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ORIGINAL ARTICLE

Activity guided characterization of antioxidant components from essential oil of Nutmeg (*Myristica fragrans*)

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Abstract Present study aimed at evaluating antioxidant and radioprotective ability of nutmeg essential oil and to isolate and identify major antioxidant components. Activity guided fractionation to identify major antioxidant compounds were carried out by dot blot tests on TLC. A new index termed as antioxidant activity values (AAV) for calculating contribution of individual compounds towards total activity is proposed. Activity guided fractionation resulted in identification of elemicin, 4-terpineol, myristicin and *trans*-sabinene hydrate as major antioxidant constituents of nutmeg essential oil. Results of DPPH assay demonstrated elemicin as most potent antioxidant compound. However, AAV suggested that 4-terpineol has greater contribution towards total antioxidant activity of oil due to its higher abundance. Essential oil is also demonstrated to possess radioprotective ability as evaluated by plasmid DNA protection assay for first time in this study.

Keywords Nutmeg essential oil · Antioxidant activity · Radioprotective ability · Antioxidant activity values · Activity guided fractionation

Introduction

In recent years there has been an increased awareness about the toxic and carcinogenic properties of synthetic additives, including antioxidants like butylated hydroxylanisole (BHA) and butylated hydroxyltoluene (BHT) (Singh et al. 2005) This has lead to greater efforts directed at isolating natural antioxidants of plant origin with low toxicity for application as food additives and preservatives (Chatterjee et al. 2007). Spices are rich in antioxidant and radioprotective activity due to the presence of compounds like flavonoids, terpenoids, lignans, sulfides, curcumins, carotenoids and polyphenolics. Some of these components display very potent antioxidant and radioprotective properties.

Oxidation of lipids is one of the basic processes causing rancidity in food products and resultant deterioration (Donelli and Robinson 1995). Products of lipid oxidation can alter organoleptic characteristics in the finished products, making them unacceptable to the consumer (Benzie 1996). Various plant essential oils including rosemary, lavender, eucalyptus, clove, oregano, and origanum are reported to exhibit strong antioxidant properties (Wei and Shibamoto 2007). Thus studies on antioxidant activity of essential oils and their role as food preservatives have currently gained considerable attention among researchers.

Active oxygen molecules, such as superoxide, hydroxyl and peroxyl radicals are implicated in oxidative stress related to the pathogenesis of various important degenerative diseases. In healthy individuals, production of free radicals is balanced by antioxidant defense mechanism. Oxidative stress is generated when balance is in favor of the free radicals as a result of depletion of antioxidant levels. Further, oxidative damage, caused by action of free radicals, may be a contributory factor in a number of chronic diseases such as cancer, inflammation, atherosclerosis and aging (Ames et al. 1993). Antioxidants can scavenge free radicals and prevent damage to tissues (Chatterjee et al. 2007). Essential oils have low density (around 0.94 g ml^{-1}) and can rapidly diffuse across cell membranes owing to their lipid solubility (Anthony et al. 2005). These properties along with their antioxidant potential can be very useful for protection of intracellular components from oxidative damage.

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Nutmeg (*Myristica fragrans*) is a spice widely used as a flavoring agent in food. It has stimulant, narcotic, carminative, astringent, aphrodisiac, hypolipidemic, antithrombotic, anti-platelet aggregation, antifungal, antidysentric and anti-inflammatory activities (Jukic, et al. 2006). Nutmeg essential oil was shown to have good radical scavenging activity (Piaru et al. 2012; Jukic et al. 2006; Tomaino et al. 2005). It was observed that the DPPH radical scavenging activity of nutmeg essential oil considerably increased after heating to 180 °C as a result of increase in myristicin and safrole contents. These compounds were proposed to be the major contributors to the antioxidant activity of nutmeg essential oil (Tomaino et al. 2005).

To the best of our knowledge the nature of the constituents that contribute to the antioxidant activity of nutmeg essential oil still remains elusive. The present study was thus directed towards activity guided isolation and identification of major constituents of the nutmeg essential oil. Property of natural antioxidants to scavenge free radicals renders them useful as radioprotective agents reducing DNA damage induced by UV and ionizing radiation (Kumar et al. 2001). Radiation treatment can produce a variety of lesions in DNA resulting in both single and double strand breaks, alteration of bases, destruction of sugar moiety and cross-linking and formation of dimers (Kada et al. 1985). No report, however, exists on the role of essential oils on reducing damage induced by ionizing radiation such as γ-radiation. Thus the potential of nutmeg essential oil as a radio protector for γ-radiation induced DNA damage was also assessed. Numerous reports exist in literature wherein activity guided isolation and identification of major active components of plant extracts has been reported. Essential oils contain a wide variety of compounds of varying structure and composition. Many of them possess considerable antioxidant properties. However these compounds are present in different relative concentrations in the oil. A compound having lower antioxidant activity but present in large amount may contribute substantially towards total activity of the essential oil. This results in difficulties in assessing the contribution of each to the total activity of the essential oil. No report however exists in literature on procedures to ascertain the contribution of each component towards total activity of the essential oil. Use of antioxidant activity value (AAV) as an index to calculate the contribution of individual compounds to the total activity of oil is proposed.

