REPORT

Gas chromatography coupled with mass spectrometric characterization of *Curcuma longa*: Protection against pathogenic microbes and lipid peroxidation in rat's tissue homogenate

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Abstract: The present study was designed to investigate the mineral content and antimicrobial activity of *Curcuma Longa* extracts and its essential oil. We also determined the lipid peroxidation inhibition activity of the ethanolic extract against sodium nitroprusside (SNP) induced thiobarbituric acid reactive species (TBARS) formation in rat's brain, kidney and liver homogenates. Major constituents of essential oil identified by gas chromatography and mass spectrometry (GCMS) were beta-sesquiphellandrene (38.69%), alpha-curcumene (18.44%) and p-mentha-1,4 (8)-diene (16.29%). Atomic absorption spectroscopy (AAS) was used for the quantitative estimation of Calcium (Ca), Magnesium (Mg), Iron (Fe), Copper (Cu), Zinc (Zn), Chromium (Cr), Nickel (Ni) and Manganese (Mn). The extract showed highest Mg (49.4mg/l) concentration followed by Ca (35.42mg/l) and Fe (1.27mg/l). Our data revealed that the ethanolic extract of *Curcuma Longa* at 1-10 mg/kg significantly inhibited TBARS production in all tested homogenates. Crude extracts and essential oil were tested against three gram positive bacteria i.e. *Bacillus subtilis*, *Bacillus atrophoeus, Staphylococcus aureus*, six gram negative bacteria i.e. *Escherichia coli, Klebsiella pneumonias, Salmonella typhi, Pseudomonas aeruginosa, Erwinia carotovora, Agrobacterium tumefaciens* and one fungal strain namely *Candida albicans* by disc diffusion assay. Essential oil showed highest anti-microbial activity as compared to the crude extracts. The present study confirms the significant antimicrobial and antioxidant potential of the studied plant, which can be considered as a diet supplement for a variety of oxidative stress induced or infectious diseases.

Keywords: *Curcuma Longa*, GCMS, AAS, antimicrobial activity and lipid per oxidation

INTRODUCTION

According to World Health Organization, about 80% of individuals use traditional medicine in the form of crude, refined extract or various compounds obtained from medicinal plants (Arutselvi *et al.,* 2012). The medicinal value of plants can be attributed to the presence of bioactive compounds that produce a specific physiological action. Alkaloids, tannins, saponins, flavonoids and phenolic compounds are the most important bioactive compounds of medicinal plants (Edeoga *et al.,* 2005).The natural products are found to be more effective with least side effects as compared to commercial antibiotics and are used an alternative remedy for treatment of various infections and pathological situation. The physico-chemical as well as biochemical characterization of such plants are mandatory as they will enhance our understanding of their mechanism of action, efficacies and related safety issues.

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Curcuma Longa, a perennial herb belongs to *Zingiberaceae* family, is cultivated in asian countries like china and India. *Curcuma Longa*, commonly called Haldi in Hindi, is a medicinal plant extensively used in Unani, Ayurvedha and Siddha medicine as an in-house designed remedy for various diseases or pathological situations. The rhizome of this plant is used medicinally; it is usually, boiled, cleaned and dried to yield a yellow to yellowish powder called turmeric. Turmeric rhizome is used as a food additive (spice), preservative and coloring agent (Arutselvi *et al.,* 2012) in various Asian countries like China and other South East Asian countries. Traditional Indian medicine utilizes turmeric powder against biliary disorders, anorexia, coryza, diabetic wounds, cough, hepatic disorder, rheumatism and sinusitis (Ammon *et al.,* 1992). The main active compounds of turmeric are curcuminoids and essential oils. Curcuminoids are reported to contain curcumin (diferuloyl methane), demethoxycurcumin and bisdemethoxycurcumin as is principal constituents (Chainani-Wn *et al.,* 2003). Curcumin (diferuloylmethane), the main yellow chemical

constituent of the plant (turmeric) posses various pharmacological potential like anti-inflammatory, anti-carcinogenic, anti-diabatic, antioxidant, antibacterial, anti-protozoal, anti-fungal, antiviral, anti-fibrotic, antiulcer and hypertensive activities (Kumar *et al*., 2011). Keeping in view its long and historically significant pharmacological background, the present study was designed to bio-chemically characterize this interesting plant, which may leads to the chemical understanding of its strong therapeutic potential. Mineral contents and presence of heavy or toxic metals are investigated in detail. It is worthy to note that the present study aimed to quantitatively investigate the various constituents of the plant and for the purpose gas chromatography coupled with mass spectrometric work was designed to chemically characterize the plant essential oil.

