EORC

International Journal of
Essential Oil Therapeutics
www.ijeot.com

Chemistry, biocidal and antioxidant activities of essential oil and oleoresins from *Piper cubeba* (seed)

G. Singh [a]*, S. Kiran [a], P. Marimuthu [a], M. P. de Lampasona [b], C. S. de Heluani [b], C. A. N. Catalán [b]

[a] *Chemistry Department, D.D.U. Gorakhpur University, Gorakhpur-273009, India*
[b] *Instituto de Química Orgánica, Universidad Nacional de Tucumán, Ayacucho 471, S.M. de Tucumán 4000, Argentina*

Abstract

The hydrodistilled *Piper cubeba* (tailed pepper) essential oil and various oleoresins individually collected by soxhlet apparatus using methanol, ethanol, petroleum benzene, diethyl ether and chloroform as solvents were tested for antimicrobial and antioxidant activities. The essential oil and oleoresins were analyzed by GC and GC/MS techniques. The main component of the essential oil was ß-cubebene (18.94 %) followed by cubebol (13.32 %), sabinene (9.60 %), α-copaene (7.41%) and ß-caryophyllene (5.28%) with many other components in minor amounts. All the oleoresins showed the presence of 85 components. The major component in all the oleoresins was cubebol (stereoisomer). The percentage of cubebol in the diethyl ether extract was 32.38, in the ethanol extract 25.51, in the petroleum benzene extract 42.89, in the chloroform extract 28.00 and in methanol extract 19.03. The essential oil and oleoresins were tested using different in vitro antioxidant and antimicrobial activities. The antioxidant activity of the essential oil and oleoresins was tested in mustard oil and the antimicrobial results were compared to commercial antifungal and antibacterial agents. Moderate to strong antimicrobial and antioxidant activities were demonstrated in the studied assays. The essential oil and oleoresins may be used as substitutes for synthetic antioxidant and antimicrobial agents after appropriate clinical trials.

Key words: *Piper cubeba*, essential oil, antioxidant activity, scavenging effect, reducing power, lipid peroxidation

Introduction

Oxidation of lipids is responsible for food rancidity [1] and leads to development of food-borne pathogens in food samples. The increasing incidence of food-borne diseases, coupled with resultant social and economic implications [2] means that there is a constant striving to produce safer food and develop new antimicrobial and antioxidant agents. However, several synthetic antioxidants like butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate (PG) are being used that are reported to possess toxic and carcinogenic properties [3]. Hence, research work has been diverted to discover new and safer antioxidants from natural origins [4-6]. Although tocopherols are natural sources of antioxidants, there are some controversial results regarding their effectiveness. Thus, efforts have been made to discover effective food preservatives by screening a broad variety of herbal extracts from various spices, aromatic herbs and other plants, since they have already been reported to possess significant antimicrobial and antioxidant compounds [7-8]. A large number of studies on the antioxidant and antimicrobial activities of essential oils and oleoresins have been reported but the chemistry and food preservative action of oleoresins is quite meager. The antioxidant and antifungal activity of black pepper has been reported [9]. There are reports on the in vivo and in vitro activities of *Piper guineense* [10] and the antimicrobial activity of green pepper [11]. There is a survey on the antimicrobial and antioxidant activities of a large number of essential oils and their individual components reported by Burt [12]; the review explained practical application of essential oils in various food systems. In addition, Chemat [13] wrote a recent report on the antioxidant activity of various essential oils. Insofar as the antioxidant activity of the tailed pepper is concerned, there is no report whatsoever

* *Corresponding author.*
E-mail address: gsingh4us@yahoo.com

to the best of our knowledge. Therefore, an attempt has been made to study the chemistry, antioxidant and biocidal activity of essential oil and various oleoresins isolated from tailed pepper.

Materials and methods

Chemicals

2,2'-diphenyl-1-picrylhydrazyl (DPPH) was bought from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany); linoleic acid from Acros (New Jersey, USA); BHT, BHA, PG and 2, 4-dinitrophenylhydrazine (DNPH) were purchased from S. D Fine-Chem Ltd, Mumbai, India; thiobarbituric acid (TBA) was received from Merck, Germany. Crude mustard oil was purchased from a local oil mill, Gorakhpur, India. All solvents used were of analytical grade.

Plant material

The seeds of tailed pepper were procured from the local market of Gorakhpur, Uttar Pradesh, India during August 2005 and voucher specimens were deposited at the Herbarium of the Faculty of Science, DDU Gorakhpur University.

Isolation of essential oil

Tailed pepper seeds were ground (250 mesh) with a domestic model electronic grinder and hydrodistilled in a Clevenger-type apparatus [14] for 3 h according to the method recommended by the European Pharmacopoeia. A light colourless volatile oil (yield 2.4 g/kg^{-1}) with a characteristic odour was obtained. It was dried over anhydrous sodium sulfate to remove traces of moisture and stored in a refrigerator in the dark at 4°C until use.

Collection of tailed pepper oleoresins

20 g of powdered seeds of tailed pepper were loaded on to a Soxhlet apparatus and extraction was carried out with various solvents (diethyl ether, ethanol, petroleum benzene, chloroform, methanol) at 60°C for 3h. The yields of oleoresins are 4.2 g/kg^{-1} (diethyl ether), 10 g/kg^{-1} (ethanol), 6.7 g/kg^{-1} (petroleum benzene), 5.8 g/kg^{-1} (chloroform) and 9.2 g/kg^{-1} (methanol). The solvent was removed and the remaining viscous extract was used for further tests.

Chemical characterization

Gas chromatography (GC)

A Hewlett Packard 5890 (Agilent Technologies, Buenos Aires, Argentina) gas chromatograph fitted with a capillary HP-5 column (5% phenyl methylsiloxane; length 30 m, inner diameter 0.32mm and film thickness 0.25 µm) whose injector and detector temperatures were maintained at 240 and 250°C respectively. Injection volume was 1µl with a split ratio 80:1; helium was used as carrier gas at a flow rate of 1.0 ml min^{-1}. The oven temperature for both essential oil was programmed as follows: 60°C for one min, rising 1.5°C/min^{-1} to 185°C then held for 1 min then again started rising at 9°C/min^{-1} to 275°C and held for 5 min. The oven temperature for oleoresins was programmed linearly as follows: 60°C to 260°C at 3°C/min^{-1} then held for 15 min at 280°C. For RI measurements, the oven temperature program suggested by Adams (60°C to 246°C at 3°C/min) was used [15].

