Food and Chemical Toxicology 106 (2017) 175–184

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Chemically characterized *Mentha cardiaca* L. essential oil as plant based preservative in view of efficacy against biodeteriorating fungi of dry fruits, aflatoxin secretion, lipid peroxidation and safety profile assessment

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ARTICLE INFO

Article history:
Received 4 April 2017
Received in revised form
18 May 2017
Accepted 21 May 2017
Available online 24 May 2017

Keywords:
Mentha cardiaca
Essential oil
Antifungal
Antiaflatoxigenic
Antioxidant
Preservative

ABSTRACT

The study reports *Mentha cardiaca* essential oil (EO) as plant based preservative against fungal and aflatoxin contamination of stored dry fruits. Mycoflora analysis of the dry fruits revealed Aspergillus favus LHP-PV-1 as the most aflatoxigenic isolate with highest Aflatoxin B_1 content. *M. cardiaca* EO showed broad fungitoxic spectrum inhibiting the tested moulds contaminating dry fruits. It's minimum inhibitory concentration (MIC), minimum aflatoxin inhibitory concentration (MAIC) and minimum fungicidal concentration (MFC) against *A. favus* LHP-PV-1 were recorded to be 1.25, 1.0 and 2.25 μL/mL respectively. The EO caused decrease in ergosterol content and enhanced leakage of Ca^{2+}, K^{+} and Mg^{2+} ions from treated fungal cells, depicting fungal plasma membrane as the site of antifungal action. The EO showed promising DPPH free radical scavenging activity (IC_{50} value:15.89 μL/mL) and favourable safety profile with LD_{50} value (7133.70 mg/kg body wt.) when estimated through acute oral toxicity on mice. Carvone (61.62%) was recorded as the major component of the oil during chemical characterisation through GC-MS. Based on strong antifungal, antiaflatoxigenic and antioxidant potential, the chemically characterised *M. cardiaca* EO may be recommended as safe plant based preservative and shelf life enhancer of food items. This is the first report on antifungal and antiaflatoxigenic activity of *M. cardiaca* EO.

1. Introduction

Dry fruits are valued for their high nutritive value because of high contents of fibers (prebiotics), phenolic, vitamins and antioxidants and are widely used as functional food ingredients in food industries (Vinson et al., 2005). Like other food commodities, dry fruits are also prone to contamination with food borne molds viz., *Aspergillus* spp., *Fusarium* spp., *Alternaria* spp., *Penicillium* spp. and their associated toxins during processing and storage. Presence of *Aspergillius* sp. such as *A. flavus, A. parasiticus*, and *A. nomius* has paid serious concern, in view of their potential to secrete aflatoxins during favourable conditions particularly in tropical regions. Aflatoxin B_1 has been recognised as the Class 1 human carcinogen category by International Agency for Research on Cancer (IARC) (WHO, & IARC, 1993). Further, the aflatoxin is a thermostable mycotoxin and may remain present even in processed and cooked food items (Prakash et al., 2012a). In addition to toxigenic effects on human systems, AFB_1 8, 9-exoepoxide, the metabolic product of aflatoxin B_1 has been reported to enhance the generation of reactive oxygen species leading to spoilage of food items due to lipid peroxidation. Hence, the presence of mold and aflatoxin contamination may cause qualitative and quantitative deterioration of food items. Tropical and subtropical regions are the main centres of food production due to growth of multiple crops annually (Dwivedy et al., 2015) but the congenial environmental conditions of these areas also facilitate huge loss of agricultural produce due to pest

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http://dx.doi.org/10.1016/j.fct.2017.05.043
0278-6915/

infestations and mycotoxins (Varma and Dubey, 2001; Wagacha and Muthomi, 2008). Although, there are some dry fruits on association of food borne fungi on dry fruits but their qualitative contamination by aflatoxins has not been paid proper attention.

Currently, a range of synthetic preservatives have been used to prevent the molds and aflatoxin contamination in food commodities. The recent reports on the negative impacts of synthetic preservatives on consumer health have caused serious concern on their continuous application as food preservatives. In addition, synthetic preservatives have also been reported to enhance the production of highly reactive oxygen molecules such as superoxide, hydrogen peroxides and hydroxyl radicals which negatively alter the functional biomolecules viz., proteins, lipids, and nucleic acids (Halliwell et al., 2000). Therefore, food industries are looking for the safer alternatives of synthetic preservatives which could enhance shelf life of food items by preventing their quantitative and qualitative losses.