Materials and methods

Materials

Nutmeg (*Myristica fragrans*) was procured from local market. It was packed and sealed in LLDPE pouches and was stored away from light under dry conditions until use. All solvents used in the present work were of analytical reagent grade procured from EMerck India Ltd. (Mumbai, India) and were redistilled before use. Myristicin, linalool, geranyl acetate, DPPH, β-carotene and linoleic acid were purchased from Aldrich Chemical Company (WI, USA). α-terpineol, BHT, deoxyribose, TBA, TCA, ascorbic acid and Tween-20 were procured from Sigma Chemical Company (St. Louis, MO, USA) while $FeCl_3$ and silica gel-60 were purchased from Merck (Darmsted, Germany). Plasmid DNA pBR-322 was procured from Bangalore Genei (Bangalore, India).

Extraction of essential oil

The nutmeg essential oil was extracted as per procedure described earlier (Nickerson and Likens 1966). In brief, 200 g of nutmeg seeds were crushed and then extracted for 2 h by simultaneous distillation extraction in Nickerson's type apparatus. Diethyl ether was used as extracting solvent. Extract was dried over sodium sulfate and ether was removed by gentle stream of nitrogen. Essential oil so obtained was stored in airtight containers at −20 °C till further use.

Gas chromatography/mass spectrometry analysis

GC/MS analysis was carried out on Shimadzu GC-MS system (GC-17A) with a QP5050A Mass Spectrometer. DB-5 capillary column (30 m×0.25 mm) with film thickness of 0.25 μm was used. The oven was programmed from 60 to 200 °C with a rate of 4 °C/min followed by rate of 10 °C/min to 280 °C. Column was finally held at 280 °C for 10 min. Split/splitless injector was used for all injections. Injector and interface temperature were set at 210 °C and 280 °C, respectively. The ionization voltage was 70 eV, and the scanning mass range was m/z 40–800. Helium was used as carrier gas at a column flow rate of 1 ml/min. Identification of the compounds was based on the comparison of their relative retention time (Kovat's Index) and mass spectra with that of Wiley and NIST library data of GC/MS system and available literature data.

DPPH radical scavenging assay

Nutmeg essential oil was analyzed for hydrogen donation or radical scavenging ability using stable DPPH radical scavenging assay as previously reported (Chatterjee et al. 2007). The initial concentration of DPPH radical was kept 60 μM in all the antioxidant radical reactions. A 0.1 ml of methanolic solution of essential oil at different concentrations ranging from 5 to 50 mg ml^{-1} was placed in test tube and 1.5 ml of DPPH methanolic solution (60 μM) was added. Methanol was used in place of essential oil solution for control. After 1 h of incubation in dark, absorbance was

taken at 517 nm. The percent of DPPH discoloration of the sample was calculated according to the formula:

$$\%\ \text{age discoloration} = (1-\text{Abs sample}/\text{Abs control}) \times 100$$

IC50, which is the amount of sample required to decrease the absorbance of DPPH by 50 % was calculated based on regression equation of percent DPPH scavenging vs. amount of essential oil. The free radical scavenging activity of each sample was expressed as trolox equivalent antioxidant capacity (TEAC). TEAC is defined as the μM of trolox having equivalent antioxidant capacity as that of 1 g of the essential oil.

Activity guided fractionation of essential oil

For screening of active compounds for radical scavenging activity in nutmeg essential oil, the dot-blot test on thin layer chromatography (TLC) using silica gel aluminum plates was carried out (Mimica-Durik et al. 2007). Pure essential oil (10 μL) was spotted on silica gel plate and TLC was developed using hexane: ethyl acetate (85:15) as solvent system. After drying, plates were sprayed with 160 μM methanolic solution of DPPH. Sprayed plates gave a purple background with bright yellow spots at the location of high radical scavenging active compounds. For identification of these compounds preparative TLC was carried out using same solvent system and compounds with corresponding Rf values were eluted with ether. Regions showing DPPH scavenging activity were further purified into individual components by preparative TLC with hexane: di-ethyl ether (85:15) as developing solvent system. All resulting TLC fractions are analyzed by GC/MS (GC-5050A Shimadzu, Japan).

Antioxidant activity by β-carotene bleaching assay

Antioxidant activity of nutmeg essential oil was evaluated by β-carotene-linoleate model (Chatterjee et al. 2007). For preparation of emulsion following reagents were mixed: 400 μL of 1 mg ml $^{-1}$ β-carotene in chloroform, 40 μL of linoleic acid and 400 μL Tween-20. Chloroform was then evaporated under vacuum using a rotary evaporator and the volume was made to 100 ml with distilled water. Mixture was vigorously shaken and saturated with oxygen. Aliquot (5 ml) of oxygenated β-carotene and 100 μL of test compound (essential oil in concentrations from 5 mg ml^{-1} to 50 mg ml^{-1} in methanol) was placed in stoppered test tubes and mixed well. The reaction mixture was incubated in a water bath at 50 °C for 1 h. 100 μL of 0.1 mg ml^{-1} and 1 mg ml^{-1} of BHT was used as positive control while methanol was used as negative control. Antioxidant activity of essential oil was calculated in terms of β-carotene bleaching using the following formula:

$$\text{Antioxidant activity} = (1-(A_0-At)/(A_00-A_0t)) \times 100$$

Where, A_0 and A_00 are the O.D. measured at the zero time of the incubation for test sample and control, respectively and At and A_0t are the O.D. values measured in the test sample and control, respectively after incubation for 1 h. Antioxidant activity was expressed as BHT equivalent antioxidant capacity.

