100 µl of diluted samples of each of varying concentrations were taken in test tubes containing 3.9 ml of DPPH solution (0.1 mM in methanol), shaken vigorously and kept in dark for 30 min at room temperature. The control was prepared as above without the EO; and methanol was used for zero adjustment. Absorbance of the samples was measured at 517 nm using a spectrophotometer (Thermo Fisher Sci., USA). Free radical scavenging activity of the test active EOs was calculated according to the following formula.

$$\% \text{Free radical scavenging} = [(A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}}] \times 100$$

Where A_{sample} is the absorbance of DPPH solution with EO and A_{blank} is the absorbance of DPPH solution in methanol without EO. BHT was used as reference standard.

-β-Carotene linoleic acid bleaching assay

β-carotene linoleic acid bleaching activity of test active EOs was evaluated following the method of Velioglu et al. (1998). Briefly, β-carotene (0.2 mg in 1 ml chloroform), linoleic acid (0.02 ml) and tween 20 (0.2 ml) were taken in round bottom flask. Then 0.2 ml of active EOs or standard BHT at 200 µg/ml (the regulatory limit

allowed in food preservation) or 0.2 ml ethanol (control) was added to the tubes. After removing chloroform at room temperature, 50 ml of distilled water was added to the mixture and shaken vigorously to form emulsion. Aliquots (2 ml) of the emulsions were taken into test tubes and placed immediately in a water bath at 50 °C. Absorbance was measured at 470 nm for every 20 min intervals for 2 h using a spectrophotometer (Thermo Fisher Scientific, USA). Antioxidant activity was expressed as percent of inhibition relative to the control using the following formula:

$$\text{Antioxidant activity\%} = \frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample or standard}})}{\text{Abs}_{\text{control}}} \times 100$$

-Ferrous (Fe^{2+}) ion chelating efficacy

The Fe^{2+} ion chelating activity of active EOs was estimated by the modified method of Dinis et al. (1994). Briefly, in tubes containing 1 ml of the test EOs at different concentrations, 2.7 ml of deionized water and 0.1 ml of 2 mM ferrous chloride solution were added and mixed thoroughly. After 3 min, the reaction was inhibited by the addition of 5 mM ferrozine (0.2 ml). The mixture was shaken vigorously and kept at room temperature for 10 min. Absorbance of the resulting solution was measured at 562 nm using a spectrophotometer (Thermo Fisher Scientific, USA). A blank was run in the same way by using distilled water instead of test EOs. EDTA and BHT were used as reference standard. Sample control was made for each test sample without adding ferrozine. The percent chelating activity was calculated using the following formula:

$$\text{Iron chelating activity\%} = \frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample or standard}})}{\text{Abs}_{\text{control}}} \times 100$$

Evaluation of cytotoxic potential

The possible cytotoxic potential of test active EOs was evaluated using brine shrimp lethality assay (Meyer et al., 1982). Briefly, artificial sea water was prepared by dissolving 38 g of sea salt in 1 L of distilled water for hatching the shrimp (*Artemia salina*) eggs. Brine shrimp eggs were incubated in artificial sea water in a specially designed two-compartment plastic tray under a 60 W lamp, providing direct light and warmth (24-26 °C). After that, 48 h were allowed for the shrimp eggs to hatch and mature as nauplii (larva). Then, in each of the nine test tubes containing 4.5 ml of artificial sea water, 10 nauplii were added.

One hundred μl of EOs at different concentrations (7.81-1000 $\mu\text{g/ml}$) was added to eight tubes. The control tube was devoid of EOs. All the tubes were incubated for 24 h at room temperature. After incubation step, number of nauplii alive was counted with help of a magnifying glass to determine 24 h LC_{50} (50% lethal concentration).

Determination of total phenolic content

To determine total phenolic content of test active EOs, Folin-Ciocalteu method (McDonald et al., 2001) was used. Briefly, 0.5 ml of test EOs (100 $\mu\text{g/ml}$) was mixed with one ml of Folin-Ciocalteu reagent (diluted 1:10 with deionized water) in test tubes, shaken vigorously and kept at room temperature for 3 min. Then, 2% Na_2CO_3 solution (3 ml) was added to the mixture and kept for 2 h with intermittent shaking for colour development. The absorbance of the resulting blue colour was measured at 760 nm. Gallic acid was used as a reference standard for plotting calibration curve. Total phenolic content was determined from the standard curve prepared with different concentrations of gallic acid. The content of total phenolic compounds was expressed as mg gallic acid equivalent/g of EO.