Gas chromatography/ mass spectrometry (GC/MS)

Analyses of tailed pepper essential oil and oleoresin were run on a Hewlett Packard 6890 GC-MS system (Agilent Technologies, Buenos Aires, Argentina) coupled to a quadrupole mass spectrometer (model HP 5973) with a HP-5MS (5% phenyl methylsiloxane, length 30m, inner diameter 0.25mm and film thickness 0.25 µm) capillary column. The injector, GC/MS interface, ion source and selective mass detector temperatures were maintained at 280, 280, 230 and 150°C respectively; ionization energy, 70 eV; injection size 1.0 µl (in split mode); carrier gas: helium at a flow rate of 1.0 ml/min^{-1}. The oven temperature for essential oil and oleoresins was programmed as follows: 60°C for 1 min, rising 1.5°C/min^{-1} to 185°C then held for 1 min, then again started rising at 9°C/min^{-1} to 275°C and held for 2 min. The oven temperature for oleoresins was programmed linearly as follows: 60°C to 280°C at 3°C/min^{-1} and then held for 15 min at 260°C.

Component identification

The components percentage was taken from capillary GC profiles with FID. Identification of the individual components of essential oil and oleoresin was based on:

a) comparison of their mass spectra and retention indices on an HP-5 column with published data [15-16]

b) computer matching with the WILEY 275 and National Institute of Standards Technology (NIST 3.0) libraries provided with the computer controlling GC/MS system

c) co-injection with authentic samples (wherever possible).

The retention index was calculated using a homologous series of *n*-alkanes.

Antifungal investigation

The antifungal activity of the essential oil and oleoresin were individually tested against a range of pathogenic fungi. All the fungi cultures were procured from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India and used for the present study. Cultures of each fungus were maintained on Czapek (DOX) agar media adjusted to pH 6.0-6.5 and the slants were stored at 5°C. The antifungal activity of the essential oil and oleoresins against fungi were undertaken using the poison food and inverted petri plate methods [18]. All tests were performed in triplicate and fungal toxicity was measured in terms of percentage mycelial inhibition calculated by following equation:

Mycelial inhibition (%) = [Control-Test)/Control] x 100

In the poison food technique, a calculated quantity (2 and 6 µl) of undiluted essential oil and oleoresin were separately mixed with 20 ml of hot culture medium (~45°C) in a previously sterilized and dried petriplate (diameter 11 cm). In the inverted petriplate method, a calculated quantity (2 and 6 µl) of essential oil and oleoresins were soaked on pre-sterilized filter paper (10 mm in diameter) and kept on the lid of the inverted petriplate. Control plates were prepared without addition of essential oil or oleoresin under the same conditions. The plates were incubated at 37°C for 6 days and zone inhibition was measured with the help of vernier calipers.

Antibacterial investigation

Antibacterial activity was tested by agar well diffusion method [19]. The bacterial strains were procured from MTCC, Chandigarh, India. In this method, a bacterial

suspension of the test microorganism (0.1ml) was spread over previously prepared and dried nutrient agar medium. The wells (10 mm diameter) were cut from agar and 50 µl of samples diluted in dimethyl sulphoxide were transferred into them. The control plates were also prepared by adding dimethyl sulphoxide in place of the essential oil or oleoresin. After incubating for 24 hr at 37°C, all plates were examined and the diameters of the zones were measured in millimeters.

Evaluation of antioxidant activity of mustard oil

To evaluate the antioxidant potential of essential oil and oleoresins, crude mustard oil (*Brassica nigra*) was selected that had an initial peroxide value 1.1 meq of O_2/kg. Mustard oil is a commonly used edible oil in the northern part of India for cooking. In the case of essential oil and oleoresins, 6 µl was added to mustard oil while in the case of synthetic antioxidants, 6 mg (BHA, BHT and PG) were added at 200 ppm concentration in 100 ml open-mouthed beakers. These mixtures were thoroughly homogenized and samples were incubated at 70°C in the dark. A blank sample was prepared under similar conditions without any additive. Three replications were performed for each sample. The antioxidant activity against mustard oil was studied by measuring peroxide [20], TBA [20], *p*-anisidine [21] and total carbonyl values [22] at fixed time intervals.

Determination of antioxidant activity in linoleic acid system

The method used for this purpose is same as proposed by Osowa and Namaki [23]. 1 ml of essential oil or oleoresin was added to a solution of linoleic acid (20 µl), 99.8% ethanol (2 ml) and 0.2 M phosphate buffer (pH7, 2 ml). The total volume was adjusted to 6 ml with distilled water. The degree of oxidation was measured every 2 days according to the ferric thiocyanate method [24]. The control sample was also prepared in same manner without additives. In the case of synthetic antioxidants BHA, BHT and PG, 1ml of 5% solution were added.

DPPH radical scavenging capacity assay

The scavenging effect on DPPH was determined according to methods developed earlier [25]. In the presence of a free radical scavenger, the unpaired electron present on nitrogen atoms becomes paired [26], resulting in absorbance loss and the solution colour fades. Various amounts of essential oil or oleoresin (5, 10, 15 and 20 µl) was mixed with 5 ml of 0.004% methanolic solution of DPPH. It was incubated for 30 min in the dark and the absorbance of the sample was read at 515 nm using a UV-visible spectrophotometer. In the case of synthetic antioxidants, 1 ml of 5% solution was added.

The percentage of the DPPH remaining was calculated as:

$$\% \text{ DPPH}_{rem} = 100 \times [\text{DPPH}]_{rem} / [\text{DPPH}] = 0$$

$\% \text{ DPPH}_{rem}$ is proportional to the antioxidant concentration.

Statistical analysis

For the essential oil or oleoresins, three samples were prepared for assay of each antioxidant attribute. The data were presented as mean + standard deviation of three determinations (data not shown). Standard analysis was performed using a one-way analysis of variance's probability; a value of $p < 0.05$ was considered significant.

