J Sci Food Agric 85:2297–2306 (2005)
DOI: 10.1002/jsfa.2255

Chemical constituents and antimicrobial and antioxidant potentials of essential oil and acetone extract of *Nigella sativa* seeds†

Gurdip Singh,[1]* Palanisamy Marimuthu,[1] Carola S de Heluani[2] and Cesar Catalan[2]

[1]Chemistry Department, DDU Gorakhpur University, Gorakhpur-273009, India
[2]Instituto de Quimica Organica, Universidad Nacional de Tucuman, Ayacucho 471, S M de Tucuman 4000, Argentina

Abstract: The antifungal, antibacterial and antioxidant potentials of essential oil and acetone extract of black cumin were investigated by different techniques. In the inverted petriplate method, the essential oil showed complete zones of inhibition against *Penicillium citrinum* at a 6 µl dose. Essential oil showed complete growth inhibition against *Bacillus cereus, Bacillus subtilis* and *Staphylococcus aureus, Pseudomonas aeruginosa* at 2000 and 3000 ppm, respectively, by the agar well diffusion method. The antioxidant activity was evaluated by measuring peroxide, TBA and total carbonyl values of rapeseed oil at fixed time intervals. Both the extract and essential oil showed strong antioxidant activity in comparison with butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). In addition, their inhibitory action in the linoleic acid system was studied by monitoring the accumulation of peroxide concentration. Their radical scavenging capacity was carried out on 2,2′-diphenyl-1-picrylhydrazyl (DPPH) radical and they showed excellent scavenging activity in comparison with synthetic antioxidants. Their reducing power was also determined, demonstrating strong antioxidant capacity of both the essential oil and extract. Gas chromatographic and gas chromatographic–mass spectrometric studies on the essential oil resulted in the identification of 38 components representing 84.65% of the total amount. The major component was *p*-cymene (36.2%) followed by thymoquinone (11.27%), α-thujene (10.03%), longifolene (6.32%), β-pinene (3.78%), α-pinene (3.33%) and carvacrol (2.12%), whereas extract showed the presence of 16 components representing 97.9% of the total amount. The major components were linoleic acid (53.6%), thymoquinone (11.8%), palmitic acid (10%), *p*-cymene (8.6%), longifolene (5.8%) and carvacrol (3.7%).

Keywords: gas chromatography–mass spectrometry; antimicrobial properties; antioxidant potential; scavenging ability; reducing power; *Nigella sativa*

INTRODUCTION

Lipid oxidation, which results in food rancidity, constitutes one of the major changes that can occur during the processing, distribution, storage and final preparation of foods. To control and delay the onset of rancidity, there is a growing demand for substances that can be introduced directly into the food product to inhibit these processes. In this context, various synthetic antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA) butylated hydroxytoluene (BHT) and propyl gallate (PG) have been approved and are routinely used as food protecting agents.[1] However, these food additives have been reported to possess possible toxic and carcinogenic properties.[2,3] Hence the recent growing public demand for replacement of synthetic with natural antioxidants has initiated intense research activity.[4–6] For instance, an ethanolic extract of bearberry leaves has been found to have potent antioxidant activity for non-nitrite-processed meats.[6] *Nigella sativa* Linn, commonly known as black cumin seed, is an annual plant that has been traditionally used in the Indian sub-continent for culinary and medicinal purposes as a natural remedy for a number of illnesses and conditions that include asthma, hypertension, diabetes, cough and influenza. Various pharmacological tests have been carried out to investigate different compounds in black cumin seeds.[7–11]Burits and Bucar[12] reported the antioxidant activity of the essential oil and the components isolated from black cumin seeds. There are reports on the antimicrobial properties of black cumin extracts.[13,14] However, little is known about the antioxidant activity of black cumin essential oil[12,14] and so far as we are

* Correspondence to: Gurdip Singh, Chemistry Department, DDU Gorakhpur University, Gorakhpur-273009, India
E-mail: gsingh4us@yahoo.com
† Part 53.
Contract/grant sponsor: Life Sciences Research Board, DDU; contract/grant number: CONICET
(*Received 20 July 2004; revised version received 24 January 2005; accepted 17 February 2005*)
Published online 28 June 2005

aware there is no report on the antimicrobial and antioxidant properties of an acetone extract of black cumin seeds. In this paper, we present studies of the antioxidant and antimicrobial potentials of black cumin essential oil and acetone extract (the latter will be referred to as 'extract' below) by different assay methods. The chemical components of both were evaluated by gas chromatographic (GC) and gas chromatographic–mass spectrometric (GC–MS) techniques.

MATERIALS AND METHODS

Plant material

The seeds of black cumin were purchased from the local market of Gorakhpur, Uttar Pradesh, India. A voucher specimen (herbarium No.123) was deposited at the Herbarium of the Faculty of Science, DDU Gorakhpur University.

Reagents

DPPH and carbendazim were purchased from Sigma (Sigma-Aldrich, Steinheim, Germany) and linoleic acid from Acros (NJ, USA). Butylated hydroxyanisole, butylated hydroxytoluene, propyl gallate and 2,4-dinitrophenylhydrazine were purchased from Sd Fine-Chem (Mumbai, India) and TBA from Merck (Darmstadt, Germany). Ampicillin and cloxacillin were purchased from Ranbaxy Fine Chemicals (New Delhi, India). Crude rapeseed oil was obtained from a local oil mill in Gorakhpur. All solvents used were of analytical grade.

Essential oil of black cumin

Black cumin seeds were ground (750 mesh) with a domestic electronic grinder and hydrodistilled in a Clevenger-type apparatus for 3 h according to the method recommended by the European Pharmacopoeia.[15] A light orange volatile oil, with a characteristic odour, was obtained with a yield of 1.2%. It was dried over anhydrous sodium sulfate and the sample was stored at 4 °C before use.

Acetone extract of black cumin

After the isolation of the essential oil, the crude material was oven dried at 50 °C for 24 h. Then, 20 g of dried material were loaded on to a Soxhlet apparatus and extraction was carried out with acetone (400 ml) at 90 °C for 3 h. The solvent was removed under reduced pressure and the remaining viscous extract was used for further tests.

GC and GC–MS analysis

The chemical analysis of black cumin oil and extract was performed by GC and GC–MS techniques.

GC

A Hewlett-Packard Model HP 5890 gas chromatograph (Analytical Technologies, Buenos Aires, Argentina) fitted with capillary column (5% phenylmethylsiloxane; length = 30 m, inner diameter = 0.32 mm and film thickness = 0.25 μm) whose injector and detector temperatures were maintained at 240 and 250 °C, respectively. The injection volume was 1 μl with a split ratio of 30:1 and helium was used as the carrier gas at a flow rate of 1.0 ml min^{-1}. The oven temperature for both essential oil and extract was programmed linearly as follows: increased from 60 to 185 °C at 1.5 °C min^{-1}, held for 1 min, then increased from 185 to 275 °C at 9 °C min^{-1}.