Gas Chromatography-Mass Spectrometry (GC-MS) analysis

The test active EOs were subjected to GC-MS analysis to identify the major chemical constituents present in EOs. The sample was subjected to GC and MS (JEOL GCMATE II, USA) equipped with a secondary electron multiplier. The column (HP5) was fused silica 50 m \times 0.25 mm I.D. The experimental conditions were 20 min at 100 °C, column temperature: 235 °C for 3 min; injector temperature: 240 °C; carrier gas: helium; and split ratio: 5:4. One μl of the sample was evaporated in a splitless injector at 300 °C and the run time was 40 min. The components were identified by GC coupled with MS. GC-MS analysis was performed at the SAIF, IIT-Madras, Tamil Nadu, India.

Statistical analysis

All the experiments were performed in triplicate. Statistical analysis of data (mean \pm SD) was performed using SPSS software, version 18.0. One-way ANOVA test followed by Tukey's range test was applied to analyze the data with level of significance set at $p < 0.05$.

Results

The IZD of test EOs against the studied food-borne bacteria are shown in Table 2 showing significant difference ($p < 0.05$). Black pepper, cinnamon, and clove oils were found to be effective ($\text{IZD} \geq 11 \text{ mm}$) against all the both Gram-positive and Gram-negative bacteria; whereas black cumin and nutmeg oils showed intermediate activity ($11 \text{ mm} > \text{IZD} \geq 8 \text{ mm}$) against Gram-positive bacteria and were found to be inactive ($\text{IZD} < 8 \text{ mm}$) against the studied Gram-negative bacteria. On the basis of MIC values, clove oil (14.58 \pm 4.79 to 27.38 \pm 7.51 $\mu\text{g/ml}$)

showed highest antibacterial activity against all the studied bacteria, followed by black pepper oil (16.66 ± 6.01 to 32.14 ± 11.57 $\mu\text{g/ml}$) and cinnamon oil (19.44 ± 6.39 to

34.52 ± 12.44 $\mu\text{g/ml}$). Based on MIC results, black cumin oil and nutmeg oil had significantly ($p < 0.05$) lower antibacterial properties than the other EOs (Table 3).

Table 1: Ethnobotanical description and yield of essential oils from spices used in the present study

| Common name | Scientific name | Family | Parts used | Yield of essential oils (%) |
|--------------|------------------------------|---------------|------------|-----------------------------|
| Black cumin | <i>Nigella sativa</i> | Ranunculaceae | seed | 0.89 |
| Black pepper | <i>Piper nigrum</i> | Piperaceae | fruit | 2.52 |
| Cinnamon | <i>Cinnamomum zeylanicum</i> | Lauraceae | stick/bark | 2.14 |
| Clove | <i>Syzygium aromaticum</i> | Myrtaceae | bud | 2.85 |
| Nutmeg | <i>Myristica fragrans</i> | Myristicaceae | seed | 1.67 |

Table 2: Inhibition zone diameter (mm) of test essential oils against food-borne *Bacillus cereus*, *Listeria monocytogenes*, *Micrococcus luteus*, *Salmonella Typhimurium*

| | Microorganisms | | | | <i>B. cereus</i> (MTCC 1272) | <i>S. Typhimurium</i> (MTCC 3224) |
|---------------------|------------------|-------------------------|------------------|-----------------------|---------------------------------|--------------------------------------|
| | <i>B. cereus</i> | <i>L. monocytogenes</i> | <i>M. luteus</i> | <i>S. Typhimurium</i> | | |
| Black cumin | 8.66 ± 0.74 | 8.61 ± 0.77 | 8.19 ± 0.74 | 4.19 ± 0.87 | 10.3 ± 1.52 | 4.33 ± 1.15 |
| Black pepper | 17.00 ± 0.78 | 16.83 ± 0.76 | 15.19 ± 1.12 | 13.04 ± 0.92 | 19.33 ± 1.52 | 14.66 ± 0.57 |
| Cinnamon | 15.27 ± 0.89 | 14.79 ± 0.93 | 14.95 ± 0.97 | 12.76 ± 0.62 | 17.33 ± 1.52 | 14.0 ± 1.00 |
| Clove | 21.72 ± 1.17 | 20.91 ± 0.88 | 19.71 ± 0.71 | 17.38 ± 0.74 | 24.66 ± 2.51 | 19.66 ± 1.52 |
| Nutmeg | 8.11 ± 1.02 | 9.00 ± 0.85 | 8.23 ± 0.88 | 5.09 ± 0.83 | 8.66 ± 1.52 | 6.33 ± 3.30 |
| Ciprofloxacin | 26.44 ± 0.78 | 22.58 ± 0.88 | 20.90 ± 0.99 | 18.14 ± 0.91 | 28.33 ± 1.52 | 20.66 ± 2.51 |
| Dimethyl sulphoxide | - | - | - | - | - | - |