Radioprotective ability of nutmeg essential oil

To evaluate the radioprotective ability of nutmeg essential oil, existing methodology (Nair and Salvi 2008) was suitably modified. 10 μL plasmid pBR322 DNA (200 ng) in potassium phosphate buffer (50 mM, pH 7.4) was mixed with 10 μL of different concentrations (0.05 mg ml^{-1} to 0.5 mg ml^{-1}) of nutmeg essential oil emulsion made in potassium phosphate buffer (50 mM, pH 7.4) with 0.004 % Tween 20. Test mixture was irradiated to a dose of 15 Gy in gamma chamber GC-220 (AECL, Ontario, Canada) having a dose rate of 7 Gy min^{-1}. After irradiation, electrophoresis was carried out in 1 g 100 ml^{-1} agarose gel. DNA bands were analyzed in a gel documentation system (Gene Genius, Syngene, United States) after staining with ethidium bromide. Closed circular and open form of plasmid was quantified by Gene Snap software (Syngene, United states). Since, Tween 20 was used for emulsifying nutmeg essential oil in buffer, different concentrations of Tween 20 (0.002 % to 0.02 %) were also tested for any potential DNA protection activity. Lowest concentration of Tween 20 which does not show any DNA protection ability was found to be 0.002 % (See Supporting data, Fig. S2).

Radioprotective activity of essential oil was calculated in terms of plasmid protection using the formula:

$$\text{Radioprotective activity} = (\%\ \text{closed form}/\%\ \text{closed form in control}) \times 100$$

Results are reported as Trolox equivalent antioxidant capacity (TEAC).

Hydroxyl radical scavenging activity

Assay for hydroxyl radical scavenging activity is normally carried out in aqueous medium. To dissolve the essential oil, 0.04 % of Tween-20 was used. Nutmeg essential oil was tested for hydroxyl radical scavenging activity in concentrations of 0.5 mg ml^{-1} to 2 mg ml^{-1}. Procedure followed for hydroxyl radical scavenging activity was essentially as

Table 1 Regression statistics for various antioxidant assays of nutmeg essential oil

Assays	R^2 ± S.E	Slope ± S.E	Intercept ± S.E	F (F_{Crit})
DPPH	0.9997±0.437	21.223±0.256	44.77±0.219	6840.502(.000146)
Beta carotene bleaching	0.988±2.495	107.28±11.553	24.51362±3.056	86.22454(0.0682)
Radioprotection	0.963±2.683	14.231±1.376312	17.089±2.088	106.928(0.000494)
Hydroxyl radical scavenging	0.924±3.012	26.758±5.389	16.275±3.690	24.648(0.0382)

described earlier (Murcia et al. 2004) The final reaction mixture volume of 1 ml was made using the following reagents: 100 μL of 20 mM deoxyribose in 10 mM potassium phosphate buffer (pH 7.4), 100 μL of 20 mM H_2O_2, 200 μL of 1:1 mixture of 20 mM $FeCl_3$ and 2 mM ethylenediaminetetraacetic acid (EDTA), 500 μL nutmeg essential oil (0.5 mg $ml^{-1}$to 2 mg ml^{-1}) in 0.04 % of Tween-20. 100 μL of 2 mM ascorbic acid was used to start the reaction. Distilled water (500 μL) acts as a negative control. Radical scavenging activity of 0.04 % Tween-20 was also assessed. The tubes were incubated at 37 °C for 1 h. After 1 h, to measure the products of hydroxyl radical attack on deoxyribose following reagents were added: 1 ml of 2 g 100 ml^{-1} trichloroaceticacid (TCA) and 1 ml of 500 mg 100 ml^{-1} thiobarbituricacid (TBA). Reaction mixture was then incubated in boiling water bath for 20 min. Chromogen formed was measured at 532 nm. The percent inhibition of hydroxyl radical was calculated according to formula:

$$\%\ \text{inhibition} = (1-A_t/A_0) \times 100$$

Where A_0 is the absorbance of control reaction (containing all reagents except test compound) and A_t is the absorbance of test compound. The results were expressed as Trolox equivalent antioxidant capacity (TEAC).

Calculation of antioxidant activity values (AAV)

For calculation of AAV values of different fractions or compounds their TEAC value or BHT equivalents were multiplied by their relative abundance in nutmeg essential oil.

$$\text{AAV} = \text{Relative abundance of compound in extract}(\%) \times \text{TEAC value or BHT equivalent}\left(\mu\text{M g}^{-1}\right) \quad (1)$$

Statistical analysis

Data collected are average of three independent determinations. All statistical calculations were performed using Origin software. One way Analysis of Variance (ANOVA) was carried out and multiple comparisons of means were done by Tukey's test. Significance level was ($p<0.05$).

Results and discussion

Yield of nutmeg essential oil obtained was 12.5 % (w/w) in present study. Thirty one components were identified by GC/MS analysis of nutmeg essential oil (see supplementary information Fig. S1, Table S1). Monoterpene hydrocarbons accounted for 90.07 % of the oil followed by oxygenated terpenes (7.39 %) and phenolic ethers (1.67 %). The composition of the oil was similar to that reported in literature (Jukic et al. 2006). DPPH and β-carotene/linoleic acid assays are widely accepted methods for antioxidant assays, however, the methodology lack in uniformity in terms of concentration of DPPH and incubation time making it difficult to compare the antioxidant strength of plant extracts. Thus, in the present study all results are represented as

Table 2 Antioxidant activities of Nutmeg essential oil and various fractions

Sample	DPPH (Trolox equivalents as μM g^{-1})	β-carotene/linoleic acid (Trolox equivalents as μM g^{-1})
Nutmeg essential oil	2.94±0.09	25.11±1.50
TLC band-I (Rf=0.17)	3.29±0.05	1.51±0.12
TLC band-II (Rf=0.26)	ND	2.33±0.15
TLC band-III (Rf=0.41)	18.19±0.5	2.89±0.01
TLC band-IV (Rf=0.67)	3.82±0.1	1.54±0.02
TLC band-V (Rf=0.81)	ND	0.81±0.17

Values are expressed as mean ± SD (n=3)

ND not detected

equivalents of standard antioxidants (Trolox or BHT). One way ANOVA analysis revealed that nutmeg essential oil demonstrated significant ($p<0.05$) antioxidant activity in all test systems. Data was fitted into linear regression models and R^2 values along with regression statistics is shown in Table 1.

