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ORIGINAL RESEARCH

Cannabis sativa leaf essential oil fractions and bioactive compounds: chemistry, functionality and health-enhancing traits

Saima Naz[1] · Waseem Ahmed[2] · Mohamed Fawzy Ramadan[3]

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Abstract

Cannabis sativa leaf essential oil (CSEO) and fractions (CSF) were investigated for their fungicidal, bactericidal, antioxidant, and cytotoxic traits. The extraction of CSEO was performed at 110 °C, 120 °C, and 130 °C through a hydrodistillation technique. A maximum extraction yield (0.035%) was obtained at 110 °C. Caryophyllene, humulene, *trans*-α-bergamotene, and *cis*-β-farnesene were the main components identified using GC–MS. The fractionation of CSEO was carried out using vacuum fractionation. Total phenolic contents (TPC) were 13.4–13.8 and 11.2–14.6 mg GAE/mL, total flavonoid contents were 14.1–14.2 and 10.7–15.3 mg CE/mL, and pro-anthocyanidin contents were 8.91–10.4 and 7.16–11.3 mg VE/mL for CSEO and CSF, respectively. Antioxidant activity assessed by scavenging DPPH· recorded 20.5–26.2% for CSEO and a maximum of 37.7% for F1 of 110 °C extracted CSEO. F4 of 130 °C extracted CSEO showed maximum inhibition of linoleic acid peroxidation. The maximum antibacterial effect was 19.3 mm, 19.6 mm, and 19.8 mm (zone of inhibition), while maximum antifungal activity was 20.1 mm, 22.1 mm, and 22.2 mm exhibited by F1, F5, and F4 for 110 °C, 120 °C, and 130 °C-extracted CSEO, respectively. Maximum brine shrimp cytotoxicity values were 20 ppm, and 25 ppm showed by F1 of CSEO collected at 110 °C. The IC_{50} of antitumor potential was 40.2 µL/mL for F4 of 130 °C extracted CSEO.

Keywords Extraction · Fractionation · Phytochemicals · Brine shrimp cytotoxicity · Antifungal · Cytotoxic · Pro-anthocyanidin · Antitumor

Introduction

Medicinal plants, spices, and herbs have been utilized for thousands of years for their medicinal traits, improving the aroma and flavor of food [1, 2]. Among the natural additives, aromatic plants' essential oils and extracts have been evaluated due to their advantages over antibiotics as growth promoter [3, 4]. In addition, they are rich in bioactive substances such as phenolics, flavonols/flavonoids, quinines, polypeptides, alkaloids, or their oxygen-substituted derivatives [5, 6]. These active constituents show therapeutic effects, such as antiseptic and antioxidant traits. Therefore, they might reduce the risk of cardiovascular, cancer, respiratory diseases, and inflammation disorders [7]. In addition, medicinal plants could delay oxidative rancidity and inhibit off-flavor development in several edible items [8]. They also contain antimicrobial constituents that inhibit microbial growth in food, especially meat products and snacks [9].

Cannabaceae or hemp is a family of flowering plants with 170 species grouped in 11 genera [10]. *Cannabis sativa* (hemp) is one of the small plant families used as a framework to evaluate specific secondary metabolites. *Cannabis sativa* is one of the different species of *Cannabaceae* [11, 12]. Hemp is a psychoactive drug cultivated as an industrial crop. *C. sativa* is one of the oldest medicinal plants and has been most investigated concerning its phytochemistry [13]. The medicinal value of *C. sativa* extracts includes

✉ Mohamed Fawzy Ramadan
mfhassanien@uqu.edu.sa; hassanienmohamed@yahoo.com

Saima Naz
saima.naz@ue.edu.pk

Waseem Ahmed
waseemuaf12@gmail.com

[1] Department of Chemistry, University of Education Lahore, Faisalabad campus, College Road Saman Abad, Faisalabad, Pakistan

[2] Department of Horticulture, The University of Haripur, Haripur 22630, Pakistan

[3] Department of Clinical Nutrition, Faculty of Applied Medical Sciences, Umm Al-Qura University, P.O. Box 7067, 21955 Mecca, Saudi Arabia

anthelmintic showed anticancer activity against cancer cell lines, antimicrobial activity, anti-nausea, and anti-vomiting [14]. In addition, it acts as an antispasmodic, anodyne and narcotic aphrodisiac apoptosis, as a hallucinogen and for rheumatism, putrefacient, antiseptic, and to treat swelling of sprained joints, intoxicant, analgesic, narcotic, stomachic, antispasmodic, anodyne, and sedative [15–17]. *Cannabis* leaves extracts alone could cure more than 25 diseases. Seeds treat tumors and cancerous ulcers [18]. Many other species grow wild in salt range areas northwest of Khushab District, Punjab, Pakistan, at 1530 m above sea level.

Extraction is the first crucial step in the preparation of plant formulations. Hydrodistillation is one of the oldest and most advanced techniques to obtain essential oil from plants [19]. Due to the influence of hot water and steam, the essential oil is freed from the oil glands in the plant tissue. Fractional distillation is the separation of a mixture into its parts or fractions, such as separating chemical compounds in oil, gas or other liquids by their boiling point by heating them to a temperature at which one or more fractions of the compound will vaporize [20, 21]. Due to its many components, vacuum fractional distillation is used to separate mixtures into various fractions based on various boiling points and the percentage quantity of components of essential oils [22]. It separates and further purifies valuable components of oils and other liquids. It operates at low temperatures and pressure, thereby avoiding the degradation of the compounds in the extract [23]. The process involves heating a mixture and vaporizing the compounds, which depends on the volatility of the compounds. Initially, the more volatile compounds are vaporized, followed by intermediately volatile compounds, and so on. At the end of the process, the less volatile or residual fraction remains in the boiler [24]. Based on the unique medical background of *C. sativa*, it was targeted for several studies. Although investigations have been done to explore the pharmacological traits of *C. sativa* extracts, there is a wide gap in knowledge about the medical attributes of *C. sativa* essential oil (CSEO) and *C. sativa* essential oil fractions (CSF). Therefore, the current investigation explored the antioxidant potential, antimicrobial attributes, antitumor and cytotoxic traits of CSEO and CSF and their separated phytochemicals.

Materials and methods

Chemicals and equipment

The main equipment used were spectrophotometer U-2001 (121-0032, Hitachi Instruments, Tokyo, Japan)/Bio-Rad, smart spec (TM) plus spectrophotometer, USA), hydrodistillation apparatus, and centrifuge (Borosil Glass Works Ltd., Mumbai, India). Aluminum chloride hexahydrate, quercetin, gallic acid (GA), trichloroacetic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH·), and dimethyl sulfoxide (DMSO) were from Sigma-Aldrich (St. Louis, MO, USA). Agar powder and potassium ferricyanide were obtained from Daejung Chemicals and Metals (Shiheung, Korea), the nutrient broth was obtained from Lab M (Lancashire, UK), and ferric chloride from BDH lab supplies (Leicestershire, UK). Methanol, *n*-hexane, and other chemicals used for antioxidant assays were from Merck (Darmstadt, Germany). Culture media and standard antibiotic discs were from Oxoid Ltd. (Hampshire, UK). Folin–Ciocalteu's reagent, nutrient agar, and sodium carbonate were from Applichem GmbH (Darmstadt, Germany).

Plant

Cannabis sativa fresh plants were collected from Soon Valley in the North West of Khushab District, Punjab, Pakistan, in September–October 2014. Dr. M. Hameed (Department of Botany, University of Agriculture Faisalabad, Pakistan) identified and authenticated plant specimens. The fresh material was immediately processed for extraction of CSEO.

Extraction of CSEO

CSEO was extracted from leaves by hydrodistillation in a batch process at 110 °C, 120 °C, and 130 °C temperatures, respectively, at atmospheric pressure. Approximately 12 kg of fresh *C. sativa* plant material was used in each batch. Continuous pressure, temperature, and gas supply were made possible. The extraction process was completed at Rose Lab Institute of Horticultural Sciences UAF (Pakistan). After extraction, CSEO was stored in black glass vials at 0–4 °C until analysis. The oil yield was reported as (%, w/w).

Fractionation of CSEO

CSEO obtained by hydrodistillation at different temperatures was distilled under two conditions with a short path molecular vacuum distillation (SPMVD). The CSEO was placed in the reception flask (100 mL), wherein fractions were obtained by modifying the temperature while pressure and flow rate are kept constant. Batch vacuum distillation was performed in a distillation column. The column was 29 mm inner diameter and 2 m high. The column has 36 theoretical stages. Fractional distillation was carried out at 760 mm Hg of pressure and by varying temperatures from 0 to 300 °C. After distillation of all essential oils extracted at three different temperatures, five fractions called F1, F2, F3, F4, and F5 were obtained at various boiling points (BP). The remaining was called Residual fraction (Rf) or residue of first distillation. Fractions of approximate volumes were

collected during the fractional distillation and analyzed using GC-FID and GC–MS.

Gas chromatograph–mass spectrometer (GC–MS) analysis of CSEO

GC–MS analysis of the CSEO was carried out using Perkin Elmer Clarus 600 GC equipped with ULTRA 1 column packed with 100% dimethyl polysiloxane (film thickness 0.25 µm, 30 m × 0.25 id) coupled with a Perkin Elmer Clarus 600C MS equipped with FID detector. Helium was the carrier at 1 mL/min. The mass transfer line and injector temperatures were set at 250 °C and 300 °C, respectively. The oven temperature was programmed from 40 °C (hold 2 min) to 270 °C at 2 °C/min, then held for 10 min and then raised to 300 °C at 10 °C/min. 1 µL diluted sample (1/100, v/v, in dichloromethane) was injected in split mode (split ratio of 120:1). Chemical constituents of CSEO were identified from their GC retention time and calculation of their retention indices based on *n*-alkanes (C_6–C_{24}). The percentages of CSEO constituents were recorded from the peak area. Compounds were identified by comparison of their mass spectra (NIST 2005 v.2.0 and Wiley Access Pak v.7, 2003 of GC–MS systems) [25, 26].