GC–MS

The essential oil and extract were subjected to GC–MS analysis using a Hewlett-Packard Model 6890 gas chromatograph (Analytical Technologies SA, Buenos Aires, Argentina) coupled to a quadrupole mass spectrometer (Model HP 5973) with a capillary column of HP-5MS (5% phenylmethylsiloxane, length = 30 m, inner diameter = 0.25 mm and film thickness = 0.25 μm). The injector, GC–MS interface, ion source and mass-selective detector temperatures were maintained at 280, 280, 230 and 150 °C, respectively. The oven temperature for essential oil was programmed linearly as follows: from 60 to 185 °C at 1.5 °C min^{-1}, held for 1 min, then from 185 to 275 °C at 9 °C min^{-1}, held for 2 min. The extract was held at 70 °C for 5 min, then increased from 70 to 220 °C at 3 °C min^{-1} and from 220 to 280 °C at 5 °C min^{-1}, then held for 5 min.

Identification of components

The components were identified on the basis of comparisons of their retention indices and mass spectra with published data[16,17] and computer matching was done with the Wiley 275 and National Institute of Standards Technology (NIST 3.0) libraries provided with the computer controlling the GC–MS system. The retention index was calculated for the volatile constituents using a homologous series of C_8–C_{16} *n*-alkanes.

Antibacterial assay

The essential oil and extract were individually tested against a panel of microorganisms including three Gram-positive bacteria, *Staphylococcus aureus* (3103), *Bacillus cereus* (430) and *Bacillus subtilis* (1790), and three Gram-negative bacteria, *Escherichia coli* (1672), *Salmonella typhi* (733) and *Pseudomonas aeruginosa* (1942). All the bacterial strains were procured from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (Chandigarh, India) and their reference numbers are given in parentheses. The bacterial cultures were grown on nutrient agar medium and stored at 4 °C. In order to prepare a bacterial strain for test, initially one loopful of bacterial culture was transferred from slant to nutrient broth solution (10 ml) and was stored at 37 °C for 24 h. The strain was prepared by adding 1 ml of broth to 9 ml of Ringer's solution. The test samples were prepared by

dissolving 2, 4 or 6 µl of essential oil or extract in 1 ml of dimethyl sulfoxide (DMSO) solution, from which 200 µl were transferred into each well.

Agar well diffusion method

The agar well diffusion method[18] was employed for the determination of antibacterial activity. Briefly, a suspension of the test microorganism (0.1 ml) was spread on a previously prepared, dried nutrient agar plate by using a sterile bent rod. The wells were 10 mm in diameter cut from the agar and 200 µl of essential oil or extract were transferred into them. The control plate without the addition of essential oil or extract containing DMSO was also maintained under the same conditions. After incubating for 24 h at 37 °C, all plates were examined for any zones of growth inhibition and the diameters of these zones were measured in millimetres.

Antifungal assay

The antifungal activity of the essential oil and extract against various pathogenic fungi, *Aspergillus flavus* (1884), *Aspergillus ochraceus* (1810), *Aspergillus terreus* (3374), *Aspergillus niger* (2479), *Fusarium moniliforme* (1893), *Fusarium graminearum* (2088), *Penicillium citrinum*, (2553) and *Penicillium viridicatum* (2007) were tested by the inverted petriplate[19] and poison food medium[20] methods. All the fungi cultures were procured from the MTCC and their reference numbers are given in the parentheses. The cultures were maintained in Czapek agar medium. Each test was replicated three times and fungi toxicity was measured in terms of percentage mycelial inhibition calculated with the following equation:

$$\text{mycelial inhibition}(\%) = [(d_c - d_t)/d_c] \times 100 \quad (1)$$

where d_c and d_t are the average diameters of the mycelial colony of the control and treated sets, respectively. In the food poison technique, a calculated quantity (2, 4, 6 µl) of undiluted essential oil (or extract, dissolved in ethanol) was mixed with 25 ml of medium (~45 °C) and poured on to a previously sterilized and dried petriplate. In the inverted petriplate method, a calculated quantity (2, 4, 6 µl) of essential oil (or extract, dissolved in ethanol) was soaked on filter-paper (10 mm in diameter) and kept on the lid of the inverted petriplate. Control plates were prepared with and without the addition of ethanol under the same conditions. The plates were incubated at 37 °C for 6 days and the zone of inhibition was measured with the help of vernier callipers.

Evaluation of antioxidant activity

In order to evaluate antioxidant potential of essential oil and extract, the crude rapeseed oil was taken, which had an initial peroxide value 2.0 meq kg^{-1}. In the case of essential oil and extract, 6 µl were added to rapeseed oil whereas in the case of synthetic antioxidants 6 mg (BHA, BHT and PG) were added to make 200 ppm. The mixtures were prepared in 100-ml open-mouthed beakers, then they were thoroughly homogenized and incubated at 70 °C in the dark. A blank sample was prepared under the same conditions without any additives. Three replications were performed for each sample. The antioxidant activity of essential oil and extract against rapeseed oil was studied by measuring peroxide, TBA and carbonyl values at fixed time intervals. In addition, their antioxidant potential was studied in linoleic acid and their individual antioxidant capacity was evaluated by DPPH assay. The reducing power of essential oil and extract was also evaluated in comparison with synthetic antioxidants.

Peroxide value

The peroxide value of all samples was measured[21] every 7 days by the Schaal oven test.[22] A known weight of rapeseed oil sample (3 g) was dissolved in glacial acetic acid (30 ml) and chloroform (20 ml), then a saturated solution of KI (1 ml) was added. The mixture was kept in the dark for 15 min. After the addition of distilled water (50 ml), the mixture was titrated against sodium thiosulfate (0.02 N) using starch as indicator. A blank titration was performed in parallel with the treated sample and the peroxide value (meq oxygen kg^{-1}) was calculated.

Thiobarbituric acid value

The test was conducted according to the methods described earlier[23,24] with minor modifications. A mixture of 10 g of rapeseed oil sample, 0.67% aqueous TBA (20 ml) and benzene (25 ml) was shaken continuously for 2 h using a mechanical shaker and then boiled in a water-bath for 1 h. After cooling, the absorbance of the supernatant was measured at 540 nm in a Hitachi (Tokyo, Japan) U-2000 spectrophotometer. A lower TBA value indicates higher antioxidant activity. The TBA value (meq malonaldehyde g^{-1}) was calculated using the following equation:

$$\text{TBA value} = (3.2 \times \text{OD})/(0.15 \times W) \quad (2)$$

where OD = absorbance of supernatant solution and W = amount of edible oil sample (in grams).