Table 3: Minimum inhibitory concentration values ($\mu\text{g/ml}$) of tested essential oils against food-borne *Bacillus cereus*, *Listeria monocytogenes*, *Micrococcus luteus*, *Salmonella Typhimurium* using microbroth dilution assay

| | Microorganisms | | | |
|--------------|-------------------|-------------------------|-------------------|-----------------------|
| | <i>B. cereus</i> | <i>L. monocytogenes</i> | <i>M. luteus</i> | <i>S. Typhimurium</i> |
| Black cumin | 77.77 ± 25.56 | 72.91 ± 25.44 | 78.57 ± 25.35 | >100 |
| Black pepper | 18.75 ± 6.43 | 16.66 ± 6.01 | 17.26 ± 6.22 | 32.14 ± 11.57 |
| Cinnamon | 19.44 ± 6.39 | 21.87 ± 5.52 | 20.83 ± 6.03 | 34.52 ± 12.44 |
| Clove | 14.58 ± 4.79 | 15.62 ± 5.52 | 14.88 ± 5.02 | 27.38 ± 7.51 |
| Nutmeg | 83.33 ± 24.25 | 79.16 ± 25.18 | 88.09 ± 21.82 | >100 |

After first screening on antibacterial susceptibility testing of EOs from five spices carried out based on IZD and MIC data, black pepper, cinnamon, and clove oils were selected for further studies. Based on sensitivity of the test food-borne bacteria, one Gram-positive (*B. cereus*) and one Gram-negative (*S. Typhimurium*) bacteria were used as indicator strains to perform the time-kill kinetic assay. Figure 1 shows the results of kill-kinetics assay of active EOs (black pepper, cinnamon, and clove oil) against the studied bacteria, where a reduction in colony count by $>3 \log_{10}$ CFU/ml at 24 h by all the active EOs

against both Gram-positive and Gram-negative bacteria in comparison to their control values was observed.

DPPH radical scavenging activities of the studied EOs, black pepper, cinnamon, as well as clove are illustrated in Figure 2 which showed a concentration-dependent antioxidant potential. The clove oil showed highest antioxidant potential (29.36 ± 1.38 to $77.28 \pm 2.05\%$), followed by BHT (24.64 ± 0.49 to $72.45 \pm 2.09\%$), black pepper oil (11.24 ± 1.36 to $64.46 \pm 1.05\%$), and cinnamon oil (4.62 ± 1.06 to $57.56 \pm 2.01\%$). Also, it was observed that clove oil exhibited highest inhibitory activity in β -

carotene bleaching, followed by BHT and black pepper oil. Cinnamon oil showed significantly ($p<0.05$) lower activity against β -carotene bleaching compared to other two EOs (Figure 3). As seen in Figure 4, all the three test active EOs exhibited concentration-dependent Fe^{2+} ion chelating activity. Clove EO showed the highest chelating activity (9.56 ± 0.08 to $72.68\pm0.10\%$), followed by black pepper EO (6.64 ± 0.01 to $62.48\pm0.01\%$), and cinnamon EO (2.13 ± 0.04 to $43.86\pm0.07\%$). The Fe^{2+} ion chelating activity of test active oils and reference standards were found to be in the following decreasing order: EDTA>clove oil>BHT>black pepper oil>cinnamon oil.

There was a close association ($R^2=0.95$) between total phenolic content and antioxidant activity. Also, a linear relationship was found ($R^2=0.75-0.90$) between total phenolic content and antibacterial activity (data not shown). According to the cytotoxicity experiment, none of the three test active EOs showed cytotoxic effects at recommended dosage level; and their 24 h LC_{50} values were found to be $>1000 \mu\text{g/ml}$ (Table 4).

GC-MS analysis of EOs revealed that eugenol, cinnamaldehyde, and β -caryophyllene were the main constituents of clove, cinnamon, and black pepper oils, respectively (Table 5).