Results and discussion

GC and GC/MS analysis

GC and GC/MS analysis of tailed pepper essential oil showed the presence of 59 components representing 98.52% of the total amount (Table 1).

Table 1. Chemical composition of tailed pepper essential oil analyzed by GC/MS.

RI#	compounds	% FID	identification†
923	α-thujene	1.85	MS, RI, co-GC
930	α-pinene	1.93	MS, RI, co-GC
945	camphene	trace	MS, RI, co-GC
970	sabinene	9.60	MS, RI, co-GC
974	ß-pinene	0.61	MS, RI, co-GC
987	myrcene	0.21	MS, RI, co-GC
1005	α-phellandrene	0.14	MS, RI
1006	3-carene	trace	MS, RI, co-GC
1014	α-terpinene	trace	MS, RI
1022	*p*-cymene	0.44	MS, RI, co-GC
1025	limonene	0.47	MS, RI, co-GC
1027	ß-phellandrene	1.70	MS, RI, co-GC
1028	1,8-cineole	1.30	MS, RI, co-GC
1053	γ-terpinene	0.09	MS, RI, co-GC
1066	*cis*-sabinene hydrate	0.70	MS, RI, co-GC
1082	terpinolene	trace	MS, RI, co-GC
1101	linalool	3.16	MS, RI, co-GC
1121	*1*-terpineol	0.62	MS, RI, co-GC
1141	camphor	0.10	MS, RI
1166	borneol	trace	MS, RI, co-GC
1175	terpinen-4-ol	0.99	MS, RI, co-GC
1180	cryptone	trace	MS, RI
1191	α-terpineol	0.26	MS, RI, co-GC
1237	cuminal	trace	MS, RI
1331	δ-elemene	0.12	MS, RI, co-GC
1343	α-cubebene	3.91	MS, RI
1364	isoledene	trace	MS, RI
1370	α-copaene	7.41	MS, RI
1378	geranyl acetate	0.05	MS, RI, co-GC
1383	ß-cubebene	18.94	MS, RI
1384	ß-elemene	1.41	MS, RI
1398	α-gurjunene	0.72	MS, RI
1407	*cis*-α-bergamotene	0.27	MS, RI
1410	ß-caryophyllene	5.28	MS, RI, co-GC
1419	aromadendrene	0.08	MS, RI
1427	α-guaiene	0.10	MS, RI
1444	α-humulene	1.97	MS, RI
1449	*allo*-aromadendrene	4.07	MS, RI
1463	cadina-1(6),4-diene	0.14	MS, RI
1467	γ-muurolene	2.43	MS, RI
1471	germacrene-D	4.70	MS, RI
1477	ß-selinene	0.56	MS, RI
1480	trans-muurola-4(14),5-diene	0.76	MS, RI

1485	bicyclogermacrene	1.51	MS, RI
1487	4-*epi*-cubebol	1.94	MS, RI
1490	α-muurolene	0.56	MS, RI
1495	germacrene A	0.19	MS, RI
1509	cubebol	13.32	MS, RI
1511	δ-cadinene	0.21	MS, RI
1513	*cis*-calamenene	0.73	MS, RI
1545	germacrene B	0.31	MS, RI, co-GC
1557	*trans*-nerolidol	0.70	MS, RI, co-GC
1565	spathulenol	0.47	MS, RI, co-GC
1568	caryophyllene oxide	0.58	MS, RI, co-GC
1578	gleenol	0.12	MS, RI
1591	viridiflorol	0.26	MS, RI, co-GC
1616	1-*epi*-cubenol	0.42	MS, RI, co-GC
1621	isospathulenol	0.12	MS, RI
	total	**98.52%**	

Trace: <0.05; # : The retention index was calculated using a homologous series of *n*-alkanes C_8-C_{18}; † :Co-GC: co-injection with an authentic sample.
For RI measurements, the oven temperature program suggested by Adams 15 (60°C to 246°C at 3°C/min) was used. Percentages (FID) are the mean of three runs and were obtained from electronic integration measurements using an HP 3395 integrator.

The major component was ß-cubebene (18.94%) followed by cubebol (13.32%), sabinene (9.60%) and ß-caryophyllene (5.28%) with many other components in minor amounts.

All the oleoresins showed the presence of 85 components (Appendix 1). The major component in all the oleoresins was cubebol (stereoisomer). The percentage of cubebol in the diethyl ether oleoresin was 32.38, in ethanol oleoresin 25.51, in petroleum benzene oleoresin 42.89, chloroform oleoresin 28.00 and in the methanol oleoresin 19.03. Other major components were ß-cubebene and germacrene D. Although chemical studies on several *Piper* species exist [27-28], there is not much detailed investigation concerning *Piper cubeba* berry oil. Our present results on the essential oil composition show some resemblance but also significant differences with the compositions reported previously [29], which are most likely due to the unknown origin of the berries (cultivar, variety, etc.) that were purchased from local markets. On the other hand, we found no reports on the chemical composition of tailed pepper oleoresins.

Antimicrobial activity

The results of antifungal activity using the inverted petriplate method and the food poison technique are given in Tables 2 and 3, respectively. Tailed pepper essential oil showed almost complete zone inhibition for *Penicillium*

Table 2. Effect of tailed pepper essential oil and various oleoresins against different food pathogenic fungi by the inverted petriplate method.

fungi	MTCC 1786		MTCC 284		MTCC 2935		MTCC 3003	
dose	2 µl	6 µl	2 µl	6 µl	2 µl	6 µl	2 µl	6 µl
	percentage zone inhibition[a]							
diethyl ether oleoresin	28	52	18	21	36.2	45	31.3	35
ethanol oleoresin	13	36	10	14	60	75	62.5	65
petroleum benzene oleoresin	35	69	(-)	2	32	40	37.5	42
chloroform oleoresin	48	83	15	22	13	40	42	53
methanol oleoresin	30	61	3.7	10	8.7	25	56	62
essential oil	90	100	12.5	35	10	12.5	65	73
carbendazim*	25	56	16	24	55	64	14	21

For all tested fungi the data was found to be highly significant (P≤ 0.05%). [a] : Average of three replicates. *: 1000 and 4000ppm solution in dimethylsulphoxide was used. *Penicillium purpurogenum* (MTCC 1786); *Fusarium oxysporum* (MTCC 284); *Fusarium proliferatum* (MTCC 2935); *Penicillium madriti* (MTCC 3003).