Evaluation of CSEO antioxidant properties

Total phenolic content (TPC)

TPC was determined using the Folin–Ciocalteu reagent. First, 0.5 mL of CSEO was mixed with 10% Folin–Ciocalteu reagent (2.5 mL). Next, the mixture was kept for 10 min at room temperature, then 2.0 mL of sodium carbonate (75% w/v) was added. In the next step, the mixture was vortexed for 15 s, heated at 40 °C in a water bath for 30 min, and then cooled in an ice bath. Finally, the absorbance at 765 nm was taken. The TPC was calculated using a gallic acid calibration curve (20–100 ppm). Results were expressed as gallic acid equivalents (GAE) per dry matter [27–29].

Total flavonoids contents (TFC)

Briefly, 0.5 mL of CSEO was added to a volumetric flask and diluted using 640 µL distilled water. In the next step, 0.3 mL of 10% $AlCl_3$ in ethanol, 0.3 mL of 5% $NaNO_2$, and 200 µL of 1 M NaOH were added at 0, 5 and 6 min intervals, respectively. The mixture was diluted with 4 mL of distilled water and mixed vigorously. The absorbance at 765 nm was recorded. TFC was calculated using a calibration curve for catechin (20–100 ppm), and TFC was expressed as catechin equivalents (mg CE/g dry weight). Samples were analyzed thrice, and the results were averaged [30, 31].

Total pro-anthocyanidin contents

The total contents of pro-anthocyanidins were determined [32] with minor modifications. First, 0.5 mL of CSEO was mixed with 3 mL vanillin-methanol solution (4%) and 1.5 mL hydrochloric acid. The solution was allowed to stand for 15 min, and absorbance was recorded at 500 nm [32, 33]. The results were expressed as mg vanillin equivalent/g dry basis.

Inhibition of linoleic acid per-oxidation

The antioxidant action of CSEO was evaluated in terms of measurement of % inhibition of per-oxidation in the linoleic acid system [34, 35]. Briefly, 50 µL oil was added to a mixture of linoleic acid (0.13 mL), 5 mL of 0.05 M sodium phosphate buffer (pH 7), and 99.8% ethanol (5 mL). The mixture was diluted to 25 mL using distilled water. The solution was incubated at 40 °C, and the oxidation was measured according to the thiocyanate method with 10 mL ethanol (75%), 0.2 mL aqueous solution of ammonium thiocyanate (30%), 0.2 mL sample solution and 0.2 mL $FeCl_2$ solution (20 mM in 3.5% HCl) being added sequentially. After 3 min stirring, the absorption was measured at 500 nm as peroxide contents. Linoleic acid was used as the positive control. The maximum per-oxidation level observed at 175 h (7 days) in the sample without antioxidants was used as a test point

$$\% \text{ Inhibition} = 100 - \left[(\text{Abs. increase of sample at 175 h} / \text{Abs. increase of control at 175 h}) \times 100\right]$$

DPPH· radical scavenging activity

The DPPH· test of CSEO was performed as described [36, 37]. A methanol solution of 0.135 Mm DPPH· was prepared, then 1.0 mL was mixed with 1.0 mL CSEO prepared in methanol containing 0.025–0.50 mg/mL of CSEO and vitamin C. The mixture was thoroughly vortexed, left in the dark for 30 min at 25 °C, then measured at 517 nm. Ability of CSEO to scavenge DPPH· radicals was calculated as % inhibition according to the following equation:

$$\% \text{ Inhibition} = (\text{Abs. control} - \text{Abs. sample})/(\text{Abs. control}) \times 100$$

Abs. control is the absorbance of the DPPH· radical + methanol; Abs. sample is the absorbance of DPPH· radical + CSEO or vitamin C. The CSEO inhibitory concentration (IC_{50}) needed to inhibit 50% DPPH· obtained from the standard curve was compared to vitamin C.

Fe^{3+} reducing power test

Fe^{3+} reducing antioxidant power (FRAP) test was used to measure the reducing capacity of CSEO. This assay was proposed [38] with modification [39, 40] that involves reducing the ferricyanide complex to the ferrous form. First, various concentrations (15–45 µL/mL) of CSEO in 1 mL distilled water were mixed with 2.5 mL of 1% potassium ferricyanide [$K_3Fe(CN)_6$], and sodium phosphate buffer (2.5 mL, 0.2 M, pH 6.6). The mixture was incubated for 20 min at 50 °C. Next, 2.5 mL of 10% trichloroacetic acid was added, and then 2.5 mL of the solution was mixed with 2.5 mL distilled water and 0.5 mL $FeCl_3$ (0.1%). The absorbance was measured at 700 nm. Increased absorbance of the mixture indicates an increase in reduction action. The experiment was performed thrice, and the results were averaged.

Antioxidant activity using ferric thiocyanate (FTC) test

The antioxidant activity of CSEO was evaluated using the ferric thiocyanate (FTC) method [41, 42]. The solution contains concentrations of CSEO (15–45 µL/mL) in 2.5 mL sodium phosphate buffer (pH 7, 0.04 M), which was added to 2.5 mL linoleic acid emulsion in sodium phosphate buffer (pH 7, 0.04 M). 5 mL linoleic acid emulsion was prepared by mixing and homogenizing 15.5 µL linoleic acid, 17.5 mg of Tween-20, and 5 mL phosphate buffer (pH 7). 5 mL control comprised 2.5 mL linoleic acid emulsion and 2.5 mL 0.04 M sodium phosphate buffer (pH 7). Mixed solution (5 mL) was incubated in a polyethylene flask at 37 °C. Peroxide levels were recorded by reading the absorbance at 500 nm (Shimadzu, UV-1208 UV–VIS Spectrophotometer, Japan) after reaction with $FeCl_2$ and thiocyanate at intervals during incubation. The absorbance of the red color was recorded at 500 nm until it reached a maximum value. This step was repeated every 5 h. The percentage inhibition values were calculated at this point (30 h). High absorbance indicates high linoleic acid emulsion peroxidation. The solution without CSEO was used as a blank sample. The % inhibition of lipid per-oxidation was calculated using the following equation:

$$\text{Inhibition of lipid per-oxidation}(\%) = 100 - \left(A_s/A_c \times 100\right),$$

where A_c is the absorbance of the control reaction, and A_s is the absorbance in the presence of CSEO [39, 41].

Antioxidant activity using thiobarbituric acid (TBA) test

A modified TBARS assay measured the potential antioxidant action using egg yolk homogenate as lipids-rich media [43]. Briefly, 0.5 mL of 10% (w/v) tissue homogenate and 0.1 mL of CSEO solution were tested in methanol (concentrations of stock solutions were 4.0, 20.0, and 40.0 g/L), were added to a test tube and made up to 1 mL using distilled water. Then, 0.05 mL of 2,2′-azobis (2-amidinopropane) dihydrochloride solution (0.07 M) was added to induce lipid per-oxidation. Then, 1.5 mL 0.8% (w/v) thiobarbituric acid in 1.1% (w/v) sodium dodecyl sulfate solution and 1.5 mL of 20% acetic acid (pH 3.5) was added. The mixture vortexed and then heated for 60 min at 95 °C. After cooling, 5.0 mL of butan-1-ol was added, then vortexed and centrifuged for 10 min at 1200×*g*. The absorbance of the upper layer was recorded at 532 nm using a spectrophotometer (Perkin-Elmer Lambda EZ 201, Roma, Italy) [44]. Values were based on the percentage antioxidant index (AI %):

$$\text{AI}(\%) = \left(1 - A_T/A_C\right) \times 100,$$

where A_C is the absorbance of the fully oxidized control, and A_T is the absorbance of the test sample.

Antimicrobial assay of CSEO and CSF

Antimicrobial activity of CSEO and CSF were screened against five bacterial strains, including *Bacillus subtilis* (*B. subtilis*) (MTCC 1790), *Staphylococcus aureus* (*S. aureus*) (MTCC 3103), *Pasturella multocida* (*P. multocida*) (ATCC 43137), *Escherichia coli* (*E. coli*) (MTCC 1672) and *Clostridium bifermentans* (*C. bifermentas*) (ATCC 19299) as well as five fungal strains *Aspergillus flavus* (*A. flavous*) (ATCC 20046), *Fusarium solani* (*F. solani*) (ATCC 36031), *Aspergillus niger* (*A. niger*) (ATCC 10152), *Alternaria alternata* (*A. alternata*) (ATCC 20084), and *Penicillium notatum* (*P.notatum*) (ATCC 28682). Bacterial strains were cultured at 37 °C overnight in nutrient agar (Oxoid, UK), while fungal strains were cultured for 48 h at 28 °C using (PDA) potato dextrose agar (Oxoid, UK).

Antimicrobial activity using disc diffusion test

The antimicrobial traits of CSEO and CSF were evaluated using the disc diffusion test [45]. Potato dextrose agar and Nutrient agar were mixed and autoclaved, then 100 µL/100 mL of inocula were added to a medium, transferred to *Petri* plates and left to solidify. Filter paper disk with 10 µL sample was placed and incubated at 37 °C and 28 °C for 24 h and 48 h, respectively. Diameters of inhibition zones were recorded in millimeters using a zone reader. Results were compared with Amoxicillin and Fungone as standard antimicrobial agents [46–48].

Minimum inhibitory concentration (MIC) of CSEO and CSF

A microdilution broth susceptibility assay was used to calculate MIC, representing the concentration inhibiting

microorganism growth [49]. Series of CSEO dilutions were prepared in a 96-well microliter plate, including 160 µL NB and SDB for bacteria and fungi, then added onto the microplates with 20 µL test solution. In the next step, inoculation was performed onto the microplates, 5×10^5 CFU/mL (confirmed by the viable count of standard microorganism suspension). The plates were incubated for 24 h at 37 °C for bacteria and 48 h at 28 °C for fungi. Rifampicin was used as an antibacterial reference, while Fluconazole was used as an antifungal reference. The growth was indicated by a white 'pellet' on the well bottom, and the calculations were performed in µg/mL [50–52].