Carbonyl value

The method developed by Frankel[25] was followed. About 4 g of rapeseed oil sample (containing essential oil or extract or synthetic antioxidants) were weighed into a 50-ml volumetric flask and made up to volume with benzene. From this 50 ml, 5 ml were removed by pipette and 3 ml of trichloroacetic acid (4.3% in benzene) and 5 ml of 2,4-dinitrophenylhydrazine (0.05% in benzene) were added sequentially. The above mixture was incubated at 60 °C for 30 min to convert free carbonyls into hydrazones. After cooling, 10 ml of potassium hydroxide (4% in ethanol) were added and made up to volume (50 ml) with ethanol. After 10 min, the absorbance was measured at 480 nm

using a UV–visible spectrophotometer. A blank was prepared under the same conditions, substituting 5 ml of benzene instead of essential oil or extract. A calibration curve was drawn using capraldehyde (50–250 μg) in 50 ml of benzene instead of the test sample. The total carbonyl values of the samples were calculated with the help of a calibration curve and expressed as milligrams of capraldehyde per 100 g of sample. Carbonyl-free benzene and alcohol were prepared using standard methods and stored as stock solutions in brown bottles; they were used for all tests.

Determination of antioxidant activity in linoleic acid system

The test samples of essential oil or extract (1 ml) were added to a solution of linoleic acid (20 μl), 99.8% ethanol (2 ml) and 0.2 M phosphate buffer (pH 7, 2 ml). The total volume was adjusted to 6 ml with distilled water. The solution was incubated at 36 °C for 12 days and the degree of oxidation was measured every 2 days with the ferric thiocyanate method,[26] with 75% ethanol (10 ml), 0.2 ml of sample solution and 0.2 ml of ferrous chloride solution (20 mM in 3.5% HCl) being added sequentially. After stirring for 3 min, the absorbance of the mixtures was measured at 500 nm in the UV–visible spectrophotometer. The control and standards were subjected to the same procedure except for the control, where there was no addition of sample, and for the standard 1 ml of sample was replaced with 1 mg of BHA, BHT or PG. A lower absorbance indicates a higher antioxidant capacity.

Radical scavenging activity

Antioxidant activities of the essential oil and extract were measured by using DPPH free radical. The DPPH assay has frequently been used for the evaluation of the antioxidant potential of various natural products.[27,28] Various amounts of essential oil or extract (5, 10, 15 and 20 μl) were mixed with 5 ml of a 0.004% methanolic solution of DPPH. The mixture was incubated for 30 min in the dark and the absorbance of the sample was read at 515 nm using the UV–visible spectrophotometer. The control and standards were subjected to the same procedure except for the control, where there was no addition of sample, and for the standard, where 5, 10, 15 and 20 μl of samples were replaced with 5, 10, 15 and 20 mg of BHA BHT or PG. The percentage inhibition (I) of DPPH was calculated using the following equation:

$$I = [(A_0 - A_1)/A_0] \times 100 \qquad (3)$$

where A_0 is the absorbance of the control reaction (containing all reagents except the test compound) and A_1 is the absorbance of the test compound.

Reducing power

The reducing power was carried out as described previously.[29] Various amounts (20, 40, 60, 80 and 100 μl) of essential oil or extract (dissolved in 2.5 ml of methanol) were mixed with 2.5 ml of 200 mM phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide and the mixture was incubated at 50 °C for 20 min. After adding 2.5 ml of 10% trichloroacetic acid, the mixture was centrifuged at 200 *g* for 10 min in a Sigma 3K30 centrifuge. The upper layer (5 ml) was mixed with 5 ml of deionised water and 1 ml of 0.1% ferric chloride and the absorbance was read at 700 nm in a UV–visible spectrophotometer. The control and standards were subjected to the same procedure except for the control, where there was no addition of sample, and for the standard, where 20, 40, 60 and 80 μl of sample were replaced with 20, 40, 60 and 80 mg of BHA or BHT. A higher absorbance indicates a higher reducing power.

Statistical analysis

For the essential oil or extract, three samples were prepared for assays of each antioxidant attribute. The data were presented as mean ± standard deviation of three determinations (data not shown). Statistical analyses were performed using a one-way analysis of variance.[30] A probability value of $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

GC–MS studies

The essential oil resulted (Table 1) in the identification of 38 components representing 84.65% of the total amount. The major component was *p*-cymene (36.2%) followed by thymoquinone (11.27%), α-thujene (10.03%), longifolene (6.32) and carvacrol (2.12%). The extract showed (Table 2) the presence of 16 components representing 97.9% of the total amount. Only the volatile constituents of the acetone extract were analysed by GC–MS. The major components were linoleic acid (53.6%), thymoquinone (11.8%), carvacrol (3.7%) and *trans*-anethole (2.5%). Burits and Bucar[12] extracted essential oil with light petroleum and further steam distilled the extract. They reported that this essential oil contains thymoquinone as the major component, whereas our essential oil contains *p*-cymene as the major component.

Antimicrobial activity

The antibacterial results obtained from both essential oil and extract are given in Table 3. Black cumin essential oil showed complete zone inhibition against *Bacillus cereus, Bacillus subtilis, Staphylococcus aureus* and *Pseudomonas aeruginosa* at 2000 and 3000 ppm by the agar well diffusion method. The oil was found to be a complete zone inhibitor against *Bacillus subtilis* and *Staphylococcus aureus* at 1000 ppm. Both essential oil and extract were found to be ineffective against *Escherichia coli* and *Salmonella typhi* at all the tested concentrations. The antibacterial activity of standard antibiotics such as ampicillin and cloxacillin was also tested and they showed moderate to good antibacterial activity for S*taphyloccoccus aureus*, *Bacillus cereus* and

Table 1. Chemical composition of *N. sativa* essential oil analysed by GC–MS

Compound	Area (%)[a]	RI[b]
α-Thujene	10.03	931
α-Pinene	3.33	941
2,4(10)-Thujadiene	0.06	951
Camphene	0.06	953
Sabinene	1.34	975
β-Pinene	3.78	980
α-Terpinene	0.47	1020
p-Cymene	36.20	1026
Limonene	1.76	1031
1,8-Cineole	tr	1035
γ-Terpinene	0.16	1064
cis-Sabinene hydrate	0.22	1069
Terpinolene	0.06	1088
Unidentified A	1.61	1092
trans-Sabinene hydrate	0.19	1098
Linalool	0.10	1099
Unidentified B	11.71	1135
Terpinen-1-ol	0.19	1139
Camphor	0.11	1144
Ethanone-1-(1,4-dimethyl-3-cyclohexen-1-yl)	0.46	1165
Terpinen-4-ol	2.37	1177
p-Cymen-8-ol	0.09	1188
α-Terpineol	0.08	1189
4,5-Epoxy-1-isopropyl-4-methyl-1-Cyclohexene	0.85	1197
Carvone	0.16	1244
Thymoquinone	11.27	1250
Bornyl acetate	0.44	1287
trans-Anethole	0.61	1283
2-Undecanone	0.09	1294
Thymol	0.13	1297
Carvacrol	2.12	1298
α-Longipinene	1.54	1355
Longicyclene	0.06	1376
Longifolene	6.32	1409
β-Caryophyllene	0.07	1420
2-Tridecanone	0.10	1497
β-Bisabolene	0.07	1505
Epizonaren	0.10	1506
Sesquiterpene alcohol acetate	0.19	—
Pimara-8(14),15-diene	0.20	—
Total[c]	84.65	