Table 3. Effect of tailed pepper essential oil and extract against different food pathogenic fungi by the poison food medium method.

fungi	MTCC 1786		MTCC 284		MTCC 2935		MTCC 3003	
dose	2 µl	6 µl	2 µl	6 µl	2 µl	6 µl	2 µl	6 µl
	percentage zone inhibition[a]							
diethyl ether oleoresin	32.5	46.2	27.5	40	32.5	37.5	31.3	65.7
ethanol oleoresin	20	25.8	36.3	51.3	40	46.3	51.3	75.2
petroleum benzene oleoresin	25.6	34.2	47.5	65.3	53	59.1	34.2	63
chloroform oleoresin	18.2	30.6	44	48.9	52	62.3	41.7	70
methanol oleoresin	36.1	38.3	12.5	39.2	54.3	68	19.7	40.3
essential oil	56.2	78.3	45	91.2	56.2	83	40.2	66
carbendazim*	20	43	15	21	34	42	-	-

For all tested fungi the data was found to be highly significant (p ≤0.05%). [a] : Average of three replicates. *: 1000 and 4000ppm solution in dimethylsulphoxide was used. -: inactive.

purpurogenum at all tested doses, whereas chloroform oleoresin exhibited 83% zone inhibition against *Penicillium purpurogenum* at 6 µl. The petroleum benzene oleoresin was found to be ineffective against *Fusarium oxysporum* at the tested dose. Other oleoresins showed minimum to moderate inhibition zones at the tested dose. In the food poison technique, the essential oil showed strong zone inhibition effect against all tested fungi, whereas the ethanol oleoresin was effective against *Penicillium madriti* at 6 µl. Other oleoresins showed minimum to moderate inhibition zones at the tested dose in this method. The higher efficacy of the essential oil could be due to higher volatility, which leads to a higher vapour concentration inside the petriplate. This might be responsible for the increase in antifungal activity with the increase in dose concentration. The oleoresins were not found effective in this method, due to their lower volatility. All the values were found to be statistically significant ($p < 0.05$).

The antimicrobial activity results (mean of three replicates) of essential oil and oleoresins obtained are given in Table 4. The essential oil exhibited more than 70% zone inhibition against *Bacillus cereus* and *Escherichia coli* at 6 µl level. Other oleoresins showed minimum to moderate inhibition zones against the tested bacterial species. Bishop and Thornton [30] reported that terpenoid phenolic and non-phenolic alcohols are the most bioactive against fungi. Chemical analysis of the essential oil and oleoresins showed that they contain non-phenolic terpenoid alcohols. The strength of inhibition and the spectrum of antimicrobial activity of the tailed pepper essential oil and oleoresins suggested that complex interaction between individual components led to 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 [30-32] and Gram-negative bacteria [33]. This type of disruption might have been occurred in the present study. The lower efficacy of this essential oil and oleoresin against some microorganisms in the present 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 oleoresin result from the synergistic action of all their components [34]. Although, the major components in essential oil and oleoresin are mostly considered to be mainly responsible for their antimicrobial property, the minor compounds may also play an important role. The synergistic or antagonistic effect of the later may significantly influence the antifungal action of the former. Such synergistic or antagonistic action probably occurred with this essential oil and oleoresin tested in the present study. From the above point of view, we believe that the methods, which we were used to evaluate antimicrobial activity, would provide additional information to assess antimicrobial properties of tailed pepper essential oil and oleoresin.

Antioxidant activity for mustard oil

The change of the peroxide value in the mustard oil system with additives is shown in Table 5. Mustard oil oxidation was measured at time intervals of 7 days during 28 days of storage. In this duration, the peroxide value of the control sample increased to 288 meq/kg^{-1}, which is significantly

Table 4. Antibacterial activities by agar well diffusion method.

test	dose (µl)	inhibition zone [a] (mm)			
		Gram-positive bacteria		Gram-negative bacteria	
		MTCC 3103	MTCC 1790	MTCC 1672	MTCC 733
diethyl ether oleoresin	2	22.3 ± 1.5	(-)	29.2 ±1.6	47.2 ± 1.1
	6	36.1 ± 1.5	13.2 ± 1.1	53.3 ± 1.9	65.2 ± 0.4
ethanol oleoresin	2	12.4 ± 1.2	18.1 ± 0.5	14.3 ±1.4	56.3 ± 1.2
	6	18.3 ± 0.6	32.0 ± 1.1	29.3 ± 0.3	100
petroleum benzene oleoresin	2	23.6 ± 0.5	36.3 ± 1.2	(-)	56.4 ± 0.8
	6	42.1 ± 0.1	73.3 ±1.4	21.4 ± 1.1	60.0 ± 0.3
chloroform oleoresin	2	17.1 ± 1.1	23.5 ± 1.3	50.0 ± 1.2	49.2 ± 1.5
	6	26.4 ± 1.5	44.9 ± 2.1	68.3 ± 0.8	84.1 ± 0.7
methanol oleoresin	2	19.2 ± 0.6	(-)	28.5 ± 1.4	32.6 ± 0.6
	6	30.2 ± 0.1	(-)	67.0 ± 0.1	41.3 ± 2.1
essential oil	2	30.6 ± 0.3	46.6 ± 1.2	42.0 ± 0.3	56.3 ± 0.1
	6	50.4 ± 1.6	72.3 ± 1.1	80.0 ± 0.3	100
ampicillin*	2	-	18.1 ± 0.7	-	44.4 ± 0.3
	6	12.6 ± 0.6	24.5 ± 0.9	14.4 ± 1.3	65.3 ± 0.2
cloxacillin*	2	-	27.3 ± 0.7	15.6 ± 0.7	28.1 ± 0.7
	6	-	31.0 ± 0.7	18.4 ± 0.7	38.2 ± 0.7

Staphylococcus aureus (MTCC 3103); *Bacillus subtilis* (MTCC 1790); *Escherichia coli* (MTCC 1672); *Salmonella typhi* (MTCC 733). For all tested fungi the data was found to be significant ($p \leq 0.05\%$). Values are means ± SD of three replicates.