Antioxidant activity and activity guided fractionation

The radical scavenging activity of nutmeg essential oil is shown in Table 2**.** TEAC for nutmeg essential oil was found to be 2.94±0.09 μM g^{-1}. A similar DPPH radical scavenging activity of nutmeg essential oil has been reported (Pillai et al. 2012; Piaru et al. 2012; Jukic et al. 2006; Tomaino et al. 2005). The active components of nutmeg essential oil were identified by dot-blot tests on TLC. TLC chromatogram of nutmeg essential oil showed five bands when stained in iodine (Rf=0.17, 0.26, 0.41, 0.67 and 0.81). However, after spraying with 160 μM DPPH four major bright yellow bands appeared (Rf=0.17, 0.41, and 0.67) (Fig. 1). Two bands in TLC chromatogram (Rf=0.26 and 0.81) did not show any yellow spots. To identify the nature of the compounds present in all five bands, preparative TLC of oil was carried out and bands corresponding to Rf 0.17, 0.26, 0.41, 0.67 and 0.81 were eluted in ether and compounds present in individual bands were further identified by GC/MS. Components identified in all five bands with their relative abundance are shown in Table 3. Major compounds identified in DPPH active bands were phenolic ethers (myristicin and elemicin), monoterpene alcohols (4-terpineol and linalool) and oxygenated monoterpenes (geranyl acetate, *trans*-sabinene hydrate).

Three bands showing DPPH scavenging activity were tested individually to ascertain their radical scavenging ability and results are shown in Table 2. Band-III (Rf 0.41) showed maximum antioxidant activity with TEAC value 18.19±0.5 μM g^{-1} followed by band-IV (Rf 0.67) and band-I (Rf 0.17) with TEAC of 3.82±0.1 and 3.29±0.05 μM g^{-1} respectively. Band-III was further subjected to TLC with hexane: diethyl ether (85:15) as developing solvent system followed by spray of 160 μM DPPH solution. Among the six constituents identified only two compounds were found to be active at Rf 0.25 and 0.3. These two compounds were identified as elemicin and 4-terpineol, respectively. Major compounds present in DPPH active regions were further purified with preparative TLC and their individual TEAC values were estimated. Comparison of TEAC values of all major compounds present in DPPH active regions showed (Table 4) that elemicin had maximum activity of (11.78±0.36 μM g^{-1}) followed by myristicin, 4-terpineol and *trans*-sabinene hydrate with values 3.24±0.14, 1.48±0.10 and 3.29±0.05 μM g^{-1} respectively. In a previous report on DPPH scavenging activity of nutmeg essential oil, myristicin and safrole were implicated as major antioxidant compounds (Tomaino et al. 2005). Interestingly, in the present study it was found that elemicin displayed a much higher DPPH radical scavenging potential with a greater TEAC value than myristicin (Table 4). Surprisingly, safrole was not detected in any of the DPPH active bands contradicting earlier report implicating safrole as possible antioxidant compound of nutmeg essential oil (Tomaino et al. 2005). Our results are however in accordance with that of Guerrini et al. (2009) who demonstrated that *Piper obliquum* essential oil containing safrole as major component (45.4 %) had poor DPPH radical scavenging activity. 4-Terpineol and *trans*-sabinene hydrate were the other two active compounds identified. DPPH radical

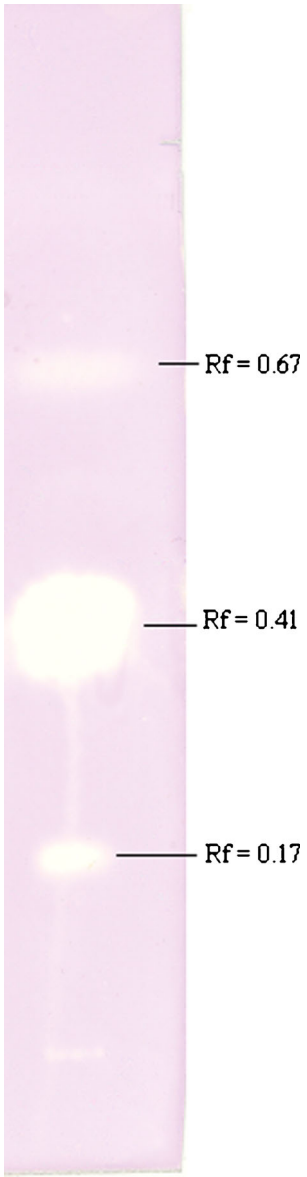

Fig. 1 TLC chromatogram of Nutmeg essential oil sprayed with 160 μM DPPH. Yellow spots against violet background show radical scavenging regions.