[a] Percentages are the means of three runs and were obtained from electronic integration measurements using a mass-selective detector; tr, trace.
[b] The retention index (RI) was calculated for all volatile constituents using a homologous series of C_8–C_{16} *n*-alkanes.
[c] The percentages of unidentified A (1.61%), unidentified B (11.17%) and sesquiterpene alcohol acetate (0.19%) are not considered in the total percentage (85.19%).

Table 2. Chemical composition of acetone extract of *N. sativa* analysed by GC–MS

Compound	Retention time (min)	Area (%)[a]	RI[b]
p-Cymene	7.50	8.60	1026
Terpinen-4-ol	13.95	tr	1177
Thymoquinone	17.34	11.80	1250
trans-Anethole	18.88	2.50	1283
2,4-Decadienal (*E*,*Z*)	19.20	1.40	1289
Thymol	19.80	0.80	1290
Carvacrol	20.22	3.70	1298
α-Longipinene	21.36	1.60	1351
α-Copaene	22.47	tr	1376
Longifolene	23.69	5.80	1402
trans-β-Caryophyllene	24.27	tr	1418
n-Pentadecane	27.65	tr	1500
β-Bisabolene	28.08	tr	1509
4-*tert*-Butylcatechol	31.53	0.10	—
Palmitic acid	47.69	10.00	—
Linoleic acid	53.27	53.60	—
Total		97.9	

[a] Percentages are the means of three runs and were obtained from electronic integration measurements using a mass-selective detector; tr, trace.
[b] The retention index was calculated for all volatile constituents using a homologous series of C_8–C_{16} *n*-alkanes.

Salmonella typhi whereas they were ineffective against *Escherichia coli* and *Pseudomonas aeruginosa*. The essential oil and extract gave antibacterial activities comparable to those of the tested standard antibiotics (Table 3).The antifungal results for both volatile oil and extract obtained by the food poison and inverted petriplate techniques are given in Tables 4 and 5. In the inverted petriplate method, the essential oil revealed good, clear zones of growth inhibition against *Aspergillus flavus*, *Fusarium graminearum*, *Fusarium monoliforme* and *Penicillium viridicatum* at all the tested doses. The essential oil showed complete growth inhibition against *Penicillium citrinum* at 6 µl. However, the extract was found to be ineffective in this method. Singh and co-workers[31,32] have already reported lower antimicrobial efficacy of acetone extracts of pepper and ginger, which could be due to the lower volatility. The higher the volatility of the aroma components of an essential oil or extract will leads to a higher vapour concentration in the surrounding air space (ie inside the petriplate). This might be responsible for the increase in antimicrobial activity with increase in dose concentration. The essential oil showed strong antifungal activity against all tested *Aspergillus* species in the poison food method. It was also found to be effective for *Fusarium graminearum* and the oil showed 100% zone inhibition at a 6 µl dose. The extract also showed more than 60% zone inhibition against *Aspergillus terreus* and *Penicillium citrinum*. For other tested fungi, both essential oil and extract exerted moderate to good zones of inhibition. All the values were found to be statistically significant ($p < 0.05$).

It has been reported[13] that *in vitro* antibacterial effects of black cumin essential oil showed pronounced activities even at 1:100 dilutions against several organisms that include *Staphylococcus albus*, *Escherichia coli*, *Salmonellae typhi*, *Shigella niger* and *Vibrio cholera*. The oil was more effective against Gram-positive than Gram-negative organisms[33] and our results were well correlated.

El-Kamali *et al*,[14] using the plate diffusion method, confirmed the above report and showed that the

Table 3. Antibacterial activity of black cumin essential oil and extract by the agar well diffusion method

Test	Concentration (ppm)[a]	Inhibition zone (mm)[b]					
		Gram-positive bacteria[c]			Gram-negative bacteria[d]		
		BS	SA	BC	EC	ST	PA
Black cumin essential oil	1000	+	+	70.4 ± 1.9	–	–	60.7 ± 0.7
	2000	+	+	+	–	–	+
	3000	+	+	+	–	–	+
Black cumin extract	1000	20.6 ± 1.5	25.1 ± 0.1	–	–	–	40.5 ± 0.3
	2000	30.0 ± 1.0	35.9 ± 0.3	–	–	-	50.4 ± 1.8
	3000	41.4 ± 0.8	40.3 ± 2.1	–	–	–	62.8 ± 0.6
Standards							
Ampicillin	1000	–	19.1 ± 0.1	–	–	–	–
	2000	12.2	22.2 ± 0.2	14.4 ± 1.4	–	–	–
	4000	15.3	24.2 ± 0.6	18.6 ± 0.1	–	12.8 ± 0.5	–
Cloxacillin	1000	–	22.8 ± 0.7	12.3 ± 0.6	–	–	–
	2000	–	26.4 ± 1.5	13.3 ± 1.1	–	12.5 ± 0.1	–
	4000	–	30.3 ± 1.1	15.8 ± 0.6	–	17.4 ± 0.2	–

[a] DMSO was used as solvent.
[b] Average of three replicates; + indicates complete inhibition and – indicates no inhibition.
[c] BS, *Bacillus subtilis*; SA, *Staphylococcus aureus*; BC, *Bacillus cereus*.
[d] EC, *Escherichia coli*; ST, *Salmonella typhi*; PA, *Pseudomonas aeruginosa.*

Table 4. Antifungal effect of black cumin essential oil and extract against different food pathogenic fungi by the poison food medium method