Cytotoxicity assay

Brine shrimp lethality assay

Eggs were hatched in a shallow glass container containing 9.5 g artificial sea salt in 250 mL distilled water. The pH of the prepared solution was adjusted to 7.0. The container was covered with aluminum foil (80%) and partially illuminated with a lamp. Hatching took about 72 h. Then shrimps were ready for the test. CSEO and CSF solutions were prepared by dissolving 100 mg oil in 1 mL DMSO. Tenfold dilutions were prepared with 10% DMSO or methanol to obtain three concentrations. Further, ten-fold dilutions were prepared using artificial seawater containing 15 swimming shrimps. The final test concentrations were 10, 100, and 1000 ppm. Test tubes used as control contained 0.5% methanol or 0.5% DMSO in artificial seawater, containing 15 swimming shrimps. Tubes were loosely covered with aluminum foil and kept at 29 °C for 24 h. The number of dead shrimps was recorded by counting motionless shrimps at the bottom of tubes. Test was performed in duplicate, and the procedure was repeated twice for oil and oil fractions. Abbott's formula determined the average percent mortality after correcting for control mortality [53, 54]

$$\% \text{ Death} = \left[(\text{Test} - \text{Control})/\text{Survivors of control}\right] \times 100,$$

LC_{50} values were determined by linear regression analysis and graphical calculations [55].

Antitumor assay

CSEO and CSF were screened for antitumor effects using the antitumor potato disc assay [56–58]. A 48-h-old culture of *Agrobacterium tumefaciens* (at 10) was used. The inoculum was prepared as three concentrations of CSEO and CSF, each separately (250, 500, 1000 µL) + 150 µL autoclaved distilled water + 750 µL of bacterial culture (1 × 108 CFU/mL). Camptothecin (30 ppm) was used as a positive control, replacing CSEO and CSF. The negative control was the inoculum without tested CSEO. The red-skinned potato was surface sterilized using 0.1% $HgCl_2$ solution. Potato discs (8 mm × 4 mm) were prepared. Autoclaved agar (1.5%) was poured into *Petri* plates and allowed to solidify. Ten discs were placed on the agar surface of each plate, and 50 µL inoculum was poured on the surface. Plates were sealed with Parafilm and incubated in the dark at 28 °C. After 21 days incubation, potato discs were stained with Lugol's solution (10% KI, 5% I_2), and tumors were counted. The experiment was performed in triplicate. Percentage tumor inhibition was calculated using following formula:

$$\% \text{ inhibition} = (1 - \text{Ns}/\text{Nc}) \times 100,$$

where Ns = average number of tumors in the sample, and Nc = average number of tumors in the negative control. More than 20% tumor inhibition was considered significant.

Statistical analysis

All tests were repeated three times, averaged, and variance was determined. Data were expressed as mean ± SE and subjected to two-way ANOVA (Graph pad Prism, version 3.0). Tukey's multiple range test was utilized to separate means ($p < 0.05$).

Results and discussion

Yield and composition of CSEO

CSEO yield obtained by hydrodistillation is given in Table 1. Hydrodistillation of the aerial parts of *C. sativa* gave a golden yellow liquid with a yield of 0.035%, 0.030%, and 0.024% at 110 °C, 120 °C, and 130 °C, respectively, based on fresh weight. The chemical compositional data of CSEO extracted by hydrodistillation are reported in Table 2. Meanwhile, 29, 36, and 45 compounds were identified in CSEO extracted at 110 °C, 120 °C, and 130 °C distillation temperatures, respectively. The major constituents (> 5%) in CSEO extracted at 110 °C were caryophyllene (44.6%), humulene (13.2%), *cis*-β-farenscene (8.68%), *trans*-α-bergamotene (7.15%) and *d*-limonene (0.11%), and The main components

Table 1 Extraction yield (%) of CSEO

Temperature (°C)	Weight (kg)	Distilled water vol (L)	Time of extraction (h)	Oil (g)	Yield (%)
110	12	21	3	4.21	0.035 ± 0.04^a
120	12	21	3	3.61	0.030 ± 0.02^b
130	12	21	3	2.88	0.024 ± 0.01^c

Means followed by different superscript letters in the same column represent significant differences among values

Table 2 Composition of CSEO extracted at various temperatures

Peak no.	Compound	Chemical class	RT	RI	Area (%)	110 °C (yield%)	120 °C (yield%)	130 °C (yield %)
1	A-pinene	Monoterpene	5.26	939.53	16,061,764	–	–	3.596 ± 0.07^{a}
2	Camphene	Monoterpene	5.635	950.57	330,201.59	–	–	0.073 ± 0.03^{a}
3	A-phellandrene	Monoterpene	7.41	966.23	9759.928	–	0.000 ± 0.08^{a}	–
4	(+)-4-carene	Monoterpene	7.511	978.11	76,258.531	0.034 ± 0.01^{c}	0.039 ± 0.06^{b}	0.555 ± 0.05^{a}
5	*p*-Cymol	Monoterpene/ Alcohol	7.761	1020.56	17,134.959	0.012 ± 0.06^{c}	0.196 ± 0.01^{b}	0.758 ± 0.02^{a}
6	D-limonene	Monoterpene	7.901	1037.28	150,320.88	0.111 ± 0.01^{c}	2.135 ± 0.07^{b}	5.130 ± 0.04^{a}
7	Cineole	Monoterpene/ether	8.001	1040.34	50,059.719	0.036 ± 0.12^{c}	1.388 ± 0.12^{b}	3.851 ± 0.04^{a}
8	*Trans*-β-ocimene	Monoterpene	8.171	1044.09	92,413.914	–	0.048 ± 0.12^{a}	0.257 ± 0.08^{b}
9	B-Ocimene	Monoterpene	8.516	1054.56	351,217.88	–	0.183 ± 0.01^{b}	0.724 ± 0.05^{a}
10	Gamma-terpinene	Monoterpene	8.906	1059.82	595,210.56	–	0.311 ± 0.08^{b}	0.844 ± 0.08^{a}
11	Nonanal	Sesquiterpene/aldehyde	10.507	1063.23	222,245.67	–	–	0.049 ± 0.14^{a}
12	Terpinen-4-ol	Monoterpene/alcohol	13.388	1178.21	21,198	0.015 ± 0.01^{c}	0.043 ± 0.12^{b}	0.279 ± 0.10^{a}
13	U.i. (unidentified)	–	13.973	1183.22	81,200.039	–	–	0.018 ± 0.10^{a}
14	U.i	–	14.914	1185.34	194,151.75	–	–	0.043 ± 0.01^{a}
15	Estragole	Monoterpene	14.229	1191.67	20,525.195	–	0.010 ± 0.01^{a}	–
16	Carvone	Monoterpene/ketone	16.079	1244.05	7571.557	–	0.003 ± 0.10^{a}	–
17	6-Ethenyl-6,9,9-trimethyl-4-methyl-idenebicyclo[5.2.0]nonane	Alkane	19.281	1267.46	299,046.25	–	0.511 ± 0.14^{a}	–
18	A-cubebene	Sesquiterpene	20.41	1308.80	43,066	0.031 ± 0.05^{a}	–	–
19	Guaia-1(10),11-diene	Sesquiterpene	21.517	1320.12	637,844.81		0.333 ± 0.11^{a}	
20	1-Ethenyl-1-methyl-4 methylidene-2-(2-methylprop-1enyl) cycloheptane	Cycloalkane	21.832	1327.45	217,560.47	–	0.113 ± 0.07^{a}	–
21	U.i	–	22.067	1345.67	113,751.63	0.084 ± 0.05^{a}	–	–
22	U.i	–	22.067	1356.22	331,999.31	–	–	0.074 ± 0.14^{a}
23	U.i	–	22.717	1378.34	2,896,410.3	–	1.516 ± 0.03^{a}	0.811 ± 0.02^{b}
24	β-Caryophyllene	Sesquiterpene	22.722	1414.33	1,304,036.5	0.963 ± 0.14^{a}	–	–
25	U.i	–	23.087	1418.43	1,206,087.8	–	0.631 ± 0.09^{a}	
26	U.i	–	23.087	1420.56	629,011.94	0.464 ± 0.07^{b}	–	0.075 ± 0.06^{a}
27	Caryophyllene	Sesquiterpene	23.262	1421.53	60,447,852	44.66 ± 0.03^{a}	44.33 ± 0.05^{b}	37.644 ± 0.06^{c}
28	(E)-β-famesene	Sesquiterpene	23.732	1423.42	622,963.38	–	–	0.139 ± 0.06^{a}
29	*Trans*-α-bergamotene	Sesquiterpene	23.893	1429.33	9,679,677	7.152 ± 0.11^{a}	6.795 ± 0.03^{b}	5.427 ± 0.14^{c}
30	U.i	–	24.178	1431.76	263,992.5	–	–	0.059 ± 0.05^{a}
31	Aromandendrene	Sesquiterpene	24.398	1435.62	573,066.69	0.423 ± 0.07^{a}	0.398 ± 0.03^{b}	0.163 ± 0.06^{c}
32	Humulene	Sesquiterpene	24.578	1452.52	17,894,736	13.22 ± 0.01^{a}	11.977 ± 0.09^{b}	9.516 ± 0.11^{c}
33	*Cis*-β-farnesene	Sesquiterpene	24.738	1455.34	11,754,096	8.685 ± 0.09^{b}	9.716 ± 0.03^{a}	8.632 ± 0.09^{c}
34	Alloaromadendrene	Sesquiterpene	24.858	1467.53	601,293.44	0.444 ± 0.06^{a}	0.401 ± 0.01^{b}	0.205 ± 0.09^{c}
35	U.i	–	24.993	1483.55	292,929.34	–	–	0.065 ± 0.05^{a}
36	Gamma-muurolene	Sesquiterpene	25.493	1487.73	938,143	0.693 ± 0.06^{a}	0.416 ± 0.03^{b}	0.344 ± 0.09^{c}
37	B-curcumene	Sesquiterpene	25.598	1491.32	231,128	0.170 ± 0.07^{b}	–	0.142 ± 0.12
38	A-curcumene	Sesquiterpene	25.728	1496.66	1,471,107	1.087 ± 0.01^{b}	0.838 ± 0.14^{c}	1.224 ± 0.11^{a}
39	Elixene	Sesquiterpene	25.838	1499.21	2,106,935	1.556 ± 0.06^{a}	0.865 ± 0.01^{b}	–
40	(+)-Valencene	Sesquiterpene	26.214	1510.55	2,341,720.5	1.730 ± 0.12^{a}	1.060 ± 0.11^{b}	0.951 ± 0.09^{c}
41	A-bulnesene	Sesquiterpene	26.639	1515.69	444,561.44	0.328 ± 0.01^{a}	0.122 ± 0.05^{b}	–
42	B-bisabolene	Sesquiterpene	26.754	1520.32	3,196,499.5	2.361 ± 0.07^{a}	2.021 ± 0.03^{b}	1.850 ± 0.09^{c}
43	U.i	–	26.949	1523.33	2,067,534.9	1.527 ± 0.05^{a}	1.238 ± 0.01^{b}	1.002 ± 0.07^{c}
44	U.i	–	27.314	1525.77	3,809,235	2.814 ± 0.03^{a}	1.987 ± 0.07^{b}	0.027 ± 0.06^{c}