Table 3 Components in different fractions of nutmeg essential oil and their relative abundance in each fraction

Compounds in different TLC fractions of Nutmeg essential oil	KI (calculated)	KI Literature	Relative percentage
TLC band-I (Rf=0.17)			
Trans-sabinene hydrate	1090	1097	100±0.00
TLC band-II (Rf=0.26)			
Cis-sabinene hydrate	1062	1068	21.6±0.54
Trans-sabinene hydrate	1090	1097	12.8±0.44
Menth-2-en-1-ol (Cis-Para)	1115	1121	10.2±0.23
Menth-2-en-1-ol (Trans-Para)	1130	1140	16.2±0.98
4-Terpineol	1173	1177	1.9±0.08
Cymen-8-ol	1174	1180	2.2±0.06
α-Terpineol	1183	1189	31.1±1.98
Cis-piperitol	1196	1193	1.7±0.18
TLC band-III (Rf=0.41)			
Cis-sabinene hydrate	1062	1068	4.0±0.23
Linalool	1087	1098	13.2±0.99
Menth-2-en-1-ol (Cis-Para)	1115	1121	12.2±0.72
4-Terpineol	1173	1177	35.8±1.43
α-Terpineol	1183	1189	6.5±0.58
Cis-piperitol	1196	1193	4.1±0.64
Elemicin	1547	1554	24.1±1.67
TLC band-IV (Rf=0.67)			
1,8 cineole	1016	1033	11.5±0.92
Borynl acetate	1271	1285	1.8±0.65
Citronellyl acetate	1337	1354	1.5 ±0.40
Geranyl acetate	1367	1383	9.1±0.96
Myristicin	1515	1520	72.7±3.32
TLC band-V (Rf=0.81)			
Sabinene	973	976	7.5±1.34
Myrcene	981	991	5.8±0.85
α-Terpinene	1003	1012	2.3±0.04
p-Cymene	1011	1026	3.1±0.45
Limonene	1019	1031	3.9±0.32
β-Phellandrene	1020	1031	1.2±0.06
γ-Terpinene	1048	1062	8.2±1.20
Cis-sabinene hydrate	1062	1068	3.5±0.67
Terpinolene	1073	1088	5.0±0.08
4-Terpineol	1173	1177	1.7±0.22
Safrole	1276	1285	21.1±3.90
α-Cubenene	1328	1351	2.5±0.35
α-Copaene	1361	1376	6.3±0.24
Trans-caryophyllene	1405	1430	12.2±2.11
Trans-α-Burgamotene	1416	1436	2.1±0.70
α-Humulene	1440	1454	2.2±0.08
Germacrene D	1466	1480	2.4±0.32
β-Bisabolone	1492	1509	2.2±0.06
δ-Cadinene	1502	1524	2.2±0.43

Values are expressed as mean± SD (n=3)

KI Kovat's Index

scavenging activity of 4-terpineol has been reported earlier (Burtis and Bucar 2000). To best of our knowledge there are no reports on radical scavenging properties of *trans*-sabinene hydrate. It is interesting to note that despite a high activity of

elemicin and 4-terpineol neither had activities comparable to Band-III (Table 4). A synergistic action of various components is thus suggested (Kelen and Tepe 2008). Other compounds (1,8-cineole, geranyl acetate, linalool and α-terpineol) identified in DPPH active bands did not showed any significant radical scavenging activity. Kelen and Tepe (2008) showed that 1,8-cineole had no radical scavenging activity while a low activity of geranyl acetate, linalool and α-terpineol was reported by Choi et al. (2000). Our results are thus in accordance with the reported literature data.

To judge the contribution of various TLC bands and individual compounds towards total activity AAV values were calculated (Fig. 2). As can be seen from figure TLC band-III (Rf=0.41) having TEAC value of 18.19 μM g^{-1} with relative abundance of 6.29 % showed maximum AAV value of 114 while Band-I (Rf=0.17) and Band-IV (Rf=0.67) showed AAV values of 0.53 and 3.55 respectively. Thus it can be concluded that Band-III containing elemicin and 4-terpineol as major active compounds mainly contributed towards total activity of the oil. AAV values of individual pure compounds were also calculated. Elemicin showed AAV value of 3.89 while myristicin and 4-terpineol showed AAV values of 2.85 and 6.65, respectively. None of the compounds showed AAV values comparable to Band-III. These results strongly indicate synergistic action of various compounds in total activity of extract. Despite the fact that elemicin had higher TEAC values, according to AAV values 4-terpineol contributed more to total activity of oil due to its higher relative abundance in nutmeg oil i.e. 4.49 % in comparison to elemicin (0.33 %). Thus, it can be concluded that elemicin, myristicin and 4-terpineol are major antioxidant compounds present in nutmeg essential oil.

β-Carotene bleaching assay

Nutmeg essential oil and all the five fractions obtained from TLC after screening with DPPH were individually tested for potential lipid peroxidation inhibitory activity. Antioxidant capacity was determined as BHT equivalents for oil and all fractions. Results are presented in Table 2. Nutmeg essential oil showed maximum lipid peroxidation inhibition activity

Table 4 Antioxidant activities of individual components in DPPH test system

Compound	DPPH (Trolox equivalents as μM g^{-1})
Elemicin	11.78±0.36
4-terpineol	1.48±0.10
Myristicin	3.24±0.14

Activity for Geranyl acetate, 1,8- cineole, Linalool and α-terpineol was not detected

Values expressed as mean±SD (n=3)