Fungus	Mycelial zone inhibition at different doses[a] of sample (%)						
	Black cumin essential oil (μl)			Black cumin extract (ppm)[b]			Carbendazim 1000 ppm[c]
	2	4	6	1000	2000	3000	
Aspergillus ochraceus	62.4 ± 0.2	72.9 ± 0.8	82.4 ± 0.2	29.4 ± 1.1	35.3 ± 1.9	41.1 ± 2.0	8.8 ± 2.1
Aspergillus niger	37.5 ± 0.1	58.8 ± 0.5	68.8 ± 0.9	25.0 ± 1.4	36.3 ± 1.5	47.5 ± 1.5	0
Aspergillus flavus	33.5 ± 1.2	53.3 ± 1.1	73.3 ± 1.5	20.0 ± 1.6	29.3 ± 0.1	36.2 ± 0.4	28.8 ± 1.8
Aspergillus terreus	46.6 ± 1.3	73.3 ± 1.1	85.3 ± 0.4	68.0 ± 0.1	76.0 ± 0.7	90.7 ± 1.0	42.5 ± 2.2
Fusarium graminearum	85.7 ± 1.2	91.4 ± 0.6	100 ± 0.7	14.3 ± 1.4	21.4 ± 1.1	28.6 ± 0.4	0
Fusarium monoliforme	25.0 ± 0.5	37.5 ± 0.2	50.0 ± 0.6	0	12.5 ± 0.7	25.3 ± 0.9	17.5 ± 1.0
Penicillium citrium	25.0 ± 1.8	37.5 ± 1.4	50.0 ± 1.5	62.5 ± 1.1	80.0 ± 0.4	96.3 ± 0.1	44.8 ± 0.2
Penicillium viridicatum	23.5 ± 1.3	49.4 ± 2.1	67.0 ± 0.1	0	14.1 ± 0.2	18.8 ± 0.8	22.5 ± 0.1

[a] Average of three replicates.
[b] Ethanol was used as solvent.
[c] Aqueous solution was used.

Table 5. Antifungal effect of black cumin essential oil and its extract against different food pathogenic fungi by the inverted petriplate method

Fungus	Mycelial zone inhibition at different doses[a] of sample (%)					
	Black cumin essential oil (μl)			Black cumin extract (ppm)[b]		
	2	4	6	1000	2000	3000
Aspergillus ochraceus	18.8 ± 0.2	31.3 ± 1.3	75.0 ± 0.1	0	5.0 ± 1.8	8.8 ± 0.0
Aspergillus niger	5.0 ± 2.0	8.8 ± 1.1	12.5 ± 0.3	2.5 ± 0.2	8.8 ± 0.2	12.5 ± 0.3
Aspergillus flavus	37.5 ± 2.1	62.5 ± 0.6	87.3 ± 0.7	37.5 ± 0.9	62.5 ± 0.3	75.0 ± 0.1
Aspergillus terrus	18.8 ± 0.8	27.5 ± 1.8	43.8 ± 1.3	0	0	0
Fusarium graminearum	25.0 ± 0.6	56.3 ± 0.9	62.5 ± 1.7	12.5 ± 0.8	25.0 ± 0.0	36.3 ± 0.5
Fusarium monoliforme	75.6 ± 0.3	85.5 ± 1.5	93 ± 1.6	12.6 ± 1.8	25.0±. 2	36.8 ± 2.1
Penicillium citrium	31.3 ± 1.4	50.0 ± 0.4	100	12.5 ± 0.8	18.8 ± 1.7	31.4 ± 0.5
Penicillium viridicatum	32.0 ± 0.3	65.7 ± 0.7	90.4 ± 1.8	5.0 ± 2.0	8.8 ± 2.2	12.5 ± 0.5

[a] Average of three replicates.
[b] Ethanol was used as solvent.

essential oil of black cumin was effective against Gram-positive bacteria such as *Staphylococcus aureus* and *Bacillus subtilis*. It has been shown that both the crude alkaloid extract and the water extract of seeds were effective against variety of organisms isolated from human patients suffering from septic arthritis, even those that were resistant to antibiotics.[34] Most of the antimicrobial activity in essential oils derived from spices and culinary herbs is believed to derive from phenolic compounds,[35,36] whereas other constituents are believed to contribute little to the antimicrobial effects.[37,38] From GC–MS studies (Tables 1 and 2), it is clear that both the essential oil and extract contain considerable amounts of phenolic compounds (thymol and carvacrol) and hence the antimicrobial activity could be due to these compounds. The antimicrobial activity of the extract could be due the presence of *p*-cymene and not due to linoleic acid. There is increasing evidence that specific compounds such as eugenol,[39] *p*-cymene[40] and thymol[40,41] play an important role in antifungal activity. Both the essential oil and extract contain *p*-cymene and thymol (Tables 1 and 2). Hence the antimicrobial activity of the essential oil and extract could be due to the presence of *p*-cymene. Bishop and Thornton[42] showed that terpenoid phenolic and non-phenolic alcohols are the most bioactive against fungi. The strength of inhibition and the spectrum of antimicrobial activity of the black cumin essential oil and extract suggest that complex interactions between individual components led to the overall activity. Reasons for the resistance of many Gram-negative bacteria will likely remain speculative until the mode of action of essential oil or extract is better understood. Disruption of the membrane by terpenes has been shown in Gram-positive[43,44] and Gram-negative bacteria.[45] This type of disruption might have been occurred in the present study. However, the mode of action of minor essential oil components is unknown. Given the difference in the cell envelope of these bacterial groups, it is plausible that access to the membrane is more restricted in Gram-negative bacteria. The contribution of these components to the antimicrobial activity of essential oil and extract clearly needs to be investigated further. The lower efficacy of the present essential oil and extract against some microorganisms in this study might have been due to the low activity of their main constituents against particular fungi or bacteria. It is likely that antifungal effects of the essential oil and extract result from the synergistic action of all their components.[46] Such synergistic or antagonistic action probably occurred with this essential oil or its extract. The chemical composition and antimicrobial properties of essential oils extracted from diverse plant species have been demonstrated using a variety of experimental methods.[47–49] Variability in qualitative and quantitative estimates of activity has been ascribed to differences in analytical techniques.[50,51] In this respect, we believe that the methods which were used to evaluate antimicrobial activity would provide additional information to assess the antimicrobial properties of black cumin essential oil and extract.

Antioxidant activity in rapeseed oil system

Figure 1 shows changes of the peroxide value in the rapeseed oil system with additives. The rapeseed oil oxidation was measured with storage for 28 days. During this time, the peroxide value of the control sample increased to 213.6 meq kg^{-1} by the final day, which is significantly higher than for the samples containing essential oil and extract. Both the essential oil and extract showed a strong antioxidative effect ($p < 0.05$) in rapeseed oil, which could be comparable to BHA and BHT effects at the 6-mg level. However, it was less effective in comparison with PG at the 6-mg level. During the oxidation process, peroxides are gradually converted into lower molecular weight compounds such as aldehydes and ketones. One such aldehyde, malonaldehyde, was measured by the TBA method. Both essential oil and extract were able to control the secondary oxidation process, which could be comparable to the effect of synthetic antioxidants at the 6-mg level (Fig 2). Singh *et al*[52] studied the antioxidative effect of ajawin essential oil and its acetone extract in linseed oil using various methods. We also measured (Fig 3) the total carbonyls present in the same time interval. The inhibitory effect of essential oil may be due to the presence of an aromatic nucleus containing polar functional groups.