Sodium Nitroprusside (SNP) is extensively used in the literature to mimic the oxidative stress since it is involved in the pathogenesis of a number of neurodegenerative disorders (Tunez *et al.,* 2010). SNP can cause oxidative stress and cytotoxicity by releasing cyanide or producing the nitric oxide (NO) and may involve complex chemical reactions with iron (Fe) which may further generate peroxy- nitrite radical (Cardaci *et al* ., 2008). The various biochemical products of SNP i.e. NO radical or Fe involved adducts, can leads to membrane damage and may cause oxidation of biologically significant molecules ranging from lipids to proteins and may also damage DNA (Valko *et al.,* 2004).

The oxidative stress has been implicated in the genesis or pathogenesis of various diseases. The plants can serve to protect the escalating effects caused by various stress related phenomenon. For the purpose the inhibitory effect of *Curcuma Longa* extract against SNP induced TBARS formation in rat's brain, kidney and liver homogenates was also studied. The extract and oil were further tested against pathogenic microbes; Both gram positive and negative bacteria and also a characteristic fungal strain. We hope that the results of the present study will help in evaluating and understanding the acclaimed medicinal properties of *Curcuma Longa.*

MATERIAL AND METHODS

Chemicals

Thiobarbituric acid (TBA) and malondialdehyde (MDA) were obtained from Sigma (USA). Iron (II) sulphate from Reagen (Brazil). All the chemicals used were pure and obtained from standard commercial supplier.

Sample collection and identification

Curcuma Longa was purchased from local market of Peshawar in February 2012. The dried plant was grinded using pestle and mortar and packed in polythene bags and placed in a dried place for further extractions. The plant species were identified by experts in PCSIR, Peshawar.

Ethanolic extract preparation for lipid peroxidation assay

The extract of *Curcuma Longa* was prepared in ethanol. Briefly, about 1g of the *Curcuma Longa* was soaked in ethanol (100ml) for a short time (5min). The mixture was centrifuged at 2,000 rpm for 10min. The supernatant (S1) was used for the elucidation of lipid peroxidation bioassay.

Preparation of tissue homogenates and thiobarbituric acid reactions

Adult male wistar rats (250-350 g) were decapitated with mild diethyl ether anesthesia and various tissues like brain, liver and kidney were quickly removed, placed on ice and weighed. The tissues were homogenized in Tris-HCl (10mM) at approximately 1,200 rpm. Low-speed supernatant (S1) was obtained after the homogenate was centrifuged at 3,000 rpm for 10 min. The lipid peroxidation assay was carried out using the modified method of Ohkawa *et al.,* (1979).

Gas chromatography & mass spectrometric analysis of the essential oils

GC-MS analysis will be performed by the method of Qureshi *et al.,* 2011.Essential oil was extracted by steam distillation method. Known weight of the sample was taken in a distillation flask (dean stark's apparatus). The flask was filled (two third) with distilled water and heated. The aromatic volatile oils from the plant sample will be trapped with the steam and was condensed by water condenser. During condensation the two layers was separated. Anhydrous Na_2SO_4 was used to remove water from the oil and finally pure oil was collected in vial. Chemical constituents were identified by comparing their retention indices and mass spectra with National Institute of Standards and Technology (NIST) library.

Metallic screening by atomic absorption spectrophotometer (AAS)

Metallic content i.e. Calcium (Ca), Nickel (Ni), Iron (Fe), Zinc (Zn), Copper (Cu), Chromium (Cr), Magnesium (Mg) and Manganese (Mn) were determined by atomic absorption spectrophotometery. Sample was prepared by washing the *Curcuma Longa* with double distilled water or deionized water. The moisture was removed and the sample (ash) was treated with standard acid mixture i.e. perchloric acid and nitric acid (1: 4). The method of Hussain *et al.,* 2009 was followed for the analysis of metallic content. 1000 mg/L solutions of all metals were obtained from Merck.