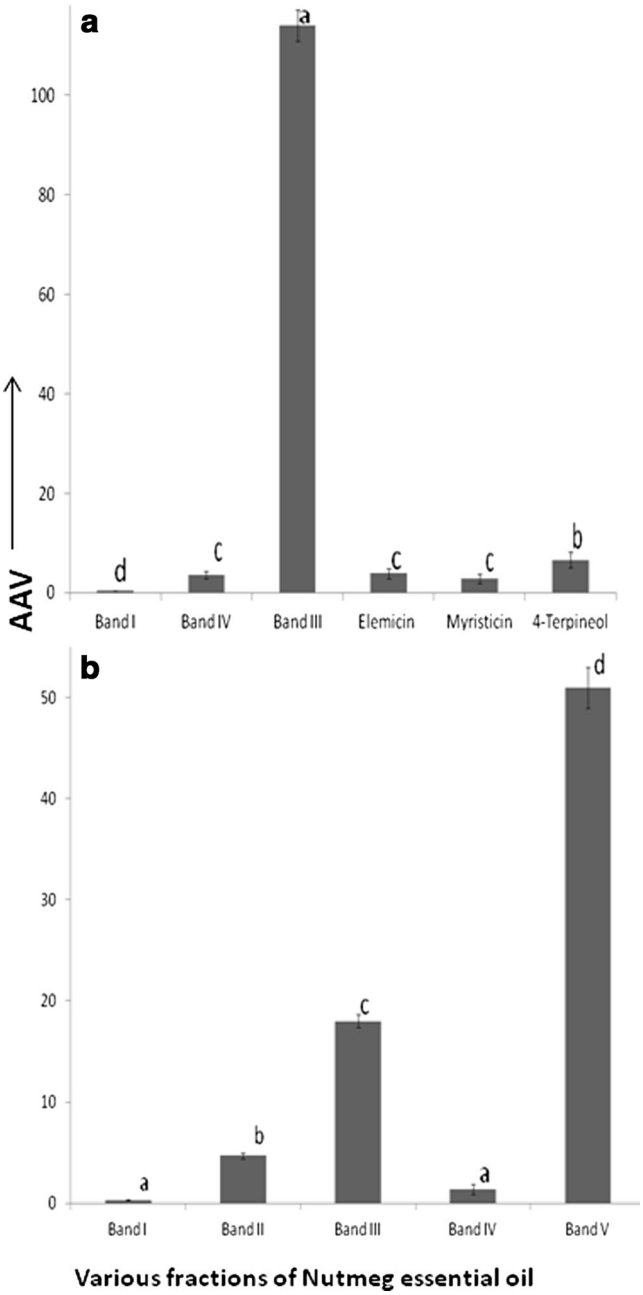

Fig. 2 Antioxidant activity value (AAV) and for various fractions and compounds of nutmeg essential oil in (**a**) DPPH radical scavenging assay (n=3) and (**b**) beta carotene bleaching assay (n=3). Different letters in superscript signifies that values are statistically significantly different (p<0.05)

with BHT equivalent of 25.11±1.50 μM g^{-1} while among the fractions tested band-III (Rf=0.41) showed maximum activity with BHT equivalent of 2.89±0.01 μM g^{-1}. Surprisingly, none of the fractions tested showed antioxidant capacity comparable to that of oil. BHT equivalent of nutmeg essential oil is approximately ten times more than all fractions tested. In earlier reports on antioxidant activity of essential oils, hydrocarbons have been suggested to be responsible for lipid

peroxidation inhibition in linoleic acid emulsion system (Singh et al. 2005). In the present study, however fraction rich in hydrocarbons (band-V Rf 0.81) showed very low activity as compared to oil with a BHT equivalent of 0.81±0.17 μM g^{-1} only. These findings strongly indicated that the high activity of nutmeg essential oil could be due to synergistic or additive effect of several different components. AAV values were further calculated for all fractions in order to estimate their contribution in total activity of oil (Fig. 2b). Band-V (Rf=0.81) showed highest AAV value of 51 while Band-III showed an AAV value of 18. Rest of the bands did not have significant AAV values. Despite the fact that band-III had higher BHT equivalents when compared to band-V it can be concluded that maximum contribution towards lipid peroxidation inhibition activity of nutmeg essential oil is contributed by band-V. Hydrocarbons showed very less activity in terms of BHT equivalents but due to their presence in large amounts in nutmeg oil, the contribution towards total activity was maximum.

Radioprotective potential of nutmeg essential oil

Exposure to gamma radiation leads to DNA strand breaks which result in conversion of plasmid DNA from supercoiled closed circular (SC) form to open circle (OC) form. Naturally occurring antioxidants have been demonstrated to provide protection against low-dose radiation including therapeutic benefits when administered post irradiation. Aqueous extract of spices have previously been shown to protect plasmid DNA from radiolytic damage (Sharma et al. 2000). Figure 3 shows the effect of different concentrations of essential oil on gamma radiation induced damage in plasmid pBR322 DNA at 15 Gy. It was seen that all SC form of plasmid got converted to OC form at dose of 15 Gy (Fig. 3, Lane 9). Further, Tween 20 at 0.002 % did not show any significant radioprotection (Fig. 3, Lane 8). Percentage of SC form remaining after irradiation treatment with different concentration of essential oil is shown in Fig. 4a. SC form of plasmid DNA showed a concentration dependent increase and at 2.3 mg ml^{-1} of nutmeg essential oil, 50 % of SC form was observed. TEAC value for nutmeg essential oil was found to be 100 μM g^{-1}. TEAC value in plasmid protection assay was found to be much better when compared to TEAC values of oil in DPPH assay. Further studies will be important in establishing potential of essential oils as intracellular reactive oxygen species (ROS) scavengers or DNA protectors during radiation dose. Radioprotective potential of lipophilic compounds is difficult to determine due to their low water solubility as assay is normally carried out in aqueous solution. Thus radioprotective action of essential oils is not generally reported. Modified method currently employed by us aided in readily assessing such activities. This is the first report on radioprotective activity of an essential oil.