[a]: average of three replicate. *: 1000 and 4000ppm of solution diluted in dimethylsulphoxide was used. - : inactive.

higher than samples containing essential oil and oleoresins. The essential oil and diethyl ether oleoresin were more effective than BHA, BHT and PG. The effectiveness of the essential oil and diethyl ether oleoresin may be due to the presence of a high ß-cubebene and sabinene content. Singh et al., [35] observed that black pepper essential oil, which also contains significant amounts of sabinene (13.0%), showed a strong antioxidant activity against linseed oil.

An early study of Chilpault et al., [36] 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 [37-39]. A limited number of methods are available in the literature regarding antioxidant activity of black cumin essential oil and extract. During the oxidation process, primary oxidation products (peroxides formed in the fats and oils) are converted into low molecular weight secondary oxidation products such as alkanes, alcohols, aldehydes and acids. They are highly reactive and initiate the oxidation chain process. Hence, the changes of the secondary oxidation products (malonaldehyde and 2-alkenals) are also measured parallel to peroxide value by thiobarbituric acid, *p*-anisidine and total carbonyl value (Table 5). All the values supported the antioxidant effect of essential oil and oleoresins in the mustard oil system. The inhibitory effect of the essential oil could be due to the presence of aromatic nuclei containing polar functional groups.

Table 5. Antioxidant activity of tailed pepper essential oil and various oleoresins in mustard oil in terms of peroxide, thiobarbituric acid, *p*-anisidine and carbonyl values.

day	7th	14th	21st	28th
peroxide value (meq/kg of O_2)				
control	110	155	208	288
BHA	88	151	174	254
BHT	87	124	157	269
PG	77	152	182	273
diethyl ether extract	75	125	135	223
ethanol extract	85	129	141	256
petroleum benzene extract	88	128	136	247
chloroform extract	88	124	140	259
methanol extract	87	126	144	236
essential oil	77	128	138	217
thiobarbituric acid value (meq/g of O_2)				
control	2.09	3.04	4.6	7.2
BHA	1.18	2.16	4.1	6.9
BHT	0.81	1.45	2.15	4.7
PG	0.54	1.07	1.63	2.63
diethyl ether extract	1.12	2.2	3.1	4.1
ethanol extract	1.39	2.9	3.4	4.4
petroleum benzene extract	1.25	2.6	3.6	4.6
chloroform extract	1.17	2.7	3.9	4.9
methanol extract	0.74	2.2	3.7	4.7
essential oil	1.17	2.1	3.3	4.3
***p*-anisidine value**				
control	14	43	51	92
BHA	6	24	32	67
BHT	9	20	38	72
PG	9	36	30	40
diethyl ether extract	3	30	32	59
ethanol extract	7	37	39	71
petroleum benzene extract	7	33	29	61
chloroform extract	6	29	26	76
methanol extract	13	27	39	67
essential oil	5	23	24	60
carbonyl value (mg)				
control	3.5	7.7	9.12	12.90
BHA	2.4	5.9	06.3	10.13
BHT	2.7	2.8	5.72	10.72
PG	2.5	3.7	7.12	10.69
diethyl ether extract	2.4	5.4	6.18	11.05
ethanol extract	2.4	5.2	6.12	11.17
petroleum benzene extract	2.4	7.2	9.02	12.05
chloroform extract	2.7	6.6	8.17	11.76
methanol extract	2.8	3.0	6.76	08.98
essential oil	2.1	2.8	5.16	08.13

Antioxidant activity in linoleic acid system

The ferric thiocyanate method was used to compare the inhibitory action of essential oil and oleoresins with those of selected standard antioxidants. In this method, the peroxide level was evaluated during the initial stage of lipid peroxidation. High absorbance is an indication of a high concentration of formed peroxides. The absorbance of linoleic acid emulsion without the addition of essential oil, oleoresins or antioxidants increased rapidly and there was a significant ($p < 0.05$) difference between the blank and the tested essential oil or oleoresins. As can be seen in Table 6, the essential oil and oleoresins were able to reduce the formation of peroxides. Chloroform oleoresin was found to be the most effective among all the additives.

DPPH radical scavenging effect

The DPPH radical scavenging activity of tailed pepper essential oil and oleoresins is shown in Table 7. The results were effective if the scavenging effect increased with concentration. The scavenging effect of the oleoresins and essential oil and synthetic antioxidant on the DPPH radical increased linearly with increasing concentration. Chloroform oleoresin, petroleum benzene oleoresin and essential oil showed excellent radical scavenging activity in comparison with synthetic antioxidants and other oleoresins. Probably a substance, present but not identified in the oleoresin by GC and GC/MS, may contribute to the improved antioxidant activity of oleoresins and this requires further investigation. Antioxidative activities observed in the essential oil and oleoresin could be due to the synergistic effect of more than two compounds that may present in the system. It has been reported that most natural antioxidant compounds often work synergistically with each other to produce a broad spectrum of

Table 6. Antioxidant effect of tailed pepper essential oil and oleoresins with standards assessed by the linoleic acid system method.

day	2th	4th	6st	8th	10	12
% inhibition of linoleic acid peroxidation						
BHA	20	36	48	59	72	94
BHT	18	32	44	50	66	83
PG	24	39	52	56	75	87
diethyl ether extract	14	16	26	51	71	80
ethanol extract	14	26	44	54	68	77
petroleum benzene extract	14	24	29	44	57	62
chloroform extract	12	16	22	31	37	45
methanol extract	22	25	33	54	58	69
essential oil	16	23	44	57	66	70

antioxidative activities that create an effective defence system against free radical attack [40]. The composition of essential oil and oleoresins are very complex; they consist of various classes of organic compounds that may possess opposite effects on the process of lipid oxidation. Based on the results obtained, it is highly possible that several compounds of different polarity may contribute to the antioxidative activity of tailed pepper essential oil and oleoresins.