Analysis of antimicrobial activity test microorganisms

Six gram negative (*E. coli, K. pneumonias, S. typi, Pseudomonas aeruginosa, Erwiniacarotovora, Agrobacterium tumefaciens*), three gram positive (*B. subtilis, B. atrophaeus, S. aureus*) bacterial species and one fungal strain i.e. *Candida albican* were used to determine the anti-microbial activity of the *Curcuma Longa* extract.

Determination of antimicrobial activity

Antibacterial activity of solvent extracts i.e. aqueous, ethanol and ethyl acetate were determined by Disc-diffusion assay in terms of diameters of inhibition zone against selected microbes. Nutrient agar media (2.8g 100 ml^{-1}) and nutrient broth (1.3g 100ml^{-1}) were prepared by dissolving in distilled water. The nutrient broth was transferred in test tubes (8-9 ml) and flasks (20-25ml). All of the apparatus and media were sterilized at 121^{o}C and 15 psi pressure for 20 minutes. Agar media was decanted into petri plates and incubated for 16 hours at 37ºC to check any contamination. The stock cultures were freshened by streaking on fresh agar plates in a laminar flow hood and incubated at 37ºC for 24 h. Next day streaked cultures were inoculated into the nutrient broth in flasks and kept in the shaking water bath (Model; GLSC-SBR- 04-28) for 16 hrs, at 200 rpm at 37ºC. The microbial cultures were standardized in test tubes by comparing with 0.5 McFarland (turbidity) Standard. 100µl of standardized microbial cultures were spread on each nutrient agar plate. These plates were then kept for absorption (15 minutes) in a refrigerator. Whattman filter paper-I discs (6mm) were placed on these agar media plates aseptically with the help of a sterile forceps. The stock solutions of all the extracts and oil in 2mg disc-1 in 12ul volume were applied on these discs in triplicates. These plates were the incubated at 37ºC overnight. Antimicrobial potential was recorded for each extract in terms of mm of zones of inhibition around each disc (Fazal *et al.,* 2012).

RESULT

The yield of the extracts (ethanolic, ethyl acetate and aqueous) were calculated and recorded as percentage of plant material. The highest extractive value was obtained for aqueous extract (6.72%) followed by ethanolic (6.24%) and ethyl acetate (4.9%) extract. Different mineral elements were quantitatively analyzed by AAS and the results are reported in table. 2 The data revealed that all analyzed metal were accumulated in *Curcuma Longa* at different concentrations. Mg (49.4mg/l) and Ca (35.425mg/l) were present in high concentration as macronutrients. Among six micronutrients, Fe (1.27mg/l) and Cr (1.188mg/l) were in highest concentrations followed by Mn (1.078mg/l), Zn (1.008mg/l), Cu (0.109mg/l) and Ni (0.096mg/l). GC/MS analysis identified 16 compounds in the essential oil representing 99.99% of the total composition. The most abundant of these compounds were beta.sesquiphellandrene (38.69%), alpha-curcumene (18.44%), p-Mentha-1, 4(8)-diene (16.29%), 1H-indene, 2.3, 3a, 4, 7, 7a-hexa-hydro-2,2,4, 4, 7, 7-hexamethyl (6.11%),alpha-caryophylle (4.72%),p-Cymen-8-ol (3.54%). The chemical constituent of *Curcuma Longa* essential oil is shown in table 3.

Our results demonstrated that SNP caused oxidative stress as apparent from increased TBRAS formation in brain, kidney and liver homogenate (fig. 1). The ethanolic extract of CL inhibited the lipid peroxidation process at the higher tested concentrations. The result of the disc diffusion test indicated that crude ethanolic, aqueous, ethyl acetate extracts and essential oil showed different degree of growth inhibition against the selected bacterial and fungus strains (table 1). Essentials oil showed highest activity against the tested microorganism as compared to crude extracts. The diameter of inhibition zone ranged from 12 to 41mm with mean index of 26.5mm and from 11.5 to 30.5mm with mean index of 21mm for essential oil and ethyl acetate extract respectively. Ethanolic and aqueous extracts inhibition zone ranged from 8 to 23mm with mean antimicrobial index of 15.5 and from 11 to 14.5mm with mean index of 12.5mm respectively. It is worthy to note that essentials oil showed highest activity against the tested microorganism as compared to crude extracts. Essential oil showed promising activity against *Staphylococcus aureus* (41mm), *Pseudomonas aeruginosa* (29.5mm), *Klebsiella pneumoniae* (22mm) and *Candida albicans* (19mm). While, least activity was observed against *Salmonella typhi* (12mm). Among the three extracts ethyl acetate showed significantly higher antimicrobial activity than the aqueous and ethanolic extracts. The crude ethyl acetate showed wider zone against *Staphylococcus aureus* (30.5mm) followed by *B. subtilis* (29mm). Ethanolic extracts was most active against *B. Subtilis* (23mm) and *B. atrophoeus*. Aqueous extract was the least active as compared to ethanol and ethyl acetate extracts. The aqueous and ethyl acetate extracts showed no activity against *Salmonella typhi*.