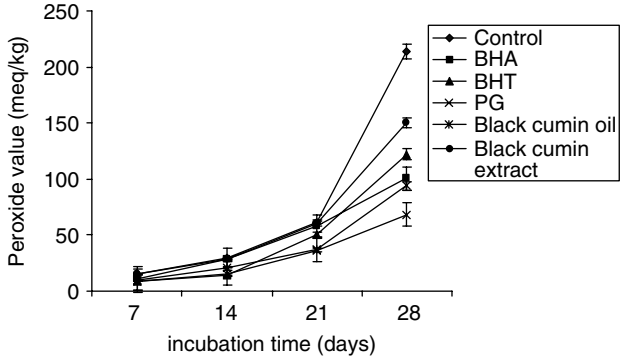

Figure 1. Stabilization of rapeseed oil by black cumin essential oil and its extract at 200 ppm with standards at 70 °C in terms of peroxide value.

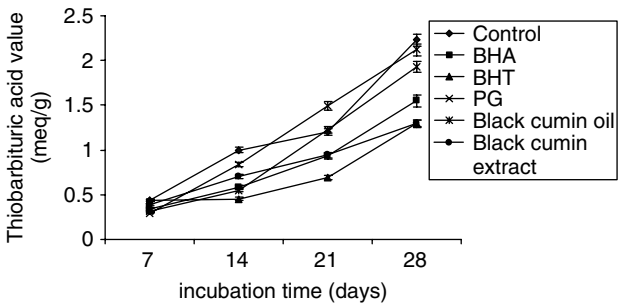

Figure 2. Secondary oxidative effect of black cumin essential oil and its extract at 200 ppm with standards at 70 °C in terms of TBA value.

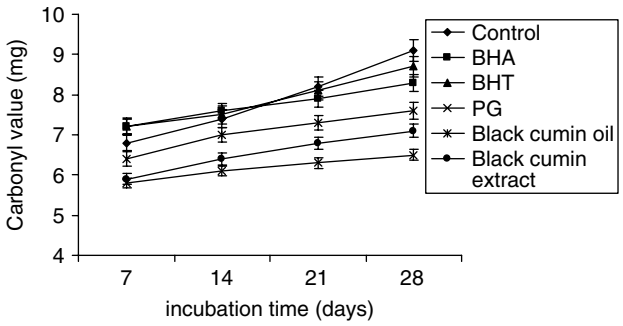

Figure 3. Antioxidative effect of black cumin essential oil and its extract at 200 ppm in terms of carbonyl value.

Antioxidant activity in linoleic acid system

To evaluate the antioxidant potential of essential oil and extract, their lipid inhibitory activities were compared with those of selected standard antioxidants by using the ferric thiocyanate method. High absorbance is an indication of a high concentration of peroxides formed. The absorbance of linoleic acid emulsion without the addition of essential oil or extract antioxidants increased rapidly. There was a significant ($p < 0.05$) difference between the blank and the tested essential oil or extract. As can be seen in Fig 4, both the essential oil and extract were able to reduce the formation of peroxides. Black cumin oil essential and extract showed considerable antioxidative properties in the linoleic acid system and were significantly ($p < 0.05$) different from the control.

Radical scavenging activity and reducing power

The decrease in absorbance of the DPPH radical due to the scavenging capability of essential oil, extract and antioxidants is illustrated in Fig 5. The scavenging effect of the essential oil (82.1–92.1%) and extract (90.37–97.18%) on DPPH radical increased linearly with increasing concentration. Both essential oil and extract showed good to excellent scavenging activity in comparison with BHA (86.58–94.7%), BHT (84.1–90.4%) and PG (84.98–93.1%) at the 5-, 10-, 15- and 20-mg levels. It has been shown that compounds isolated from black cumin (including thymoquinone, carvacrol, *trans*-anethole and 4-terpineol) have appreciable free

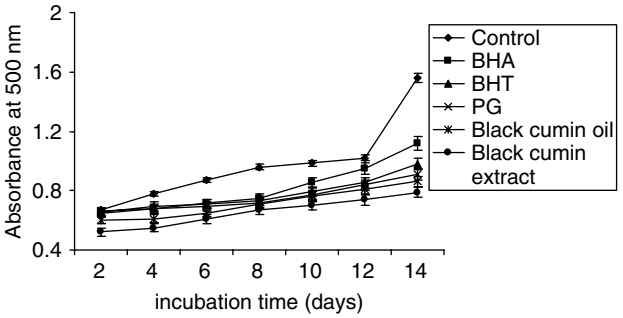

Figure 4. Antioxidative activity of black cumin essential oil and its extract compared with standard antioxidants (BHA, BHT and PG) at 5×10^{-3} ppm in the linoleic system.

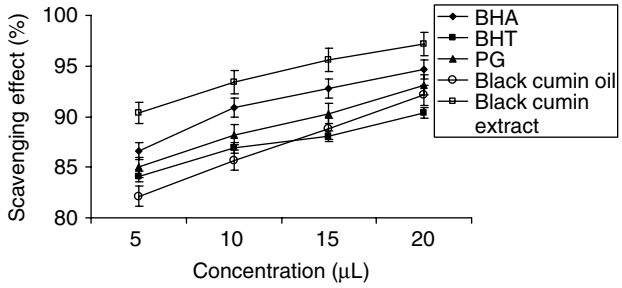

Figure 5. Radical-scavenging ability of essential oil and its extract on 2,2′-diphenyl-1-picrylhydrazyl radical.

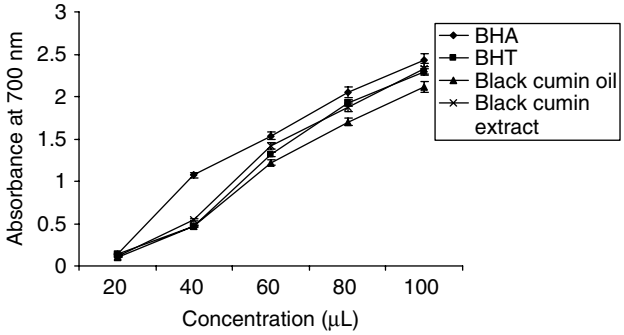

Figure 6. Reducing power of essential oil and its extract in comparison with commercial antioxidants BHA and BHT.

radical scavenging properties.[12] The reducing powers of both essential oil and extract were excellent and increased with increasing concentration, and they showed moderate to good reducing ability in comparison with BHA and BHT (Fig 6). The reducing powers of essential oil and extract might be due to their hydrogen-donating ability.[53] The components present in the essential oil and extract could act as good reductants, which could react with free radicals to stabilize and terminate radical chain reactions.