Table 2 (continued)

Peak no.	Compound	Chemical class	RT	RI	Area (%)	110 °C (yield%)	120 °C (yield%)	130 °C (yield %)
45	1,6-Dimethyl-4-propan-2-yl-1,2,3,7,8,8a-hexahydronaphthalene	Alkene	27.634	1528.92	244,463.66	–	–	0.054 ± 0.06^{a}
46	U.i	–	27.734	1533.61	685,533.81	0.506 ± 0.12^{b}	–	1.794 ± 0.03^{a}
47	(−)-A-gurjunene	Sesquiterpene	27.849	539.11	637,696.38	–	–	0.142 ± 0.09^{a}
48	U.i		28.044	1541.83	2,819,940.8	2.083 ± 0.03^{a}	0.757 ± 0.11^{c}	1.340 ± 0.06^{b}
49	Caryophyllene oxide	Sesquiterpene/oxide	28.374	1543.21	1,173,483.5	–	–	0.262 ± 0.10^{a}
50	U.i	–	28.614	1545.27	198,081.06	–	–	0.044 ± 0.12^{a}
51	U.i	–	28.80	1549.66	76,050.289	–	–	0.017 ± 0.10^{a}
52	U.i	–	29.53	1555.23	9,156,048	6.765 ± 0.12^{a}	6.386 ± 0.11^{b}	5.466 ± 0.01^{c}
53	U.i	–	30.13	1567.63	289,741.53	0.214 ± 0.05^{a}	0.195 ± 0.11^{c}	0.204 ± 0.12^{b}
54	U.i	–	30.515	1575.44	2,139,913.8	1.581 ± 0.01^{a}	0.000 ± 0.12	1.206 ± 0.05^{b}
55	Cedrenol	Sesquiterpene/alcohol	30.765	1577.32	3,221,597.3	–	1.686 ± 0.03^{a}	
56	U.i	–	31.101	1581.81	150,363.7	–	–	0.033 ± 0.14^{a}
57	U.i	–	31.236	1582.82	849,279.38	–	–	0.190 ± 0.07^{a}
58	U.i	–	31.246	1583.11	24,609.959	–	0.012 ± -0.11^{a}	–
59	U.i	–	33.567	1585.91	5,438,936.5	–	–	1.217 ± 0.11^{a}
60	2-Nonadecanone	Monoterpene/ketone	38.709	1592.63	71,862	0.053 ± 0.03^{a}	–	–

1. Values are mean ± standard deviation of three samples of each CSEO, analyzed individually in triplicate. Means followed by different superscript letters in the same row represent the significant difference ($p < 0.05$)

2. Compounds are listed in order of elution from ULTRA Dimethyl polysiloxane packed capillary column

3. Retention indices relative to *n*-alkanes (C_6–C_{24}) on the MS Clarus 600 column

4. RT, identification based on retention time; RI, identification based on retention index; MS, identification based on comparison of mass spectra. t = traces < 0.05

obtained at 120 °C distillation temperature were caryophyllene (44.3%), humulene (11.9%), *cis*-β-farnesene (9.71%), *trans*-α-bergamotene 6.79% and d-limonene (2.13%). Similarly, the principal components of CSEO extracted at 130 °C were caryophyllene (37.6%), humulene (9.51%), *cis*-β-farnesene (8.63%), *trans*-α-bergamotene (5.42%) and *d*-limonene (5.13%).

In addition, CSEO contained substantial amounts of various minor constituents. The minor components (< 5%) included α-pinene (3.59%), unidentified compounds (u.i., 2.81–0.01%), β-bisabolene (1.85–2.36%), cedrenol (1.68%), (+)-valencene (0.95–1.73%), α-curcumene (0.83–1.22%) and α-curcumene (0.08–1.22%). The main differences between oils extracted at various temperatures were caryophyllene, humulene, *cis*-β-farenscene, *trans*-α-bergamotene, and limonene. At a higher temperature of 130 °C, 45 components were analyzed compared to 29 components identified in CSEO extracted at 110 °C. This might be because almost all constituents of essential oils are unstable at high temperatures, and degradation into other components occurs. Therefore, to obtain high-quality oil, distillation should be carried out at low temperatures [59–62].

Table 3 Fractionation of CSEO collected at various BP ranges using vacuum fractional distillation

No	110 °C HD		120 °C HD		130 °C HD	
	BP (°C)	Vol (mL)	BP (◦C)	Vol (mL)	BP (°C)	Vol (mL)
F1	150–180	22	140–170	24	130–160	20
F2	180–210	12	170–200	10	160–190	14
F3	210–230	5	200–230	7	190–220	8
F4	230–250	30	230–260	35	220–250	40
F5	250–270	15	260–290	14	250–280	16
Rf	> 270	16	> 290	10	> 280	15

Fractional separation and analysis

Bioassay-guided fractionation is a common approach to investigating plant extract, essential oils, and their fractions [63–65]. Table 3 shows the collection of various fractions of CSEO at various temperatures by hydrodistillation. The short-path molecular distillation or vacuum distillation allowed different molecules or components of CSEO in different fractions (F1, F2, F3, F4, F5, and Rf) to be concentrated. The hydrodistilled essential oil extracted at 110 °C, 120 °C and 130 °C distributed into five main fractions with boiling point ranges (150–180, 180–210, 210–230, 230–250, and 250–270 °C), (140–170, 170–200, 200–230, 230–260 and 260–290 °C), (130–160, 160–190, 190–220, 220–250, 250–280), respectively, and a residual fraction containing fewer terpene components or low volatile components with the higher boiling points as > 270, > 290 and > 280 °C respectively.

The chemical compositions of the studied CSEO fractions that are characterized by maximum antimicrobial and antioxidant activities are given in Table 4. The results are of fractions having maximum levels of most active components. The active compounds of CSEO were identified by GC–MS analysis. Table 4 shows four major components of 110 °C distilled isolated fraction (F1). These major compounds were humulene 42.4%, α-pinene 28.8%, *cis*-β-farnesene 19.9%, and one minor component, β-bisaboline 8.74%, that accounted for 100% of the total fraction collected. For 120 °C distilled active fraction (F5), the major components were humulene 34.2%, *trans*-α-bergamotene 26.1%, humulene 34.2%, *cis*-β-farnesene 13.6%, and the minor components, are alloaromadendrene, 8.44%, β-curcumene 7.10%, gamma-muurolene 3.17%, and α-curcumene 2.10%. For 130 °C hydrodistilled CSEO, the most active fraction (F4) was caryophyllene 52.6%, humulene 17.0%, and *cis*-β-farnesene 12.6%. Vacuum distillation allowed a different proportion of CSEO molecules in different fractions to be concentrated. Differences in the compositions between fractions are based on the difference between the boiling points (BP) of CSEO.

Table 4 Composition of CSEO fractions collected by vacuum fractionation

Peak #	Compound	Class	RT	RI	Area (%)	(Yield %)
Fraction 1 CSEO extracted at 110 °C						
1	A-pinene	Monoterpene	5.26	939.53	5,373,294.5	28.87 ± 0.08^{b}
2	Humulene	Sesquiterpene	24.578	1452.52	17,894,736	42.47 ± 0.01^{a}
3	*Cis*-β-farnesene	Sesquiterpene	24.738	1455.34	11,754,096	19.92 ± 0.09^{c}
4	Beta bisabolene	Sesquiterpene	26.74	1520.32	4,208,737	8.74 ± 0.10^{d}
Fraction 5 of CSEO extracted at 120 °C						
1	*Trans*-α-bergamotene	Sesquiterpene	23.893	1429.33	9,679,677	26.15 ± 0.11^{a}
2	Humulene	Sesquiterpene	24.608	1452.52	17,894,736	34.22 ± 0.01^{b}
3	*Cis*-β-farnesene	Sesquiterpene	24.758	1455.34	11,754,096	13.60 ± 0.09^{c}
4	Alloaromadendrene	Sesquiterpene	24.858	1467.53	601,293.44	8.44 ± 0.06^{d}
5	U.i		24.993	1483.55	292,929.34	5.22 ± 0.03^{e}
6	*Gamma*-muurolene	Sesquiterpene	25.493	1487.73	938,143	3.17 ± 0.06^{f}
7	B-curcumene	Sesquiterpene	25.598	1491.32	231,128	7.10 ± 0.07^{d}
8	A-curcumene	Sesquiterpene	25.728	1496.66	1,471,107	2.10 ± 0.01^{f}
Fraction 4 of CSEO extracted at 130 °C						
1	Caryophyllene	Sesquiterpene	23.262	1421.53	60,447,852	52.66 ± 0.03^{a}
2	Aromandendrene	Sesquiterpene	24.408	1447.76	543,785.56	9.11 ± 0.06^{d}
3	Humulene	Sesquiterpene	24.588	1452.52	27,610,816	17.01 ± 0.06^{b}
4	*Cis*-β-farnesene	Sesquiterpene	24.748	1455.34	21,479,936	12.68 ± 0.01^{c}
5	UI		26.214	1510.55	2,341,720.5	5.36 ± 0.12^{e}
6	B-bisaboline	Sesquiterpene	26.74	1520.32	4,208,737	3.18 ± 0.03^{a}

1. Values are mean ± standard deviation of three samples of each CSEO fraction, analyzed individually in triplicate. Means followed by different superscript letters in the same column represent the significant difference ($p < 0.05$)

2. Compounds are listed in order of elution from ULTRA Dimethyl polysiloxane packed capillary column

3. Retention indices relative to *n*-alkanes (C_6–C_{24}) on the MS Clarus 600 column

4. RT, identification based on retention time; RI, identification based on retention index; MS, identification based on comparison of mass spectra. t = traces < 0.05