Table 4: Cytotoxic potential (mortality%) of black pepper, cinnamon, and clove oils in brine shrimp lethality assay *

| Dose ($\mu\text{g/ml}$) | Black pepper | Cinnamon | Clove |
|---------------------------|--------------|----------|-------|
| Control | 0 | 0 | 0 |
| 7.81 | 0 | 0 | 0 |
| 15.62 | 0 | 0 | 0 |
| 31.25 | 0 | 0 | 0 |
| 62.50 | 0 | 0 | 0 |
| 125 | 0 | 0 | 0 |
| 250 | 0 | 0 | 0 |
| 500 | 10 | 20 | 20 |
| 1000 | 30 | 30 | 40 |

* The LC_{50} for all treatments is $>1000 \mu\text{g/ml}$.

Table 5: Major constituents of black pepper, cinnamon, and clove oil obtained by GC-MS analysis

| No. | Retention time (min) | Molecular weight | Formula | Compound | % |
|------------------|----------------------|------------------|--|--|-------|
| Black pepper oil | | | | | |
| 1 | 11.12 | 203.8929 | $\text{C}_{15}\text{H}_{24}$ | β -caryophyllene | 43.47 |
| 2 | 12.5 | 221.8666 | $\text{C}_{15}\text{H}_{26}\text{O}$ | α -bisabolol | 3.72 |
| 3 | 12.95 | 203.9031 | $\text{C}_{15}\text{H}_{24}$ | Humulene | 3.86 |
| 4 | 13.67 | 219.8568 | $\text{C}_{15}\text{H}_{24}\text{O}$ | caryophyllene oxide | 14.65 |
| 5 | 14.08 | 221.8666 | $\text{C}_{15}\text{H}_{26}\text{O}$ | Cadinol | 2.83 |
| 6 | 16.07 | 235.8215 | $\text{C}_{15}\text{H}_{24}\text{O}_2$ | murolan-3,9(11)-diene-10-peroxy | 3.18 |
| 7 | 17.5 | 255.8341 | $\text{C}_{16}\text{H}_{32}\text{O}_2$ | n-hexadecanoic acid | 4.45 |
| 8 | 19.28 | 283.8413 | $\text{C}_{18}\text{H}_{36}\text{O}_2$ | octadecanoic acid | 5.26 |
| 9 | 22.55 | 278.7268 | $\text{C}_{16}\text{H}_{22}\text{O}_4$ | 1,2-benzenedicarboxylic acid, mono(2-ethylhexyl)ester | 2.72 |
| Cinnamon oil | | | | | |
| 1 | 11.4 | 131.8743 | $\text{C}_9\text{H}_8\text{O}$ | Cinnamaldehyde | 63.82 |
| 2 | 11.88 | 163.6201 | $\text{C}_{10}\text{H}_{12}\text{O}_2$ | Eugenol | 9.57 |
| 3 | 12.68 | 203.8828 | $\text{C}_{15}\text{H}_{24}$ | β -caryophyllene | 7.21 |
| 4 | 22.95 | 278.7150 | $\text{C}_{16}\text{H}_{22}\text{O}_4$ | 1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester | 3.27 |
| Clove oil | | | | | |
| 1 | 11.05 | 163.8388 | $\text{C}_{10}\text{H}_{12}\text{O}_2$ | Eugenol | 72.46 |
| 2 | 11.50 | 164.8521 | $\text{C}_{10}\text{H}_{12}\text{O}_2$ | Isoeugenol | 2.12 |
| 3 | 12.33 | 203.8828 | $\text{C}_{15}\text{H}_{24}$ | β -caryophyllene | 3.73 |
| 4 | 12.98 | 205.7933 | $\text{C}_{12}\text{H}_{14}\text{O}_3$ | eugenyl acetate | 4.18 |
| 5 | 14.28 | 221.8560 | $\text{C}_{15}\text{H}_{26}\text{O}$ | tau muurolol | 2.83 |