El-Ghorab[54] studied the scavenging activity of supercritical fluid-extracted black cumin seed essential oil on 1,1′-diphenyl-2-picrylhydrazyl radical and observed remarkable radical scavenging activity of the essential oil. An early study of Chilpault *et al*[55] demonstrated the antioxidant activity of 32 spices and herbs and their solvent extracts both in edible oils and in oil-in-water emulsions. A large number of methods have been developed in order to evaluate antioxidant activity.[56–58] A limited number of methods are available in the literature regarding antioxidant activity of black cumin essential oil and extract. As different testing methods provide particular, but limited, information about antioxidant activity, we applied different methods to evaluate antioxidant activity in this investigation. This would provide more information about the antioxidant potential of both the essential oil and extract. Phenolic groups play an important role in antioxidant activity,[59,60] hence, the presence of phenolic compounds such as carvacrol and thymol in the essential oil and carvacrol, thymol and 4-*tert*-butylcatechol in the extract are responsible for the antioxidant activity. It has been

reported that most natural antioxidant compounds often work synergistically with each other to produce a broad spectrum of antioxidative activities that creates an effective defence system against free radical attack.[61]

CONCLUSION

Both the essential oil and the acetone extract exhibited a broad spectrum of antimicrobial activity against the tested microorganisms. On the basis of the results, it was concluded that both the essential oil and extract provide antioxidant activity equivalent to or higher than those of synthetic antioxidants and could be a better natural antioxidant.

ACKNOWLEDGEMENTS

We are thankful to the Head, Chemistry Department, DDU Gorakhpur University, Gorakhpur, for providing laboratory facilities. Thanks are also due to the Life Sciences Research Board, DRDO, New Delhi for financial assistance and the Universidad Nacional de Tucuman, Argentina, for spectral investigations through a CONICET grant. Professor KDS Yadav of our department is also thanked for providing spectroscopic facilities.

REFERENCES

1 McMullen LM, Hawrysh C and Tokarska B, Ascorbyl palmitate efficiency in enhancing the accelerated storage stability of canola oil. *J Food Sci* **56**:1651–1659 (1991).
2 Ito N, Fukushim S and Tsuda S, Carcinogenicity and modification of carcinogenic response by BHA, BHT and other antioxidants. *Cri Rev Toxicol* **15**:109–150 (1985).
3 Barlow SM, Toxicological aspects of antioxidants used as food additives, in *Food Antioxidants*, ed by Hudson BJF. Elsevier, London, pp 253–307 (1990).
4 Banias C, Oreopoulou V and Thomopoulos CD, The effect of primary antioxidants and synergistics on the activity of plant extracts in lard. *J Am Oil Chem Soc* **69**:520–524 (1992).
5 Hras AR, Hadolin M, Knez Z and Bauman D, Comparison of antioxidative and synergistic effects of rosemary extract with α-tocopherol, ascorbyl palmitate and citric acid in sunflower oil. *Food Chem* **71**:229–233 (1992).
6 Pegg RB, Amarowicz R and Barl B, Application of plant polyphenolics in model and meat systems, in *Proceedings of the 47th International Congress of Meat Science and Technology, Cracow, Poland*, Vol II. pp 234–235 (2001).
7 El-Dakhakhny M, Studies on the Egyptian *Nigella sativa* L. *Arzneim Forsch* **15**:1227–1229 (1965).
8 Marrozzi FJ, Kocialski AB and Malone MH, Studies on the antihistaminic effects of thymoquinone, thymohydroquinone and quercetin. *Drug Res* **20**:1574–1577 (1970).
9 Khanna T, Zaidi FA and Dandiya PC, CNS and analgesic studies on *Nigella sativa*. *Fitoter* **5**:407–410 (1993).
10 Mahfouz M, Abdel-Maquid R and El-Dakhakhny M, The effect of nigellone therapy on the histaminopexic power of the blood sera of asthmatic people. *Arzneim Forsch* **15**:1230–1231 (1996).
11 Akhtar AH, Ahmed KD, Gilani SN and Nazir A, Antinuclear effects of aqueous extract of *Nigella sativa* and *Pongamia pinnata* in rats. *Fitoter* **3**:195–199 (1996).
12 Burits M and Bucar F, Antioxidant activity of *Nigella sativa* essential oil. *Phytother Res* **14**:323–328 (2000).
13 Agrawal R, Kharya MD and Shrivastava R, Antimicrobial and anthelmintic activities of the essential oil of *Nigella sativa* Linn. *Indian J Exp Biol* **17**:1264–1265 (1979).
14 El-Kamali HH, Ahmed AH, Mohammed AS, Yahia IA, El-Tayeb IH and Ali AA, Antimicrobial properties essential oil from *Nigella sativa* seeds, *Cymbopogan citatus undulata* aerial parts. *Fitoter* **34**:77–78 (1988).
15 *European Pharmacopoeia*, Vol 1. Maissoneue, Sainte Ruffine (1983).
16 Massda Y, *Analysis of Essential Oils by Gas Chromatography and Mass Spectrometry*. Halsted/Wiley, New York (1976).
17 Adams RP, *Identification of Essential Oil Components by Gas Chromatography/Quadrupole Mass Spectrometry*. Allured Publishing Corporation, Carol Stream, IL (2001).
18 NCCLS (National Committee for Clinical Laboratory Standards), *Performance Standards for Antimicrobial Disc Susceptibility Test*, 6th edn. Wayne, USA (1997).
19 Rao GP and Shrivastava AK, *Curr Trends Sugarcane Pathogens* **4**:364–374 (1994).
20 Ramdas K, Suresh G, Janardhanan N and Masilamani S, Antifungal activity of 1,3-disubstituted symmetrical and unsymmetrical thioureas. *Pestic Sci* **52**:145–151 (1998).
21 Horwitz W (ed), *Official Methods of Analysis of the Association of Official Analytical Chemists*, 13th edn. Association of Official Analytical Chemists, Washington, DC (1980).
22 Bandoniene D, Gruzchene D and Venkutonis PR, Antioxidant activity of sage extracts in rapeseed oil irradiated with UV rays. *Nahurng/Food* **2**:105–108 (2002).
23 Ottolenghi A, Interaction of ascorbic acid and mictochondrial lipids. *Arch Biochem Biophys* **79**:355–358 (1959).
24 Kikuzaki H and Nakatani N, Antioxidant effects of some ginger constituents. *J Food Sci* **58**:1407–1410 (1993).
25 Frankel EN, *Lipid Oxidation*. The Oily Press, Aberdeen, p 30 (1998).
26 Mitsuda H, Yasumoto K and Iswami K, Antioxidative action of indole compounds during the autooxidation of linoleic acid. *Eiyo to Shokuryo* **19**:210–214 (1996).
27 Cotelle N, Bernier JL, catteau JP, Pommery J, Wallet JC and Gaydou EM, Antioxidant properties of hydroxyflavones. *Free Rad Biol Med* **20**:35–43 (1996).
28 Lee SK, mbwambo ZH, Chung H, Luyengi L, Gamez EJC, Mehta RG, Kinghorn AD and Pezzuto JM, Evaluation of the antioxidant potential of natural products. *Combin Chem High Throughput Screen* **1**:35–46 (1998).
29 Oyaizu M, Studies on products of browning reactions: antioxidative activities of products of browning reactions prepared from glucosamine. *Jpn J Nutr* **44**:307–315 (1986).
30 Sokal RR, *Introduction to Biostatistics*. Freeman, San Francisco, pp 164–185 (1973).
31 Singh G, Maurya S, Catalan C. and de Lampasoma MP, Studies on essential oils, Part 42: Chemical, antifungal and antioxidant and sprout suppressant studies of ginger oil and its oleoresin. *Flavour Frag J* **20**:1–6 (2005).
32 Singh G, Marimuthu P, Catalan C. and de Lampasoma, MP, Chemical, antioxidant and antifungal activities of volatile oil of black pepper and its acetone extract. *J Sci Food Agric* **84**:1878–1884 (2004).
33 Ali BH and Blunder G, Pharmacological and toxicological properties of *Nigella sativa*. *Phytother Res* **17**:299–305 (2003).
34 Morsi NM, Antimicrobial effect of crude extracts of *Nigella sativa* on multiple antibiotics-resistant bacteria. *Acta Microbiol Pol* **49**:63–74 (2000).
35 Lambert BJW, Skandamis PN, Coote PJ and Nychas GJE, A study of minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. *J Appl Microbiol* **91**:453–462 (2001).
36 Maritini H, Weidenborner M, Adams S and Kunz B, Eugenol and carvacrol: the main fungicidal compounds in clove and savory. *Ital J Food Sci* **1**:63–67 (1996).
37 Shelef L, Antimicrobial effect of spices. *J Food Saf* **6**:29–44 (1983).
38 Nychas GJE, *Natural Antimicrobials from Plants*. Chapman and Hall, New York (1995).