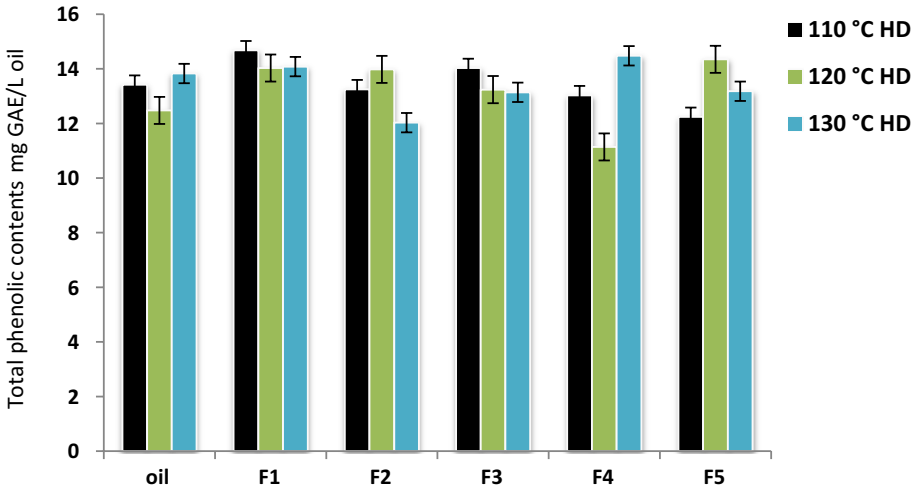

Fig. 1 Total phenolic content of CSEO and CSF

Antioxidant activity

Antioxidants react with free radicals, neutralizing them and reducing their damaging impact on the body [66]. TPC ranged from 13.4 to 13.8 mg GAE/mL for hydro-distilled CSEO, as shown in Fig. 1. For 110 °C extracted CSEO, the isolated fraction with higher TPC was F1, followed by F3 > F4 > F2 > F5. For 120 °C extracted CSEO, F5 has maximum TPC followed by F1 > F2 > F3 > F4. For 130 °C extracted CSEO, F4 had higher TPC followed by F1 > F5 > F3 > F2.

TFC ranged from 14.1 to 14.2 mg CE/mL for hydro-distilled extracted CSEO, as shown in Fig. 2. For 110 °C extracted CSEO, the isolated fraction with higher TFC was F1, followed by F5 > F4 > F2 > F3. For 120 °C extracted CSEO, F5 has maximum TPC followed by F2 > F4 > F3 > F1. For 130 °C extracted CSEO, F4 had higher TPC followed by F5 > F2 > F1 > F3.

Total pro-anthocyanidin contents (TPACC) ranged from 8.91 to 10.46 mg VE/mL for hydro-distilled extracted CSEO, as shown in Fig. 3. For 110 °C extracted CSEO, the isolated fraction with higher TPACC was F1, followed by F4 > F5 > F2 > F3. For 120 °C extracted CSEO, F5 has maximum TPC followed by F1 > F4 > F2 > F3. For 130 °C extracted CSEO, F4 had higher TPACC followed by F1 > F5 > F2 > F3.

Phytochemicals like phenolics, flavonoids, proanthocyanidins, and flavonols are primary or natural antioxidants. Fractions usually have a more concentrated amount of these chemicals than the rest of the oil. This could also be due to the difference in the method used to extract the sample. The concentration of phenolics in this study is comparable to [67, 68] for the essential oil of commercial chamomile and thyme.

The antioxidant effect of extracted CSEO and CSF was determined according to the FTC [41]. The percentage of total antioxidant contents by the FTC method ranged from 31.4 to 47.1 for hydrodistilled extracted CSEO, as shown in Fig. 4. For 110 °C extracted CSEO, the isolated fraction with higher TAC was F1, followed by F5 > F4 > F2 > F3.

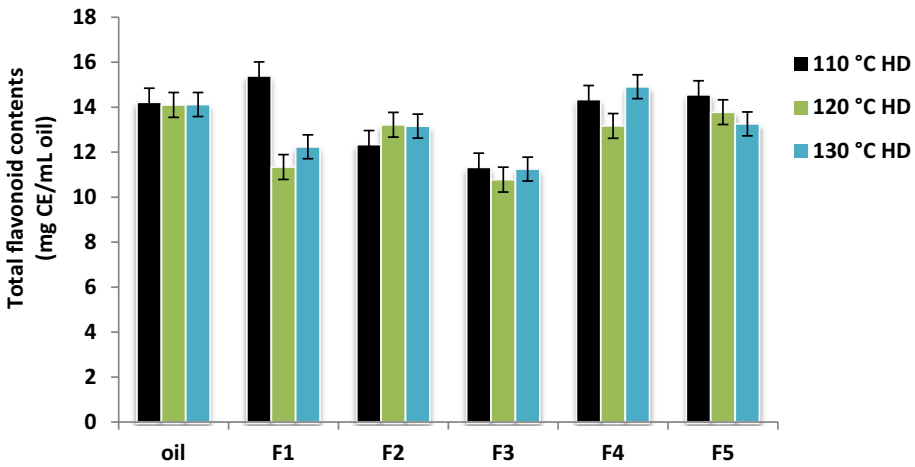

Fig. 2 Total flavonoid content of CSEO and CSF

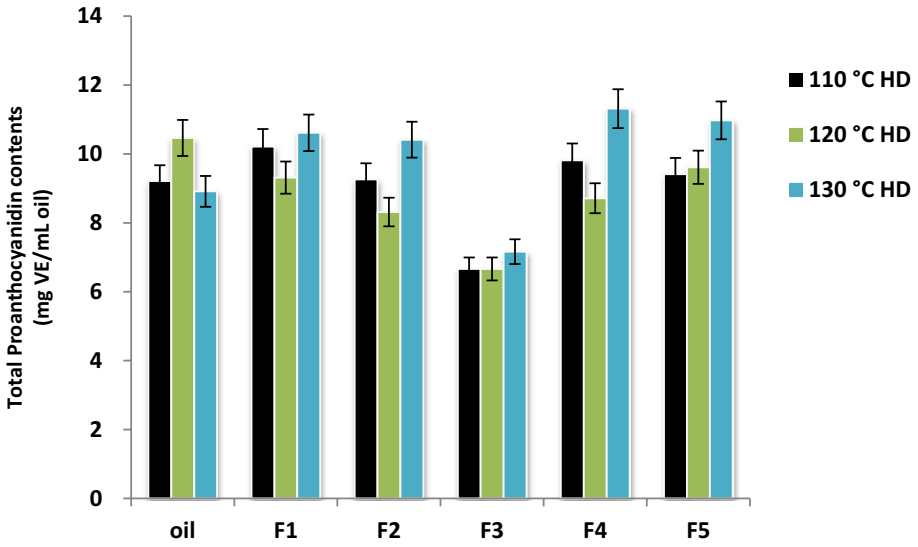

Fig. 3 Total pro-anthocyanidin contents of CSEO and CSF

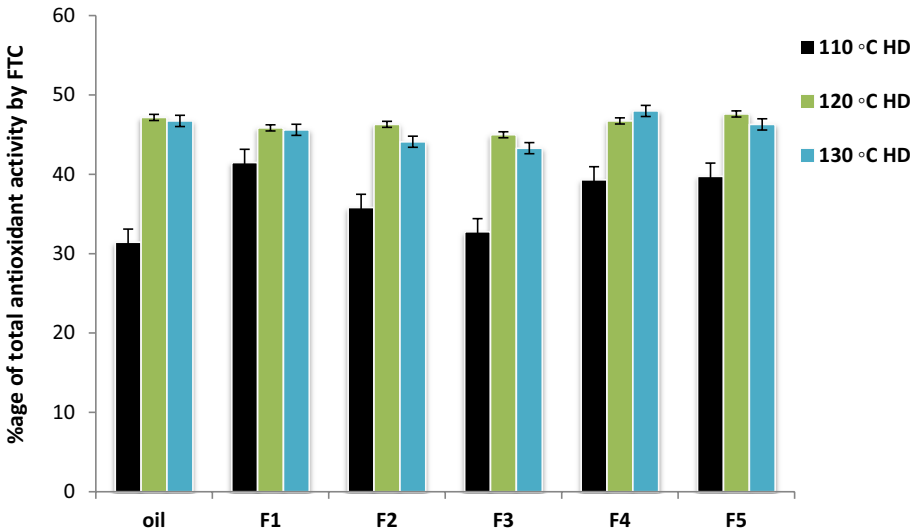

Fig. 4 Total antioxidant activity of CSEO and CSF by FTC method

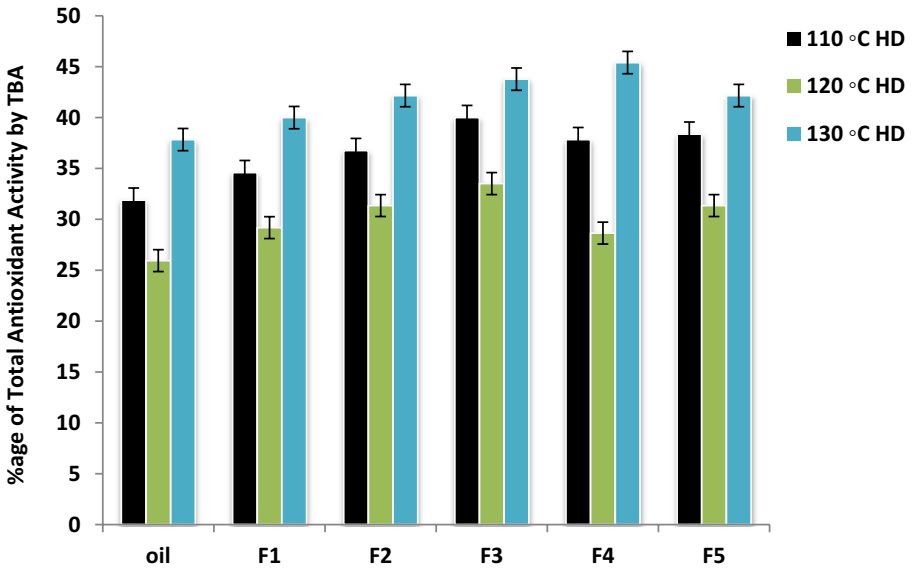

Fig. 5 Total antioxidant activity of CSEO and CSF by TBA method

For 120 °C extracted CSEO, F5 has maximum TAC followed by F4 > F2 > F1 > F3. For 130 °C extracted CSEO, F4 had higher TAC followed by F5 > F1 > F2 > F3. A modified TBARS assay [69–71] was used to measure the potential antioxidant activity of CSEO and CSF. The percentage of total antioxidant contents by the TBA method ranged from 19.2 to 27.1 for CSEO, as shown in Fig. 5. For 110 °C extracted CSEO, the isolated fraction with higher TAA was F1, followed by F5 > F2 > F4 > F3. For 120 °C extracted CSEO, F5 has maximum TAA followed by F4 > F1 > F3 > F2. For 130 °C extracted CSEO, F4 had higher TAA followed by F1 > F5 > F2 > F3.