Table 7. Radical scavenging activity of tailed pepper essential oil and oleoresins on 2, 2´-diphenyl-1-picrylhydracyl radical.

	% DPPH radical scavenging activity			
	5 µl	10 µl	15 µl	20 µl
control	27	48	64	76
BHA	29	43	51	60
BHT	31	49	52	69
PG	31	72	84	89
diethyl ether extract	14	65	75	80
ethanol extract	35	68	82	96.7
petroleum benzene extract	37	79	92	99.2
chloroform extract	29	43	61	70
methanol extract	36	68	80	93
essential oil	77	128	138	217

Conclusion

Summarizing these results, it can be concluded that the essential oil and all oleroresins of tailed pepper exhibited a broad spectrum of antimicrobial activity against the tested microorganisms and that they could be a better natural antioxidant for stabilising mustard oil. More studies are needed to clarify the antioxidant mechanisms of the essential oil and oleoresins. On the basis of the above results, it was observed that the essential oil and oleoresins provided equivalent or higher antioxidative activity as compared to synthetic antioxidants.

Acknowledgements

Thanks to Head, Chemistry Department, DDU Gorakhpur University, Gorakhpur for providing laboratory facilities. and to the Life Sciences Research Board, DRDO, New Delhi for financial support. Prof. K. D. S. Yadav of our department is also acknowledged for providing spectral facility. Work in Tucuman was supported by grants from Consejo Nacional de Investigaciones Cientificas y Tecnicas, Argentina (CONICET, PIP 5176) and ANPCYT-Universidad Nacional de Tucuman (PICTO 2004 No. 503).

References

1. Harman DA. Theory based on free radical and radiation chemistry. *J Gerontol*. 1956;11:298-300.
2. Sockett PN. Food-borne Escherichia O157:H7 Disease costs in the United States and Scotland. Food Policy. 1985;107:335-47.
3. Ito N, Fukushim S, Tsuda H. Carcinogenicity and modification of carcinogenic response by BHA, BHT and other antioxidants. *Crit Rev Toxicol.* 1985;15:109-50.
4. Jung CH, Seog HM, Choi IW, Cho HY. Antioxidant activities of cultivated and wild Korean ginseng leaves. *Food Chem.* 2005;92:535-40.
5. Katalinic V, Milos M, Kulisic T, Jukic M. Screening of 70 medicinal plant extracts for antioxidant capacity and total phenols. *Food Chem*. 2006;94:550-57.
6. Singh G, Marimuthu P, de Heluani CS, Catalan AN. Antioxidant and biocidal activities of Carum nigrum essential oil, oleoresin and their selected components. *J Agric Food Chem*. 2006;54:174-81.
7. Baratta MT, Dorman HJD, Deans SG, Figueiredo AC, Baroso JG, Ruberto G. Antimicrobial and antioxidant properties of some commercial essential oils. *Flav Fragr J.* 1998;13:235-44.
8. Lambert BJW, Skandamis, PN, Coote PJ, Nychas GJE. A study of minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. *J Appl Microbiol.* 2001; 91: 453-462.
9. Singh G, Marimuthu P, Catalan C, de Lampasona MP. Chemical, antioxidant and antifungal activities of volatile oil of black pepper and its acetone extract. *J Sci Food Agric*. 2004;84:1878-84.
10. Ekanem AP, Wang M, Simon JE, Obiekezie AI. In vivo and in vitro activities of the seed extract of Piper guineense Schum. and Thonn. against skin and gill monogenean parasites of goldfish (Carassius auratus). *Phytother Res.* 2004;18:793-79.
11. Pradhan KJ, Variyar PS, Bandekar JR. Antimicrobial activity of novel phenolic compounds from green pepper (Piper nigrum L.). *Lebens-Wissen Technol*. 1999;32:121-23.
12. Burt S. Essential oils: their antibacterial properties