Hydroxyl radical scavenging activity

Among all oxygen radicals, hydroxyl radical ($OH^{\cdot}$) is most reactive and is implicated in damage of various biomolecules. Radical scavenging activity of nutmeg essential oil for hydroxyl radical has not been investigated yet. In present study, nutmeg essential oil showed high hydroxyl radical scavenging activity that enhances with concentration (Fig. 4b). Oil showed antioxidant activity even at a low concentration of 0.25 mg ml^{-1}. TEAC values for this test system was found to be 308.8±14.08 μM g^{-1}. Among all the test systems, nutmeg essential oil gave best TEAC value for hydroxyl radical scavenging assay. Compounds identified in present study and having DPPH radical scavenging like elemicin, 4-terpineol, myristicin and *trans*-sabinene hydrate may be responsible for OH radical scavenging potential of nutmeg essential oil. High hydroxyl radical scavenging activity of oil could explain the radio protective effects of oil.

Conclusion

Nutmeg essential oil tested here showed potent DPPH radical scavenging activity and prevented linoleic acid

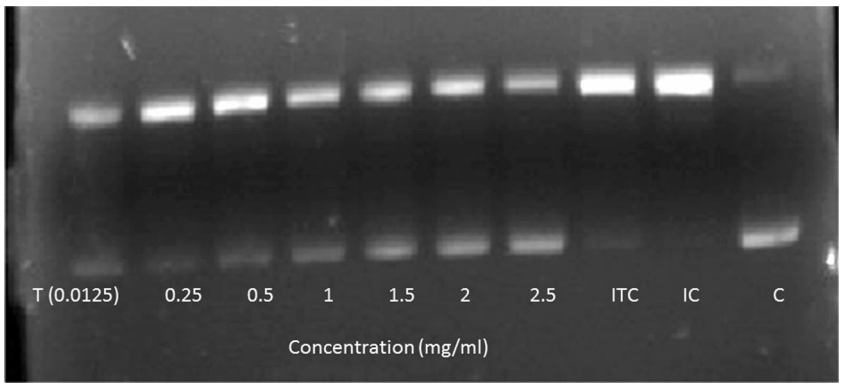

Fig. 3 Radioprotective effect of nutmeg essential oil on plasmid pBR322 DNA. (T – Trolox, ITC- Irradiated Tween Control, IC- Irradiated Control, C- control)

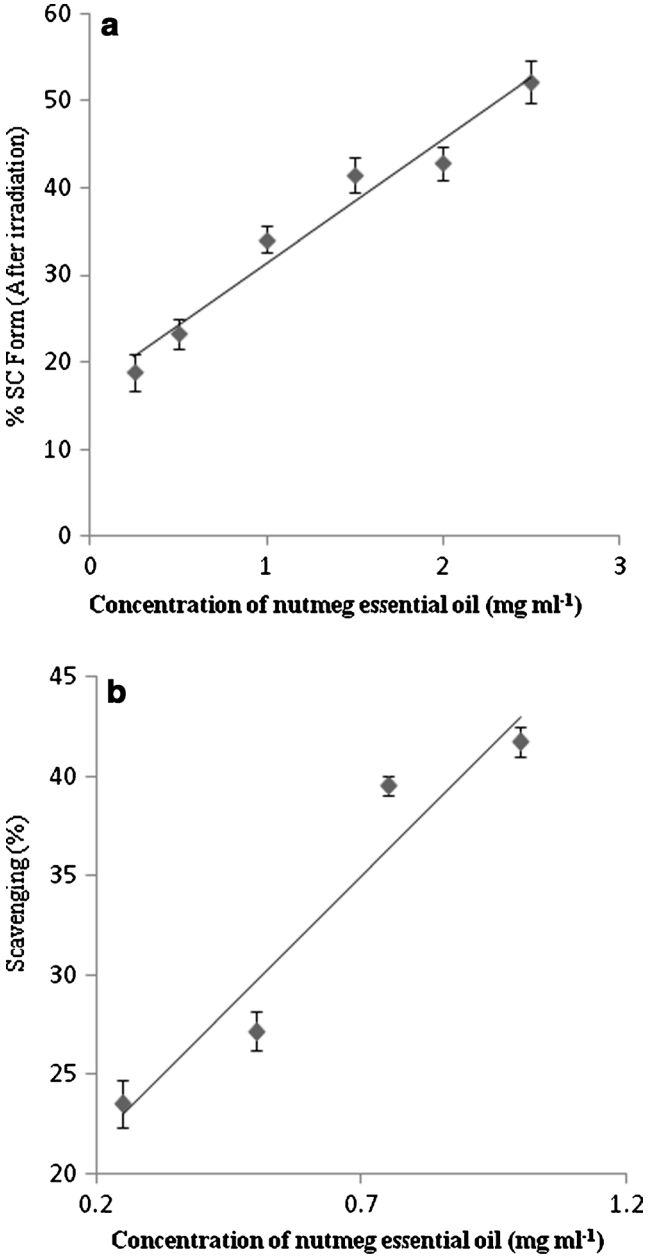

Fig. 4 Radioprotective effect and Hydroxyl radical scavenging activity of nutmeg essential oil. **a** Concentration of nutmeg essential oil vs. percent supercoiled (SC) form of plasmid pBR322 DNA after irradiation dose of 15 Gy (n=3) and (**b**) Hydroxyl radical scavenging activity of nutmeg essential oil (n=3)