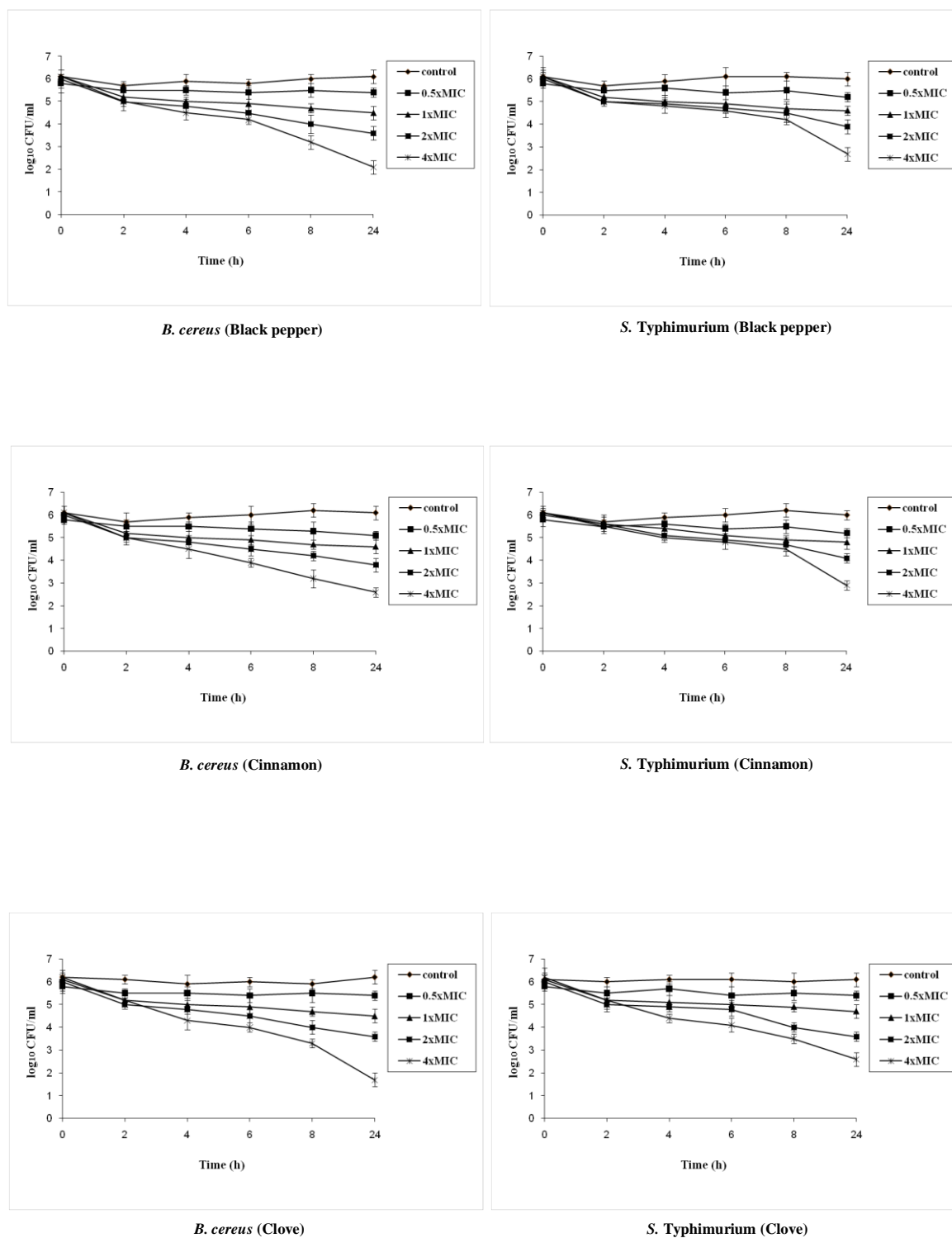


Figure 1: Time-kill curves of black pepper, cinnamon and clove essential oils against food-borne bacteria *Bacillus cereus* and *Salmonella Typhimurium*

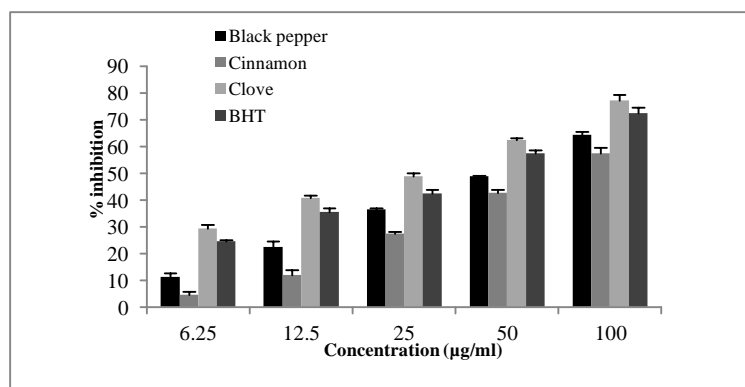


Figure 2: DPPH radical scavenging activity of black pepper, cinnamon, and clove oils