The effect of temperature and mode of extraction on total phytochemical contents and total antioxidant activity of CSEO was significant ($p < 0.05$). Also, a significant difference ($p < 0.05$) in total antioxidant activity and fractions contents was observed. F1, F5, and F4 contained higher TPC, TFC, and TPACC and similarly higher antioxidant capacity in hydro-distilled *C. sativa* oil fractions. Extraction temperature affects the chemical composition and bioactivities of the essential oil and oil fractions. Different oil yields and components of essential oils were obtained at different temperatures. Meanwhile, bioactive components that showed maximum biological effects are present in oil extracted at lower temperatures or in fractions obtained at a specific range of temperatures.

Plant phytochemicals' antioxidant activities also happen by inhibiting the production of free radicals, scavenging free radicals, or chelating the transition metal [72]. Prevention of chain initiation by scavenging reactive species is considered an essential antioxidant mode of action [73]. DPPH· radical scavenging effects of CSEO and CSF had depicted in Fig. 6. Ranges of CSEO inhibition were 20.5–26.2%, 20.1–35.9%, and 36.1–41.8% for HD, SD, and SCFE essential oils, respectively.

For 110 °C extracted CSEO, the isolated fraction with higher % DPPH· scavenging activity was F1, followed by F4 > F2 > F3 > F5. For 120 °C extracted CSEO, F5 has maximum activity followed by F4 > F2 > F1 > F3. For 130 °C extracted CSEO, F4 had maximum activity followed by F2 > F5 > F1 > F3. Hence, F1, F5, and F4 were the most active scavengers of DPPH·. Nevertheless, it is not easy to attribute the radical scavenging activity of the CSEO to one or a few major components because essential oils are a complex mixture of active compounds. However, phenolics exhibited maximum antioxidant traits [74]. The antioxidant effect of essential oil fractions showed a higher radical scavenging effect than the whole essential oil, indicating the possible synergistic interaction between different components of essential oils. The literature revealed a moderate radical scavenging impact of mentha, strawberries and citrus essential oils comparable to our findings [75–77]

Reducing power is a novel antioxidant defense system; electron transfer or hydrogen atom transfer is responsible for this property [78, 79]. The reducing power of CSEO and oil fractions is shown in Fig. 7. The reducing effect was measured over concentration ranges of 0.1–0.3 mg/mL. Results exhibited an increase in activity when concentration increased. Reducing potentials ranged from 2.44 to 2.75 for hydrodistilled CSEO. For 110 °C extracted CSEO, the isolated fraction with maximum reducing potential was F1, followed by F4 > F5 > F3 > F2. For 120 °C extracted CSEO, F5 has maximum potential followed by F4 > F1 > F2 > F3. For 130 °C extracted CSEO, F4 had higher reducing potential followed by F5 > F3 > F2 > F1. No earlier reports or past data are available about the reducing potential of CSEO or fractions. However, literary works are available about the reducing ability of different essential oils of different plants, i.e., *Marrubium vulgare*, oregano, pomegranate peel, chamomile (*Matricaria chamomilla*) flowers and *Mentha piperita*

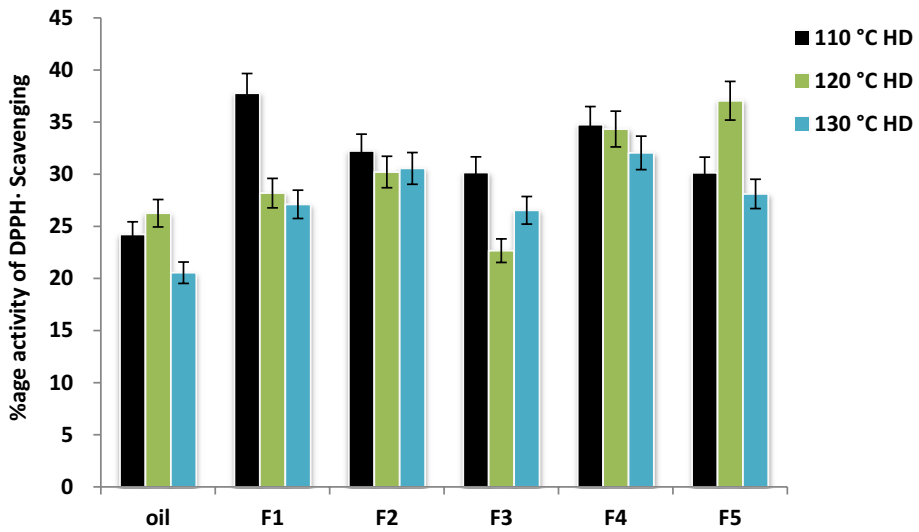

Fig. 6 DPPH· scavenging activity of CSEO and CSF

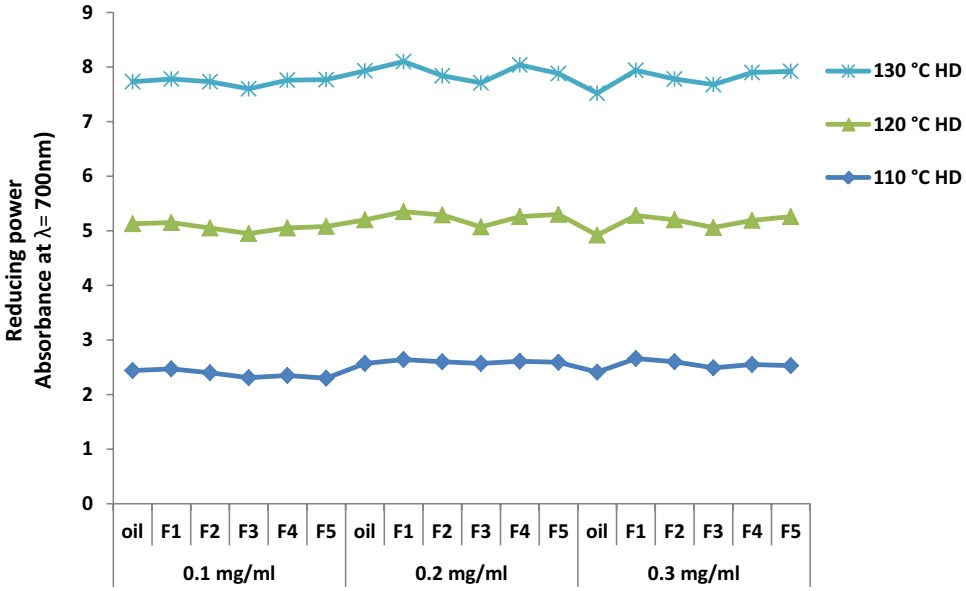

Fig. 7 Reducing power of CSEO and CSF

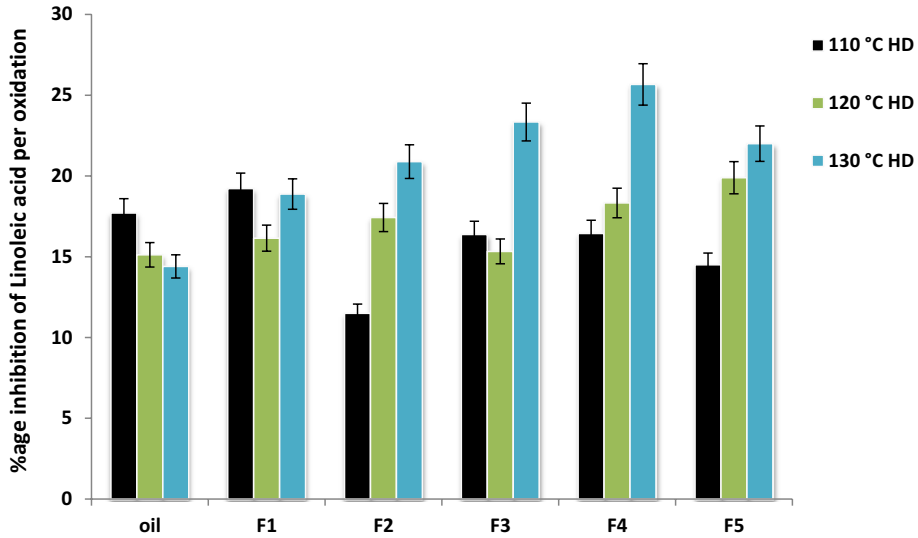

Fig. 8 %age inhibition of linoleic acid peroxidation by CSEO and CSF

leaves essential oils [80–85]. This available data could be comparable to our present results.

Figure 8 depicted the inhibition of per-oxidation of linoleic acid after 360 h of an incubation period. BHT was used as a positive control to compare the antioxidant impact of essential oil and fractions. Inhibition of linoleic acid peroxidation percentages ranged from 14.4 to 17.1% for CSEO. For 110 °C extracted CSEO, the isolated fraction with maximum inhibition % was F1 followed by F4 > F3 > F5 > F2. For 120 °C extracted CSEO, F5 has maximum % inhibition followed by F4 > F2 > F1 > F3. For 130 °C extracted CSEO, F4 had higher % followed by F5 > F3 > F2 > F1.

Antimicrobial activity

The CSEO and CSF were applied to bacterial strains, and a zone reader measured the inhibition zones (mm). From the data given in Fig. 9, it is accomplished that zones of inhibition in mm against *E. coli* ranged from 12.8 to 15.2 mm, against *P. multocida*, ranged from 12.6 to 14.8 mm, against *S. aureus* range ranged from 14.3 to 16.3 mm, against *B. subtilis* ranged from 12.2 to 13.4 mm and against *C. bifermient* as activity ranged from 13.7 to 14.6 mm for hydrodistilled CSEO. For 110 °C, 120 °C, and 130 °C CSF, the maximum inhibition zone against all strains of bacteria were F1, F5, and F4, respectively.