DISCUSSION

The ash of *Curcuma Longa* showed the presence of important minerals element in different concentrations. Mg, Ca, Fe were in maximum concentrations followed by Mn, Zn, Cu, Ni. These inorganic elements play an important role in various and diverse physiological processes involved in human health. Calcium plays a crucial role in regulating various biological processes like excitability, neurotransmitter release, gene transcription and synaptic plasticity (Clapham, 1995). Magnesium acts as activators for various enzymes involved in carbohydrate metabolism and synthesis of nucleic acids (DNA and RNA). Decrease magnesium concentration causes increased irritability of the nervous system and cardiac arrhythmias (Singh and Jain, 2006). Zn is an essential constituent of a number of enzymes such as alcohol dehydrogenase, carbonic anhydrase and procarboxy peptidase. Zinc deficiency result in growth retardation and skin lesions (Chatterjee and Shinde, 1995). Similarly, Ni is reported to involve in the synthesis of haemoglobin in the bone marrow. Iron is the most well known in biological system and performs a wide range of

biological functions. It is an essential constituent of cytochromes, haemoglobin, myoglobin and certain enzymes such as catalase and per oxidases (Chatterjee and Shinde, 1995).

The GC-MS result of *Curcuma Longa* revealed the presence of different monoterpene and sesquiterpene varying in concentrations. The major components of oil were beta-sesquiphellandrene (38.69%), alpha-curcumene (18.44%), p-Mentha-1, 4(8)-diene (16.29%), 1H-indene, 2.3, 3a, 4, 7, 7a-hexa-hydro-2, 2, 4, 4, 7, 7-hexamethyl (6.11%) and alpha-caryophyllene (4.72%). Contrasting results are reported by Raina *et al.,* in terms of chemical composition of rhizome oil. The major constituent of the present study were beta-sesquiphellandrene (38.69%), alpha-curcumene (18.44%), p-Mentha-1, 4(8)-diene (16.29%) in contrast to alpha. turmeron (44%), beta turmeron (18.5%) as reported by Raina *et al.,* 2005. While in another reportβ- sesquiphellandrene (22.8%), terpinolene (9.5%), alpha-curcumene (7,8%) were identified as the major components of the tested plant (Priya *et al.,* 2012). These different results may reflect the differences in geographical location, soil composition and various other human or natural factors. Beta-sesquiphellandrene and alpha-curcumene are classified as sesquiterpene compounds. This class of compounds has been known for diverse biological activities in the literature like such as antimicrobial, cytotoxic, anti-inflammatory, antiviral, antifungal activities (Chaturvedi, 2011). However there has been scarcity in literature for biological activities of the isolated major compounds identified in the current approach. We thus hypothesized that the detected antimicrobial activities of our essential oil sample might be due to the presence of these major components.

Literature have demonstrated that degradation of SNP can produce nitric oxide radical (NO) and other possible oxidizing species (Bates *et al*., 1990; Arnold *et al*., 1984). The NO is a potent short lived (30 s) radical and may damage neurons and glial cells in association with other ROS or RNS (Pryor and Squadrito, 1995). By closer inspection of the chemical structure of SNP, it is apparent that it may have a free iron (Fe) coordination site for a water molecule, which could trigger the generation of highly reactive oxygen species, such as OH● via the Fenton reaction (Graf *et al.,* 1985). It should also be noted that SNP molecules may help in some iron complexes forsmation i.e. pentacyanoferrate complex (Bates *et al.,* 1990; Arnold *et al.,* 1984), which could lead to oxidative reactions involved in ROS generation. The fenton reaction is one of the most studied mechanistic reactions of lipid per oxidation. In this study, we have demonstrated that SNP, a NO donor, can induce lipid peroxidation *in vitro*, in rat's tissue homogenates (fig. 1). The result presented in (figs.1) indicated that the *Curcuma Longa* extract exerted an antioxidant effect on *in vitro* SNP induced lipid peroxidation in rat's tissue homogenate. A plausible explanation for the SNP induced TBARS inhibition could be the direct interaction between *Curcuma Longa* extract with SNP or its derivatives.