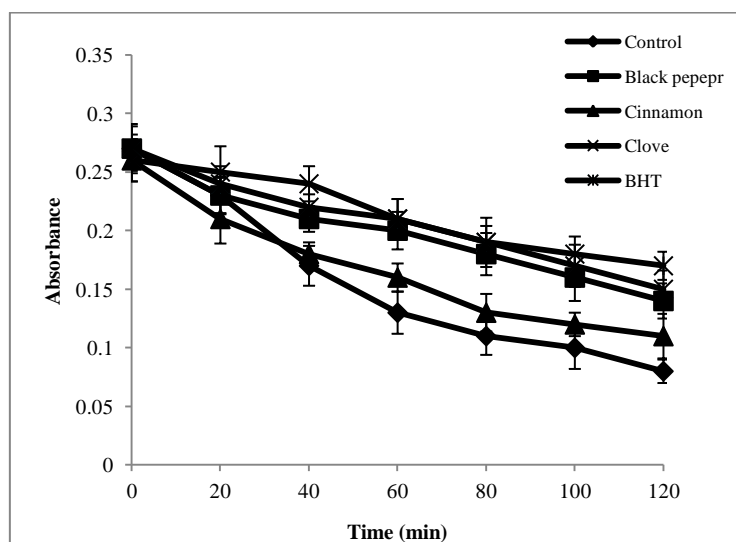


Figure 3: β -Carotene linoleic acid bleaching activities of black pepper, cinnamon, and clove oils

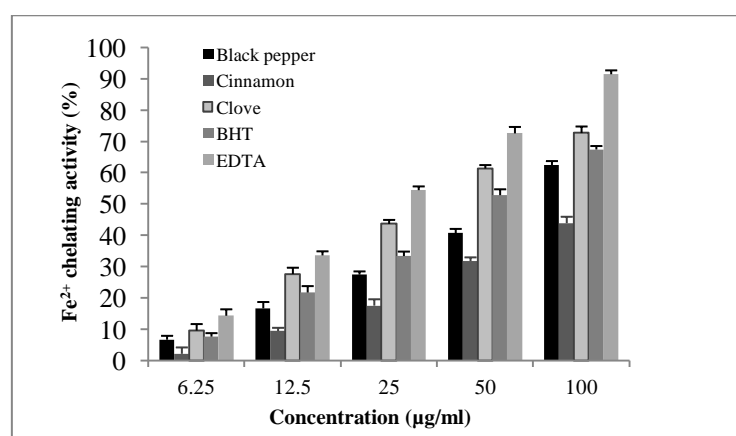


Figure 4: Fe^{2+} ion chelating activities of black pepper, cinnamon, and clove oils

Discussion

In the present investigation, we found considerable antimicrobial and antioxidant potential of black pepper, cinnamon, and clove EOs which had not yet been comparatively reported by other workers. Apart from microbial spoilage, food items are prone to oxidative deterioration during storage condition; and cumulative effect of these two events results in significant changes to food quality. Furthermore, reports on the toxic and carcinogenic potential of currently used synthetic antioxidants like BHT and BHA constrain their continuous application as food preservatives (Prakash and Kiran, 2016). Despite the worldwide development of new ranges of preservation techniques, the microbiological spoilage and contamination as well as oxidation of food components pose major challenges for food safety and quality (Lucera et al., 2012). Therefore, a new source of antimicrobial and antioxidant compounds is needed for the shielding of food matrices against contamination and oxidative deterioration.

To achieve our goal, at first a screening was performed on antibacterial susceptibility testing of EOs from five spices based on IZD and MIC data against Gram-positive and Gram-negative food-borne bacteria. Test EOs that showed IZD ≥ 11 mm (Bauer et al., 1966) and MIC < 100 $\mu\text{g/ml}$ (Chlipala et al., 2010) were screened against both the studied Gram-positive and Gram-negative bacteria and selected for further studies. Time-kill kinetics assay revealed that all the test active EOs showed bactericidal activity against both Gram-positive and Gram-negative bacteria. The rate and extent of bacterial killing of clove oil was found to be higher than black pepper and cinnamon oil. Our results are similar with the findings of other researchers where EOs of clove oil showed higher antibacterial potential than black pepper and cinnamon oils against a number of microbes (Chouhan et al., 2017; Liu et al., 2017).