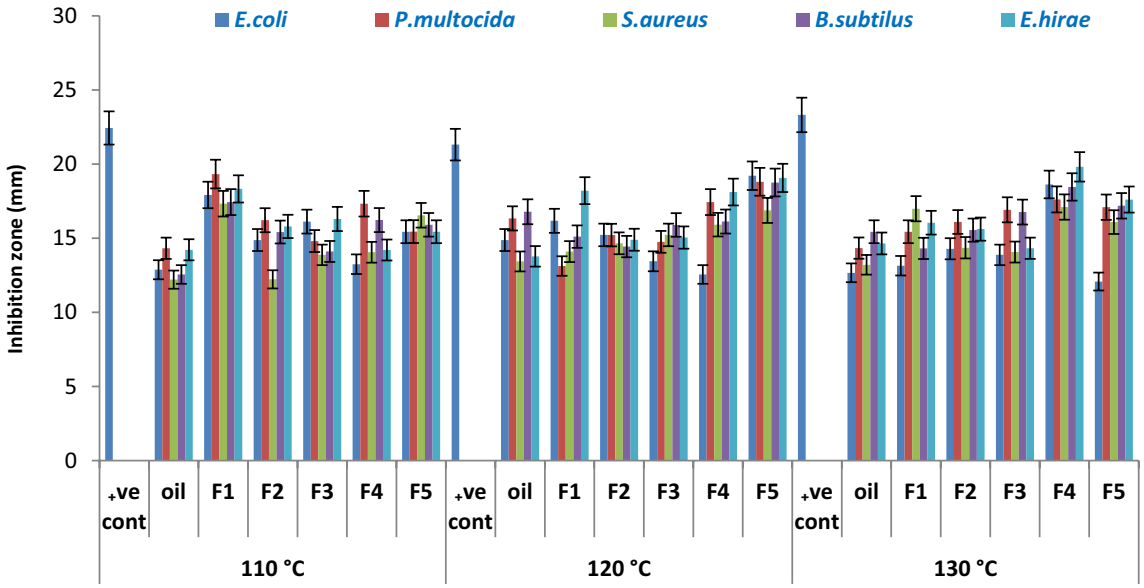

Fig. 9 Antibacterial activity of CSEO and CSF

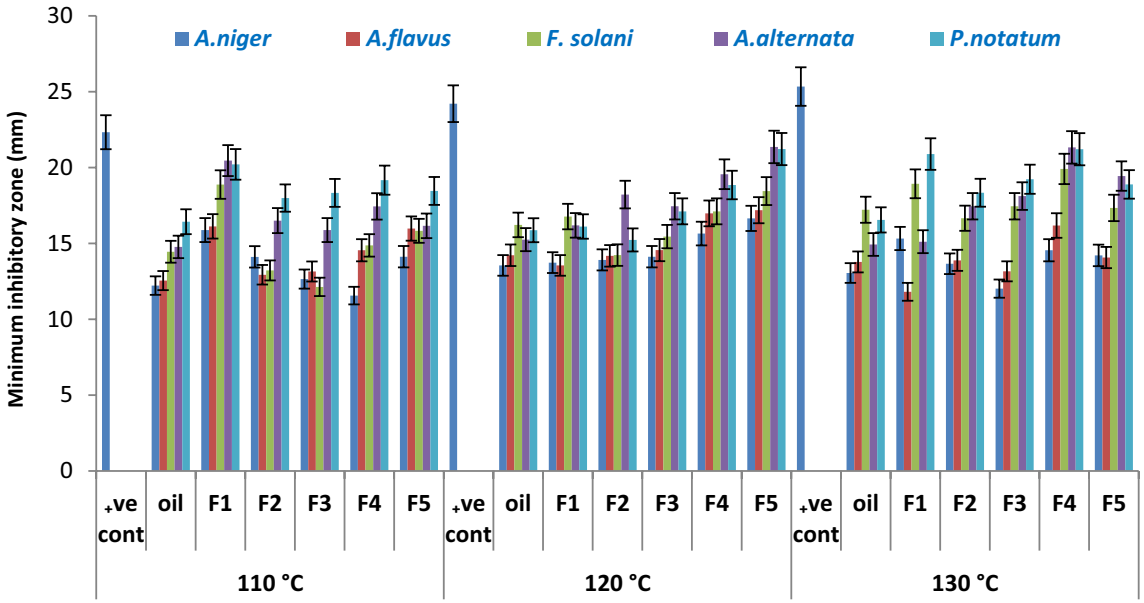

Fig. 10 Antifungal activity of CSEO and CSF

Antifungal tests of CSEO and CSF were carried out by the disk diffusion method [86] as reported [87–89] with minor modifications. From the data given in Fig. 10, it is accomplished that zones of inhibition in mm against *A. niger* ranged from 12.2 to 13.5 mm, against *A. flavus* ranged from 12.5 to 14.2 mm, against *P. notatum* ranged from 15.8 to 16.5 mm, against *A. alternate* ranged from 14.7 to 15.2 mm and against *F. solani* ranged from 14.4 to 17.2 mm. For 110 °C, 120 °C, and 130 °C CSF, the maximum inhibition zone against all tested strains of fungus were F1, F5, and F4, respectively. The effect of temperature and mode of extraction on the antifungal potential of CSEO was significant ($p < 0.05$). Also, a significant difference ($p < 0.05$) in the antifungal activity of fractions was observed.

CSEO and CSF are rich in monoterpenes and sesquiterpenes. Sesquiterpenes were detected at higher levels, as shown in the GCMS analysis of CSF in Table 4. F1is rich in α-pinene, humulene, *cis*-β-farnesene and β-bisaboline. F5 is rich in sesquiterpenes as shown in

Table 4, *trans*-α-bergamotene, humulene, *cis*-β-farnesene, alloaromadendrene, gamma-muurolene, β-curcumene and α-curcumene. Major and minor components of F4 fraction were caryophyllene, aromandendrene, humulene, *cis*-β-farnesene, and β-bisaboline. F2 consisted of monoterpenes as a major part of *d*-limonene, cineole, *trans*-β-ocimene, and gamma-terpinene. All these fractions were collected and isolated as the best antibacterial compared to other fractions and oils. The antifungal activity could be attributed to the presence of some components in CSEO and CSF, such as humulene, *cis*-β-farnesene, β-bisaboline, *trans*-α-bergamotene, alloaromadendrene, γ-muurolene, β-curcumene, α-curcume, caryophyllene, aromadendrene, *d*-limonene, cineole, *trans*-β-ocimene, and γ-terpinene.

Minimum inhibitory concentration (μg/mL) of CSEO and CSF against bacterial strains was achieved by microdilution