oxidation. TLC screening of antioxidant compounds lead to identification of elemicin, 4-terpineol, myristicin and *trans*-sabinene hydrate as major antioxidant components of essential oil. AAV values proposed in this study reaffirm the role of synergistic actions among various components towards total activity of oil. Interestingly, none of the fractions tested gave results comparable to oil in linoleic acid test system. Nutmeg essential oil has potential for further use as an antioxidant in lipid rich food products. Since nutmeg essential oil has been shown to have many components with strong antioxidant activity they have potential of being tested as possible drugs for diseases resulting from oxidative stress. Potential radioprotective effects of essential oil on plasmid DNA is demonstrated here for first time. Owing to their low density and lipid solubility essential oils can easily penetrate cell membranes. In this regard use of essential oils as DNA protective agents against unwanted radiation exposure holds promise.

References

Ames BN, Shigrnaga MK, Hagen TM (1993) Oxidants, antioxidants, and the degenerative diseases of aging. Proc Natl Acad Sci 90: 7915–7922

Anthony J, Fyfe L, Smith H (2005) Plant active components- a resource for antiparasitic agents? Trends Parasitol 21:462–468

Benzie IFF (1996) Lipid peroxidation: a review of causes, consequences, measurement and dietary influences. Int J Food Sci Nutr 47:233–261

Burtis M, Bucar F (2000) Antioxidant activity of *Nigella sativa* essential oil. Phytother Res 14:323–328

Chatterjee S, Niaz Z, Gautam S, Adhikari S, Variyar PS, Sharma A (2007) Antioxidant activity of some phenolic constituents from green pepper (*Piper nigrum* L.) and fresh nutmeg mace (*Myristica fragrans*). Food Chem 101:515–523

Choi HS, Ukeda H, Sawamura M (2000) Radical scavenging activities of citrus essential oils and their components: detection using 1,1-Diphenyl-2-picrylhydrazyl. J Agric Food Chem 48:4156–4161

Donelli JK, Robinson DS (1995) Free radicals in foods. Free Radic Res 22:147–176

Guerrini A, Sacchetti G, Rossi D, Paganetto G, Muzzoli M, Andreotti E, Tognoline M, Maldonado ME, Bruni R (2009) Bioactivities of *Piper aduncum* L. and *Piper obliquum* Ruiz and Pavon (Piperaceae) from eastern ecuador. Environ Toxicol Pharmacol 47:39–48

Jukic M, Politeo O, Milo M (2006) Chemical composition and antioxidant effect of free volatile aglycone from Nutmeg (*Myristica fragrans* Houtt.) compared to its essential oil. Croat Chem Acta 79:209–214

Kada T, Kaneko K, Matsuzaki S, Hara Y (1985) Detection and chemical identification of natural bioantimutagens. A case of green tea factor. Mutat Res 150:127–132

Kelen M, Tepe B (2008) Chemical composition, antioxidant and antimicrobial properties of the essential oils of three *Salvia* species from Turkish flora. Bioresour Technol 99:4096–4104

Kumar SS, Devasagayam B, Jayashree B, Kesavan PC (2001) Mechanism of protection against radiation-induced DNA damage in plasmid pBR322 by caffeine. Int J Radiat Biol 77:617–623

Mimica-Durik N, Samojlik I, Jovin E, Bozin B (2007) Antimicrobial and antioxidant properties of rosemary and sage (*Rosmarinus officinalis* L. and *Salvia officinalis* L., Lamiaceae) essential oils. J Agric Food Chem 55:7879–7885

Murcia MA, Egea I, Romojaro F, Parras P, Jimeanez AM, Martinez-Tomea M (2004) Antioxidant evaluation in desert spices compared with common food additives. Influence of irradiation procedure. J Agric Food Chem 52:1872–1881

Nair CKK, Salvi VK (2008) Protection of DNA from gamma radiation induced strand breaks by epicatechin. Mutat Res 58:48–54

Nickerson GB, Likens ST (1966) Gas chromatographic evidence for the occurrence of hop oil components in beer. J Chromatogr 21:1–5

Piaru SP, Mahmud R, Majid AMSA, Ismail S, NinMan C (2012) Chemical composition, antioxidant and cytotoxicity activities of the essential oils of *Myristica fragrans* and *Morinda citrifolia*. J Sci Food Agric 92:593–597

Pillai S, Majid AMSA, Nassar ZD (2012) Antioxidant and antiangiogenic activities of the essential oils of *Myristica fragrans* and *Morinda citrifolia*. Asian Pac J Trop Med 5:294–298

Sharma A, Gautam S, Jadhav SS (2000) Spice extracts as dose-modifying factors in radiation inactivation of bacteria. J Agric Food Chem 48:1340–1344

Singh G, Marimuthu P, Heljjani CS, Catalan C (2005) Antimicrobial and antioxidant potential of essential oil and acetone extract of *Myristica fragrans* Houtt. (Aril part). J Food Sci 70:M141–M148

Tomaino A, Cimino F, Zimbalatti V, Venuti V, Sulfaro V, De Pastquale A, Saija A (2005) Influence of heating on antioxidant activity and the chemical composition of some spice essential oils. Food Chem 89:549–554

Wei A, Shibamoto T (2007) Antioxidant activities and volatile constituents of various essential oils. J Agric Food Chem 55:1737–1742