Antioxidant activity of EOs is another biological property of great interest because it may preserve foods from the toxic effects of oxidants. Moreover, EOs have free radical scavenging property which in turn may play an important role in some disease prevention such as brain dysfunction, cancer, heart disease, and immune system decline. Increasing evidence has suggested that these diseases may result from cellular damage caused by free radicals. If EOs are able to scavenge some free radicals, they can also act as anti-inflammatory agents, because one of the inflammatory responses is the oxidative burst that occurs in diverse cells such as monocytes, neutrophils, eosinophils, and macrophages (Aruoma, 1998). Therefore, after performing the antibacterial activity screening, test active EOs (black pepper, cinnamon, and clove oils) were also subjected to *in vitro* antioxidant

potential evaluation using DPPH free radical scavenging, β -carotene-linoleic acid bleaching and Fe^{2+} ion chelating methods. The reduction capacity of DPPH radical is determined by the decrease in absorbance induced by antioxidants (Kumar and Pandey, 2014). In DPPH method, we observed that all the test active EOs exhibited concentration-dependent radical scavenging activity. Clove oil was found to have greater radical scavenging activity followed by black pepper and cinnamon oils suggesting that clove oil has greater hydrogen donating power than other EOs tested. The extent of β -carotene bleaching can be diminished or prevented by the presence of an antioxidant that reacts with free radicals formed in the system (Kamnath and Rajini, 2007). In the present study, we found that the test active EOs inhibited β -carotene-linoleic acid bleaching activity. Clove oil was found to be most effective followed by black pepper and cinnamon oils. These findings indicated that clove oil had maximum capability to hinder free radicals in chain reaction produced during oxidation of linoleic acid over other tested active oils. It is well recognized that oxidation of lipids in food is stimulated by the action of pro-oxidant such as Fe^{2+} ion. Besides, Fe^{2+} ion catalyses the breakdown of lipid peroxides, which leads to the formation of volatile oxidation products responsible for off-flavour and off-odour formation in foods (Rice-Evans et al., 1997). Thus, the chelation of Fe^{2+} ion often reduces lipid oxidation. In our present investigation, we, therefore, evaluated the possible Fe^{2+} ion chelating efficacy of test active EOs. It was observed that clove oil exhibited highest iron chelating efficacy than black pepper oil whereas cinnamon oil did not show any promising Fe^{2+} ion chelating activity which are in agreement with the findings indicated by Amorati et al. (2013) and Loizzo et al. (2015).

In the current study, it was found that all the test active EOs showed no cytotoxic potential at recommended dosage level which indicated that these oils could generally be considered as safe as according to Meyer et al. (1982) crude plant extract is considered to be non-toxic, if the LC_{50} value > 1000 $\mu\text{g/ml}$.

In the present research, a linear relationship that was observed between total phenolic contents and antibacterial as well as antioxidant activity of test active EOs, suggesting that phenolic group containing constituents of EOs may have important role for antioxidant and antibacterial activities. Plant phenolics are known to have multifunctional properties due to their characteristic structural features; and biological activities related to antibacterial and antioxidant property may be correlated well with total phenolic content of plant extracts/EOs (Ahn et al., 1993; Craft et al., 2012; Daglia, 2012).

According the data obtained from GC-MS analysis of our studied samples, eugenol, cinnamaldehyde, and β -

caryophyllene were found to be the major constituents of test active EOs which probably had the most important role for antibacterial and antioxidant activities. It is well documented in the literature that the biological properties of the EOs are generally determined by their major components, including two groups of distinct bio-synthetical origin. Terpenes and terpenoids comprise the main groups whereas aromatic and aliphatic constituents comprise the other group, all characterized by low molecular weight (Pichersky et al., 2006).

Conclusion

The present study provides some evidence about considerable antibacterial and antioxidant potential of clove, cinnamon, and black pepper EOs. These EOs might indeed be used both as natural broad-spectrum antibacterial and antioxidant agents for preventing food spoilage and contamination as well as oxidation of food components which subsequently may help in developing safe and effective natural food preservatives.

Author contributions

S.P. and A.Bh. designed the study and conducted the experimental work; A.B. and R.R.C. analysed the data and wrote the manuscript. All authors revised and approved the final manuscript.

Conflicts of interest

There are no conflicts of interest.

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