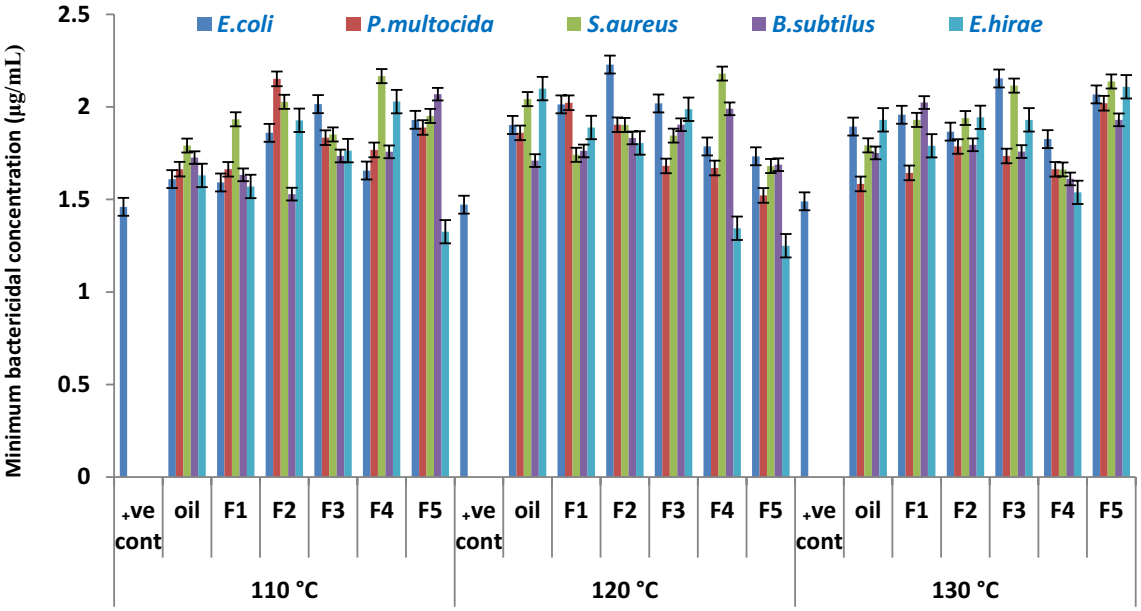

Fig. 11 Minimum bactericidal concentration of CSEO and CSF

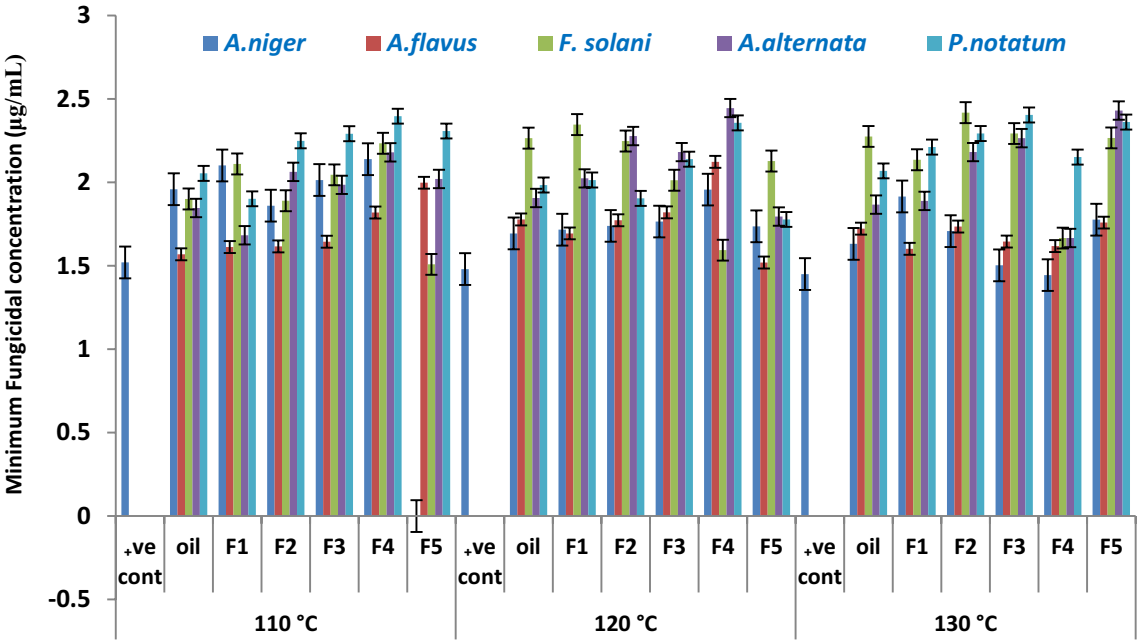

Fig. 12 Minimum fungicidal concentration of CSEO and CSF

broth assay in 96-well microplates. From the data given in Fig. 11, it was accomplished that the minimum inhibitory concentration in µg/mL required to kill *E. coli* was found to be ranged from 1.61 to 1.79 µg/mL against *P. multocida* ranged from 1.58 to 1.86, against *S. aureus* ranged from 1.79 to 2.04 µg/mL, and against *B. subtilis* ranged from 1.53 to 1.65 µg/mL. For 110 °C, 120 °C, and 130 °C CSF, the minimum inhibitory concentration against all tested strains of bacteria were F1, F5, and F4, respectively. From the data given in Fig. 12, it was accomplished that the minimum inhibitory concentration in µg/mL required to kill the fungal strain *A. niger* ranged from 1.53 to 1.69 µg/mL, against *A. flavus,* ranged from 1.57 to 1.78 µg/mL, against *A. alternate* ranged from 1.85 to 1.91 (µg/mL), and against *F. solani* activity ranged from 1.81 to 2.15 µg/mL. For 110 °C, 120 °C, and 130 °C CSF, the minimum fungicidal concentration against all tested strains of fungus were F1, F5, and F4, respectively. High antifungal activity of *Cuminum cyminum* L. against *Aspergillus* species was reported [90, 91]. Volatile oil showed a broad range of antifungal effects, inhibiting some nail-infecting fungi such as *A. fumigatus*, *A. ustus*, *Aspergillus niger*, *A. flavus*, *M. canis*, *M. audouini*, *C. albicans*, *E. flooccosum*, *M. nanum*, *T. tonsurons*, *M. gypseum*, *R. nigricans*, and *T. violaceum* [92–95].

Cytotoxicity

The brine-shrimp assay finds the lethality of samples toward brine-shrimp larvae. The shrimp nauplii have been used for several experiments in which various samples are tested at 10–1000 µg/mL in vials containing 5 mL of brine and ten nauplii [96]. The current study used the brine shrimp lethality assay to assess the cytotoxic effect of CSEO and CSF. The results obtained are tabulated in Table 5. Results showed that all CSEO and CSF were toxic to brine shrimps. The maximum and minimum mortality (98% and 9%) was found at 1000 ppm and 10 ppm, respectively, with the LC_{50} of 17.8% and 55.5%. For hydrodistilled CSEO, minimum LC_{50} was found in 110 °C CSEO and F1 with LC_{50} (20 and 25 ppm) and LC_{90} (180 and 187 ppm), respectively. The

Table 5 Cytotoxicity of CSEO against brine shrimps

	Percent death at 24 h				
	10 (ppm)	100 (ppm)	1000 (ppm)	LC_{50} (ppm)	LC_{90} (ppm)
CSEO					
110 °C					
F1	25 ± 0.01	50 ± 0.01	96 ± 0.01	20.00 ± 0.01^{a}	180.00 ± 0.04^{a}
Oil	20 ± 0.01	48 ± 0.02	94 ± 0.03	25.0 ± 0.01^{b}	187.50 ± 0.03^{b}
12 0 °C					
F5	15 ± 0.02	48 ± 0.03	95 ± 0.05	33.33 ± 0.02^{b}	187.50 ± 0.01^{b}
Oil	14 ± 0.07	45 ± 0.07	94 ± 0.01	35.71 ± 0.01^{b}	200.0 ± 0.02^{b}
13 0 °C					
F4	10 ± 0.01	45 ± 0.02	92 ± 0.02	50.0 ± 0.03^{d}	200.0 ± 0.01^{d}
Oil	11 ± 0.01	42 ± 0.02	90 ± 0.02	45.45 ± 0.03^{d}	214.2 ± 0.02^{d}

Means followed by different superscript letters in the same column represent significant differences among values

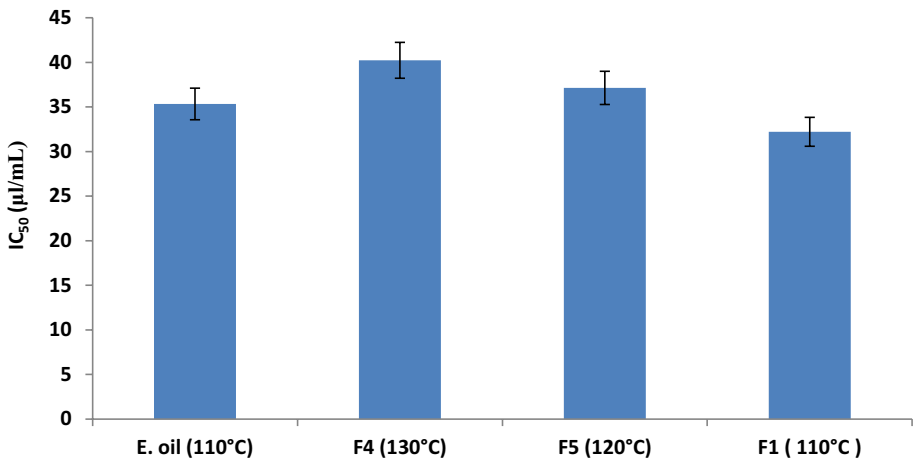

Fig. 13 IC_{50} of CSEO and CSF against potato tumor growth

Table 6 Percentage tumor inhibition of CSEO and CSF

Concentration (µL)	Sample code	The average number of tumors per disc	%age of tumor inhibition
250	CSEO (110 °C)	10 ± 0.02^{b}	33.33 ± 0.01^{b}
	F4 (130 °C)	12 ± 0.03^{d}	20.00 ± 0.03^{d}
	F5 (120 °C)	11 ± 0.01^{c}	26.67 ± 0.04^{c}
	F1 (110 °C)	9 ± 0.04^{a}	40.00 ± 0.01^{a}
500	CSEO (110 °C)	8 ± 0.02^{b}	46.67 ± 0.02^{b}
	F4 (130 °C)	10 ± 0.03^{d}	33.33 ± 0.03^{d}
	F5 (120 °C)	9 ± 0.01^{c}	40.00 ± 0.04^{c}
	F1 (110 °C)	7 ± 0.02^{d}	53.33 ± 0.01^{a}
1000	CSEO (110 °C)	5 ± 0.04^{c}	60.00 ± 0.04^{b}
	F4 (130 °C)	7 ± 0.01^{d}	53.33 ± 0.03^{d}
	F5 (120 °C)	6 ± 0.02^{c}	66.67 ± 0.02^{c}
	F1 (110 °C)	4 ± 0.03^{a}	73.33 ± 0.03^{a}
	−ve control	15 ± 0.01	0.00 ± 0.04
	+ve control	3 ± 0.01	80.00 ± 0.01

Means followed by different superscript letters in the same column represent significant differences among values

degree of lethality was directly proportional to the concentration of CSEO and CSF. Maximum mortalities occurred at a concentration range of 150–215 ppm, whereas the least mortalities were at 10–30 ppm, with almost 50% mortalities at 100 ppm concentration. No mortality was noted in the control tubes indicating that CSEO was responsible for the mortality (Fig. 13).

The observed activity variations might be due to their chemical composition. The possible cytotoxicity of CSEO may be due to the presence of bioactive constituents, including cis-β-farnesene, β-bisaboline, *trans*-α-bergamotene, alloaromadendrene, γ-muurolene, β-curcumene, α-curcume, caryophyllene, aromadendrene, *d*-limonene, cineole, *trans*-β-ocimene, and γ-terpinene makes it a non-specific cell targeting cytotoxic. Overall results showed that pinene, humulene, *cis*-β-farnesene, and β-bisaboline in F1 from 110 °C extracted essential oil is more toxic than other components.

Crown gall antitumor activity

The results of the percent inhibition of tumors in potatoes are shown in Table 6. The %t inhibition results showed that all samples had an anti-crown gall effect. Tumor inhibition was noted in a concentration-dependent mode. At higher concentrations (1000 µL), maximum antitumor activity was observed. Inhibition increased with increasing concentration. Meanwhile, 73% inhibition was recorded for the F1 from 110 °C extracted oil, followed by 60% inhibition of CSEO (110 °C), which was > 66.6% for F5 (120 °C) and > 53.3% for F4 (130 °C). A similar trend was found in lower concentrations (250 µL and 500 µL) of CSEO and CSF. The highest cytotoxic activity (IC_{50}) was found in F1 from 110 °C extracted oil fraction.

Conclusion

The present study explored the antimicrobial, antioxidant, antitumor, and cytotoxic potential of CSEO extracted by hydrodistillation. Maximum antioxidant, antimicrobial and antitumor potential was observed for F1, F5, and F4 of CSEO, which might be correlated to their higher percentage of sesquiterpenoid and monoterpenoid contents compared to pure essential oil. Based on our findings, CSEO and CSE could be used in several food and non-food products. Furthermore, *C. sativa* essential oil can further be used for anticancer medicines and drug formulations to prevent other neurodegenerative diseases. This brief study can be helpful for pharmacists and health workers to use *C. sativa* essential oil isolated components for human medicines and drugs after further purification by using the latest separation techniques. Moreover, NMR techniques can identify unidentified components in GC–MS analysis, and this will be an excellent addition to the library of chemical components isolated from plants.

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Data availability The datasets generated during the current study are made available by the corresponding author upon reasonable request.

Code availability Not applicable.

Declarations

Competing interests The authors declare no competing interests.

Research involving human and/or animal participants This study does not contain any studies with human participants or animals performed by any authors.

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