and potential application a review. *Int J Food Microbiol.* 2004;94:223– 53.
13. Chemat F, Abertvian M. Dangles O. Essential oils as antioxidants. *Int J Essential Oil Ther*. 2007;1:4-15.
14. Clevenger European Pharmacopoeia. Maissoneuve, SA: Sainte Ruffine ; 1983.
15. Adams RP. Identification of essential oil components by Gas Chromatography/Quadrupole Mass Spectrometry. Allured Publishing Corporation: Carol Stream; 2001.
16. Massda Y. Analysis of essential oils by Gas Chromatography and Mass Spectrometry. Halsted/Wiley: New York; 1976.
17. Ramdas K, Suresh G, Janardhanan N, Masilamani S. Antifungal activity of 1, 3- disubstituted symmetrical and unsymmetrical thioureas. *Pest Sci*. 1998;52:145-51.
18. Amvam Zolla PH, Biyiti L, Tchoumbougnang F, Menut C, Lamaty G, Bouchet P. Aromatic plant of tropical Central Africa. Part XXXIII, Chemical composition and antifungal activity of thirteen essential oils from aromatic plants of Cameroon. *Flav Fragr J.* 1998;13:107-14.
19. NCCLS (National Committee for Clinical Laboratory Standards). 6th ed. Performance standard for antimicrobial disc susceptibility test, Approved standard, M2-A6. Wayne, 1997.
20. Bandoniene D, Venskutonis PR, Gruzdiene D, Murkovic M. Antioxidant activity of Sage (Salvia offcinalis L.), Savory (Satureja hortensis L.) and Borage (Borago officinalis L.) extracts in rapeseed oil. *Euro J Food Res Technol*. 2004;104:286-92.
21. AOCS official Methods, p-anisidine value. In: Official methods and recommended practices of the American Oil Chemist's Society, 5th ed. AOCS Press: Illinois; 1998.
22. Frankel EN. Lipid oxidation. The Oily Press: Dundee, U. K; 1998.
23. Osawa T, Namaki MA. Novel type antioxidant isolated from leaf wax of Eucalyptus leaves. *Agric Biol Chem.* 1983;45:735-39.
24. Kikuzaki H, Nakatani N. Antioxidant effect of some ginger constituents. *J Food Sci.* 1993;58:140710.
25. Cuendet M, Hostettmank K, Potterat O. Iridoid glucosides with free radical scavenging properties from Fagraea blumei. *Helv Chim Acta.* 1997:80;1144-52.
26. Dejian H, Boxin O, Prior RL. The chemistry behind antioxidant capacity assays. *J Agric Food Chem.* 2005;53:1841-56.
27. Srinivas PV, Madusudana J. Isopiperolein B: an alkamide from Piper nigrum. *Phytochem.* 1999;52:952-58.
28. Tsukamoto S, Tomise K, Miyakawa K, Cha BC, Abe T, Hamada T, Ohta T. CYP3A4 inhibitory activity of new bisalkaloids, dipiperamides D and E, and cognates from white pepper *Bioorg Med Chem*. 2002;10:2981-85.
29. Sumathykutty MA, Madhusudana Rao J, Padmakumari KP, Narayaran CS. Essential oil constituents of some Piper species. *Flav Fragr J*. 1999;14:279-82.
30. Bishop CD, Thornton IB. Evaluation of the antifungal activity of the essential oils of Monadra citriodora var. citriodora and Melaleuca alternifolia on post-harvest pathogens. *J Essential Oil Res*. 1997;9:77-82.
31. Ultee A, Kets EPW, Smid EJ. Mechanisms of action of carvacrol on the food-borne pathogen Bacillus cereus. *Appl Environ Microbiol*. 1999;65:4606-10.
32. Cox SD, Mann CM, Markham JL, Bell HC, Gustafson JE, Warmington JR, Wyllie JG. The mode of antimicrobial action of Malaleuca alternifolia (tea tree oil). *J Appl Bacteriol*. 2002;88:170-75.
33. Helander IM, Alakomi HL, Latva-kala K, Mattila Sandholm T, Pol I, Smid EJ, Gorris LGM, von Wright A. Characterization of the action of selected essential oil components on gram negative bacteria. *J Agric Food Chem*. 1998;46:3590-95.
34. Dubey NK, Kishore N, 1987. Fungitoxicity of some higher plants and synergistic activity of their essential oils. *Tropical Sci.* 1987;27:23-27.
35. Singh G, Marimuthu P, Catalan C, de Lampasona MP. Chemical, antioxidant and antifungal activities of volatile oil of black pepper and its acetone extract. *J Sci Food Agric.* 2004;84:1878-84.
36. Chilpault JR, Mizuno GR, Lundberg WO. The antioxidant properties of spices in foods. *Food Technol*. 1956;10:209-12.
37. Ragnarsson JO, Labuza TP. Accelerated shelf life testing for oxidative rancidity in foods. *Food Chem.* 1997; 8: 291-308.
38. Robards K, Prenzier PD, Tucker G, Swatsitang P, Glover W. Phenolic compounds and their role in oxidative processes in fruits. *Food Chem*. 1999;20:401-36.
39. Khal R, Hilderbrant AG. Methodology for studying antioxidant activity and mechanism of action of antioxidants. *Food Chem Toxicol*. 1986;24:1007-14.
40. Lu F, Foo LY. Phenolic antioxidant component of evening primrose. In: Ong ASH, Niki E, Packer L. Nutrition, lipids, health and diseases. Champaign: American Oil Chemists Society Press; 1995.
41. Badheka LP, Prabhu BR, Mulchandani NB. Dibenzylbutyrolactone lignans from Piper Cubeba. *Phytochem.* 1986;25:485.
42. Haque E, Rahman A, Amin A, Shekher HU. Alkamide and a Lignan from the stem bark of Piper chaba (Piperaceae). *J Pharmaceut Sci.* 2004;3:1-2.
43. Mass Spectra in the NIST computer data base.
44. Prabhu BR, Mulchandani NB. Lignans from Piper cubeba. *Phytochem*. 1985;24:329.
45. Xia YM, Cao ZP, Peng K, Reng ZF, Pan XF. An efficient Synthetic Method of Nordihydroguaiaretic acid (NDGA). *Chinese Chem Lett*. 2003;14:359-60.

Table 7. Radical scavenging activity of tailed pepper essential oil and oleoresins on 2, 2´-diphenyl-1-picrylhydracyl radical.