39 Juglal S, Govinden R and Odhav B, Spice oils for the control of co-occurring mycotoxin producing fungi. *J Food Protect* **65**:683–687 (2002).

40 Soliman KM and Badeaa RI, Effects of oil extracted from some medicinal plants on different mycotoxigenic fungi. *Food Chem Toxicol* **40**:1669–1675 (2002).

41 Zambonelli A, Zechini D' Aulerio A, Bianchi A and Albasini A, Effects of essential oils on phytopathogenic fungi *in vitro*. *J Phytopathol* **144**:491–494 (1996).

42 Bishop CD and Thornton IB, Evolution of antifungal activity of the essential oils of *Mandra citriodora* var.*citriodora* and *Melaleuca alternifolia* on postharvest pathogens. *J Essent Oil Res* **9**:77–82 (1997).

43 Ultee A, Kets EPW and Smid EJ, mechanisms of action of carvacrol on the food-borne pathogen *Bacillus cereus*. *Appl Environ Microbiol* **65**:4606–4610 (1999).

44 Cox SD, Mann CM, Markham JL, Bell HC, Gustafson JE, Warmington JR and Wyllie JG, The mode of antimicrobial action of *Malaleuca alternifolia* (tea tree oil). *J Appl Bacteriol* **88**:170–175 (2000).

45 Helander IM, Alakomi HL, Latva-kala K, Mattila Sandholm T, Pol I, Smid EJ, Gorris LGM and Von Wright A, Characterization of the action of selected essential oil components on Gram negative bacteria. *J Agric Food Chem* **46**:3590–3595 (1998).

46 Dubey NK and Kishore N, Fungitoxicity of some higher plants and synergistic activity of their essential oils. *Trop Sci* **27**:23–27 (1987).

47 Conner DE and Beuchat LR, Effects of essential oils from plants on growth of food spoilage yeasts. *J Food Sci* **49**:429–434 (1984).

48 Deans SG and Ritchie G, Antibacterial properties of plant essential oils. *Int J Food Microbiol* **5**:165–180 (1987).

49 Conner DE, naturally occurring compounds, in *Antimicrobials in Foods*, ed by Davidson PM and Branen AL. Marcel Dekker, New York, pp 441–468 (1993).

50 Zaika LA, Spices and herbs: their antimicrobial activity and its determination. *J Food Saf* **9**:97–118 (1988).

51 Mann CM and Markham JL, A new method for determining the minimum inhibitory concentration of essential oils. *J Appl Bacteriol* **84**:538–544 (1998).

52 Singh G, Maurya S, Catalan C and de Lampasoma MP, Chemical, antifungal, antioxidative studies of ajwain oil and its acetone extract. *J Agric Food Chem* **52**:3292–3296 (2004).

53 Hyang-Sook C, Song HS, Ukeda H and Sawamura M, Radical-scavenging activities of citrus essential oils and their components: detection using 1,1-diphenyl-2-picrylhydrazyl. *J Agric Food Chem* **48**:4156–4161 (2000).

54 El-Ghorab AH, Supercritical fluid extraction of the Egyptian rosemary (*Roamarimus officinalis*) leaves and *Nigella sativa* L. seeds volatile oils and their antioxidant activities. *J Essent Oil Bearing Plants* **6**:67–77 (2003).

55 Chilpault JR, Mizuno GR and Lundberg WO, The antioxidant properties of spices in foods. *Food Technol* **10**:209–212 (1956).

56 Ragnarsson JO and Labuza TP, Acclerated shelf-life testing for oxidative rancidity in foods. *Food Chem* **2**:291–308 (1977).

57 Robards K, Prenzier PD, Tucker G, Swatsitang P and Glover W, Phenolic compounds and their role in oxidative processes in fruits. *Food Chem* **66**:401–436 (1999).

58 Khal R and Hilderbrant AG, Methodology for studying antioxidant activity and mechanism of action of antioxidants. *Food Chem Toxicol* **24**:1007–1014 (1986).

59 Huang SW and Frankel EN, Antioxidant activity of tea catechins in different lipid systems. *J Agric Food Chem* **40**:3033–3038 (1997).

60 Baratta MT, Dorman HJD, Deans SG, Figueiredo AC, Baroso JG and Ruberto G, Antimicrobial and antioxidant properties of some commercial essential oils. *Flavour Frag J* **104**:286–292 (1998).

61 Lu F and Foo LY, Antioxidant activities of polyphenols from sage (*Salvia officinalis*). *Food Chem* **75**:197–202 (2001).