Essentials oil and crude extracts of *Curcuma Longa* exhibited different degree of inhibition against the selected microbes i.e. *P. aeruginos, E. carotovora, A. tumefaciens, B. subtilis, B. atrophaeu, S. aureus, E. coli, K. pneumonias, S. typi* and *Candida albican* (table 1). The result of the present study are in accordance to *Rambir et al.,* 2002 which showed that *Curcuma longa* rhizome extracts showed antibacterial activity against *Sra phylococcus aureus*, *Staphylococcus epidermidis, Escherichia coli, Pseudomonas aeruginosa and Salmonnella typhimurium*. Similarly, Shagufta *et al.,* 2010 reported that among all the tested bacterial strains, *Bacillus subtilis* was the most susceptible to *Curcuma longa* extracts. *Curcuma longa* extracts and essential oil showed better activity against gram positive bacteria than gram negative bacteria which may be due to the structural differences in the cell wall and cell membrane of the tested microbes. Gram negative bacteria have outer membrane which makes them more resistant to extracts and oil. The ethyl acetate and ethanolic extracts of *Curcuma Longa* were more efficient in its antibacterial activity than the aqueous extract. The reason is that the antimicrobial component of the *Curcuma Longa* rhizome is more soluble in ethyl acetate and ethanol as compared to water. The ethanolic extracts demonstrated better results than the aqueous as being organic dissolves more organic compounds resulting in the release of greater amount of active antimicrobial components (Darout *et al.,* 2000). Turmeric shows antibacterial activity against *E. coli*, *B. subtilus* and *S. aureus* due to the presence of a phenolic compound i.e. curcuminoid (Chandrana *et al.,* 2005). The mechanism action for antimicrobial activity of spices involves the hydrophobic and hydrogen bonding of phenolic compounds to membrane proteins, membrane distraction, and annihilation of electron transport systems and cell wall disruption (Bodhav *et al.,* 2000). The anti-microbial potential of aqueous extracts could be attributed to anionic components such as thiocyanate, chlorides, nitrate and sulphates in addition to various other compounds present in plants (Darout *et al.,* 2000).

CONCLUSION

The present study demonstrated that *Curcuma Longa* may serve as supplement of macro and micro elements in the body. Furthermore the inhibition of lipid peroxidation and the strong antimicrobial activity of essential oil and crude extracts of *Curcuma Longa* suggest that it can be used as safe alternative to treat infectious disease. However, further studies are required to determine and characterize the exact chemical components responsible for its remarkable biological efficacy.

Table 1: Antimicrobial activity of *Curcuma Longa* essential oil and different extracts against pathogenic microbes

S #	Organisms	Zone of inhibition in mm			
		Aqueous	Ethyl Acetate	Ethanol	Essential Oil
A	Gram Positive Bacteria				
1	Bacillus Subtilis	14	29	23	12.5
2	Bacillus Atrophaeus	12.5	23.75	21	16
3	Staphylococcus Aureus	11	30.5	19	41
B	Gram Negative Bacteria				
1	Eshericha Coli	13	11.5	N.D	17.5
2	Klebsiella Pneumonia	12.5	15.5	N.D	22
3	Salmonella Typimurium	N.D	N.D	8	12
4	Pseudomonas Aeruginosa	14.5	18.5	13	29.5
5	ErwiniaCarotovora	12	16.5	12.5	14.5
6	Agrobacterium Tumefaciens	11.5	17.5	N.D	14
C	Fungal Strain				
1	Candida Albican	12.5	18	11.5	19

Table 2: Elemental analysis of *Curcuma Longa* by using AAS

S #	Analyte	Wavelength	Mean (mg/L)	Std. Dev	% RSD
1.	Mn	279.5	1.078	0.0193	1.79
2.	Cr	357.9	1.188	0.0341	2.87
3.	Fe	248.3	1.270	0.0144	1.13
4.	Ni	232.0	0.096	0.0584	61.12
5.	Zn	213.9	1.008	0.0100	0.99
6.	Cu	324.8	0.109	0.0231	21.15
7.	Ca	422.8	35.425	0.0031	0.22
8.	Mg	285.2	49.4	0.0511	2.59