RI#	compound	DE %	E %	PB %	CL %	M %	identification†
922	α-thujene	0.13	trace	trace	0.11	trace	MS, RI, co-GC
930	α-pinene	0.13	trace	0.06	0.10	trace	MS, RI, co-GC
968	sabinene	0.73	0.13	0.35	0.52	0.12	MS, RI, co-GC
974	ß-pinene	0.03	trace	trace	trace	trace	MS, RI, co-GC
986	myrcene	trace	trace	trace	trace	trace	MS, RI, co-GC
1003	α-phellandrene	0.04	trace	trace	trace	trace	MS, RI
1013	α-terpinene	trace	trace	trace	trace	trace	MS, RI
1020	*p*-cymene	trace	trace	trace	trace	trace	MS, RI, co-GC
1025	limonene	trace	trace	trace	trace	trace	MS, RI, co-GC
1027	ß-phellandrene	0.33	0.20	0.18	0.26	0.04	MS, RI, co-GC
1028	1,8-cineole	trace	trace	trace	trace	trace	MS, RI, co-GC
1051	γ-terpinene	trace	0.05	trace	trace	trace	MS, RI, co-GC
1066	*cis*-sabinene hydrate	0.11	0.12	0.14	0.13	0.10	MS, RI, co-GC
1082	terpinolene	trace	trace	trace	trace	trace	MS, RI, co-GC
1097	linalool	0.28	0.41	0.36	0.33	0.26	MS, RI, co-GC
1099	*trans*-sabinene hydrate	0.09	0.11	0.13	0.12	0.10	MS, RI, co-GC
1174	terpinen-4-ol	0.07	0.19	trace	0.09	0.08	MS, RI, co-GC
1189	α-terpineol	trace	0.07	trace	trace	trace	MS, RI, co-GC
1338	δ-elemene	0.11	0.09	0.11	0.10	trace	MS, RI, co-GC
1347	α-cubebene	4.83	2.86	2.30	6.41	1.22	MS, RI
1349	eugenol	trace	0.10	trace	trace	trace	MS, RI
1373	α-copaene	3.21	3.09	3.25	2.87	1.92	MS, RI
1387	ß-cubebene	9.95	5.66	9.81	7.12	4.32	MS, RI
1390	ß-elemene	trace	trace	trace	trace	trace	MS, RI
1401	α-gurjunene	0.28	0.26	0.29	0.25	0.17	MS, RI
1415	ß-caryophyllene	2.42	2.42	2.48	2.16	1.59	MS, RI, co-GC
1423	ß-gurjunene	0.10	trace	trace	trace	trace	MS, RI
1427	*trans*-α-bergamotene	0.06	0.03	trace	trace	trace	MS, RI
1429	α-guaiene	0.05	trace	trace	trace	trace	MS, RI
1443	*cis*-muurola-3,5-diene	0.28	0.31	0.17	0.27	0.11	MS, RI
1450	α-humulene	1.07	1.14	1.18	1.00	0.77	MS, RI
1456	*allo*-aromadendrene	1.99	2.08	2.12	1.79	1.35	MS, RI
1467	*trans*-cadina-1(6),4-diene	0.33	0.52	trace	0.41	trace	MS, RI
1473	γ-muurolene	1.37	1.45	1.40	1.27	0.90	MS, RI
1493	4-*epi*-cubebol	2.86	4.40	3.35	2.48	1.77	MS, RI
1479	germacrene-D	4.58	5.12	5.24	3.91	3.45	MS, RI
1477	ß-selinene	0.56	0.25	0.24	0.21	0.17	MS, RI
1487	*trans*-muurola-4(14),5-diene	0.41	0.56	0.28	0.41	0.21	MS, RI
1495	α-muurolene	0.49	3.45	0.48	0.46	0.99	MS, RI
1497	α-bulnesene	0.08	0.10	trace	trace	trace	MS, RI
1504	ß-bisabolene	0.18	0.25	0.22	0.16	0.13	MS, RI
1508	γ-cadinene	0.13	0.10	trace	0.17	trace	MS, RI
1512	cubebol	trace	trace	trace	trace	6.91	MS, RI
1524	cubebol stereoisomer*	32.38	25.51	42.89	28.00	19.03	MS, RI
1526	*trans*-γ-bisabolene	0.23	0.26	0.26	0.21	trace	MS, RI
1531	*trans*-cadina-1(2),4-diene	1.02	0.88	0.39	1.14	1.30	MS, RI
1544	elemol	0.12	nd	0.14	0.12	0.08	MS, RI

1544	unidentified I	-----	5.93	-----	-----	-----	-----
1553	germacrene B	0.06	trace	0.11	trace	0.10	MS, RI
1560	unidentified II	-----	8.56	-----	-----	-----	-----
1560	*trans*-nerolidol	2.43	1.55	2.82	2.29	1.87	MS, RI
1564	ledol	0.12	0.14	0.14	0.11	0.10	MS, RI
1572	germacrene-D-4-ol + spathulenol	0.80	0.33	1.19	0.70	0.44	MS, RI
1568	caryophyllene oxide	0.13	0.34	0.17	0.13	0.22	MS, RI, co-GC
1582	gleenol	1.00	1.17	0.22	0.94	0.77	MS, RI
1594	viridiflorol	2.06	0.21	2.41	1.94	1.55	MS, RI, co-GC
1615	junenol	0.42	0.52	0.47	0.39	0.38	MS, RI
1625	*1-epi*-cubenol	0.80	1.43	0.93	0.74	0.65	MS, RI
1627	isospathulenol	trace	trace	trace	trace	trace	MS, RI
1640	torreyol	0.25	0.31	0.29	0.23	0.19	MS, RI, co-GC
1657	bulnesol	0.07	0.09	trace	trace	0.14	MS, RI, co-GC
1667	trans-ß-asarone	0.10	0.19	trace	trace	trace	MS, RI, co-GC
-----	palmitic acid	1.08	1.14	1.41	1.23	2.19	MS, co-GC
-----	linoleic acid	0.70	0.72	0.80	0.59	0.72	MS, co-GC
-----	oleic acid	0.21	0.16	0.25	0.22	0.39	MS, co-GC
-----	unidentified lignan III	0.45	2.18	1.41	6.55	7.89	-----
-----	unidentified lignan IV	0.34	0.26	0.41	0.49	1.08	-----
-----	unidentified lignan V	0.13	trace	trace	0.20	0.10	-----
-----	unidentified lignan VI	nd	0.43	nd	nd	0.08	-----
-----	piperine	0.46	0.52	0.37	0.62	0.91	MS [45]
-----	unidentified lignan VII	nd	0.18	nd	nd	trace	-----
-----	unidentified lignan VIII	3.25	0.91	1.00	4.43	1.22	-----
-----	unidentified lignan IX	0.67	0.41	0.42	1.12	3.28	-----
-----	cubebin	1.05	1.24	1.66	1.57	2.78	MS [44]
-----	hinokinin	3.88	2.99	2.21	6.92	11.19	MS (Ref. NIST)[43]
-----	kusumokinin	0.39	0.37	0.17	0.89	1.28	MS 42]
-----	cubebinone	0.42	0.14	0.12	0.69	0.51	MS[41]
-----	unidentified lignan X	0.36	0.33	0.12	0.60	0.94	-----
-----	clusin	0.09	0.12	0.32	0.15	0.28	MS [41]
-----	dihydrocubebin	0.15	trace	nd	trace	0.29	MS
-----	yatein o isoyatein	0.64	0.34	0.28	1.33	1.59	MS [41]
-----	isoyatein o yatein	0.17	trace	nd	0.41	0.44	MS [41]
-----	sitosterol	0.19	0.12	0.15	0.21	0.53	MS, co-GC
-----	cubebininolide	0.16	trace	trace	0.40	0.44	MS [41]
-----	unidentified lignan XI	nd	trace	nd	0.28	0.25	-----
	Total	**88.9%**	**78.6%**	**94.3%**	**85.3%**	**77.0%**	

DE: diethyl ether oleoresin; E: ethanol oleoresin; PB: petroleum benzene oleoresin; CL: chloroform oleoresin; M: methanol oleoresin.