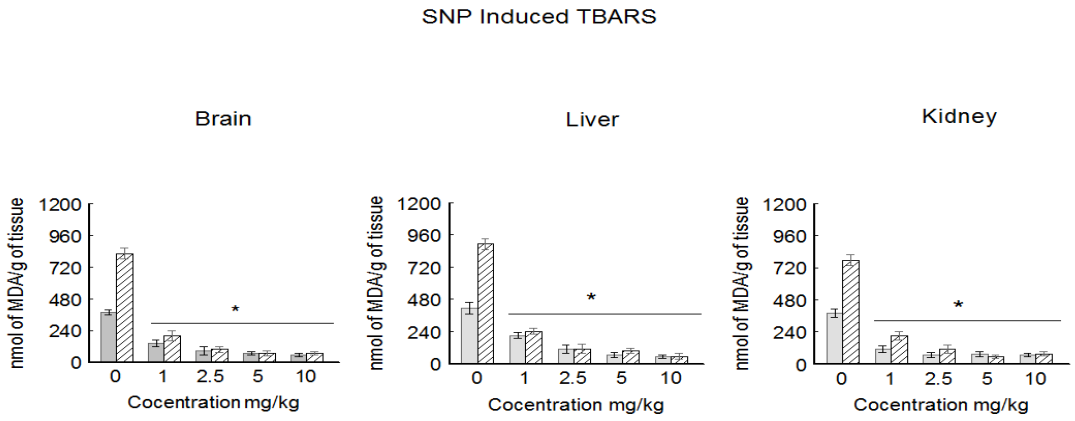
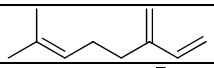
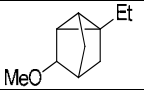
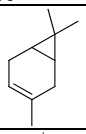
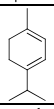
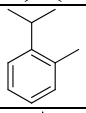
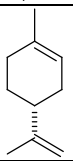
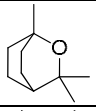
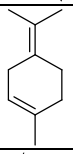
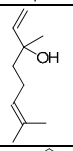
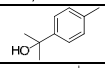
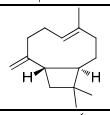
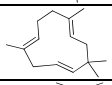
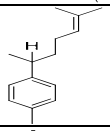
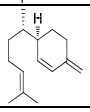
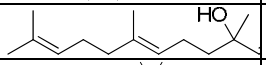
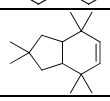


Fig. 1: Effect of ethanolic extract of *Curcuma longa* on basal (Shaded) or SNP-induced TBARS (Line Bars) production in rat's tissue homogenates. The values are expressed as nmol of MDA per gram of tissue. Data are expressed as means ± S.E.M. (n=5-7). Letter (a) shows main effect SNP while asteric/s shows main effect of *Curcuma Longa* extract at $p<0.05$.

Table 3: Chemical composition of *Curcuma Longa* essential oil

S #	Name	Structure	R. Time	Area	Conc. (%)
1.	Beta.-Myrcene		13.231	3433	0.25
2.	Tricyclo[2.2.1.0(2,6)heptane,1,3.3-trimethyl		14.106	12129	0.90
3.	3-Carene		14.215	5214	0.39
4.	Alpha.-Terpinen		14.772	10356	0.77
5.	o-Cymene		15.282	34407	2.55
6.	D-Limonene		15.543	4334	0.32
7.	Eucalyptol		15.728	20752	1.54
8.	p-Mentha-1,4(8)-diene		19.238	219605	16.29
9.	Beta.-Linalool		20.464	5883	0.44
10.	p-Cymen-8-ol		26.275	47729	3.54
11.	Caryophyllene		38.332	36704	2.72
12.	Alpha.-Caryophyllene		39.951	63583	4.72
13.	Alpha.-Curcumene		41.256	248595	18.44
14.	beta.-Sesquiphellandrene		43.092	521577	38.69
15.	Nerolidol		44.636	31276	2.32
16.	1H-Indene,2,3,3a,4,7,7a-hexahydro-2,2,4,4,7,7-hexamethyl		52.600	82343	6.11

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