Recently, many traditionally used plant based products are included in frontier areas of research in food security and green consumerism programmes in view of their cost-effectiveness and favourable safety profile to human and live stocks (Burt, 2004). In this context, essential oils of different traditionally used plants are recognized as vital tool in formulation of novel plant based preservatives against losses of food items due to fungal and aflatoxin contamination as well as free radical generation (Kocić-Tanackov et al., 2012; Prakash et al., 2012b). Different essential oils and their active components have been included in GRAS (Generally Recognized As Safe) category (Burt, 2004). Inclination of the modern society is currently towards green consumerism (Smid and Gorris, 1999) desiring least use of synthetics as grey chemicals in food commodities. Some essential oil based preservatives viz., DMC Base Natural' comprising 50% EO from rosemary, sage and citrus and 50% glycerol and carvone, a monoterpene of the essential oil of *Carum carvi* are commercially available on large scale as food additives (Burt, 2004).

Mentha cardiaca L., var. *Mukta* commonly known as Scotch Spearmint, luxuriantly grows in tropical and subtropical countries. It is widely used as an ingredient in food flavouring, toothpaste, mouthwash, chewing gum and candy etc. (Atal and Kapur, 1982; Guenther, 1949). In addition, the plant has been traditionally used in Indian system of medicine to treat digestive ailments, fevers and headaches (Sole. "Mentha X Gracilis", 2014).

Based on the above background, the present investigation has been designed to explore the molds and aflatoxin B_1 contamination in the selected dry fruit samples and to further investigate the preservative potential of chemically characterised *M. cardiaca* EO in terms of its antifungal, antiaflatoxigenic and antioxidant efficacy. In addition, the possible mode of antifungal action of the EO has also been investigated by assessing ergosterol content in the plasma membrane and its impact on cellular ion contents of test fungus so as to recommend its possible application as a plant based preservative.

2. Materials and methods

2.1. Chemicals and equipment

All the chemicals used in study were procured from SRL (Sisco Research Laboratories) Pvt. Ltd. Mumbai, HiMedia Laboratories Pvt. Ltd., Mumbai, and Genuine Company Mumbai, India. The major equipments used were hydro-distillation apparatus, (Merck Specialities Pvt. Ltd., Mumbai, India), centrifuge, UV transilluminator (Zenith Engineers, Agra, India), spectrophotometer (Hitachi 2900), Atomic Absorption Spectrophotometer (Perkin Elmer AAnalyst 800), pH meter (Systronics μpH System 361) and GC-MS (Perkin Elmer Turbomass Gold MA, USA). Glasswares were obtained from Borosil Glass Works Limited, Mumbai (India).

2.2. Procurement of dry fruits

Frequently used dry fruits (stored for six months) viz. *Pistachia vera, Juglans regia, Buchnania lanzan, Sesamum indicum, Pinus gerardiana, Anacardium occidentale, Prunus amygdalus, Prunus armeniaca, Vitis vinifera, Phoenix dactilifera, Cocos nucifera, Linum usitatissimum, Nelumo nucifera, Helianthus annuus, Punica granatum, Ficus carica, Citrullus lanatus* and *Cucumis melo* were procured from the local market (Gola Deena Nath) of Varanasi., India The fruits were stored at room temperature in sterile polythene bags to prevent further contamination.

2.3. Moisture content and pH of raw materials

To determine the moisture content of the dry fruits, individual samples were weighed and then dried at 85 °C till their weight became constant and the difference in weight was calculated following Mandeel (2005). For pH, the samples were finally grounded by using a sterilised mixer grinder, thereafter their distilled water suspension (1:10) was prepared and stirred for 24 h in 200 ml beaker. The pH of the suspension was measured by using an electronic pH meter (Mandeel, 2005).

The moisture content was calculated from the following formula

$$\%\ \text{moisture content} = \frac{\text{Fresh wt. of dry fruit} - \text{Dry wt. of dry fruit}}{\text{Fresh wt. of dry fruit}} \times 100$$

2.4. Mycoflora analysis of procured samples

Mycoflora analysis of collected dry fruits was performed by serial dilution and direct plating methods. For serial dilution method, 1 g of each dry fruit sample was suspended in 10 ml sterile 0.85% saline solution in a 250-mL Erlenmeyer flask and was thoroughly homogenised on an electric shaker with a constant speed for 15 min. Three fold serial dilutions were then prepared following Aziz et al. (1998). One mL of dry fruit suspension (3 time dilution) was inoculated to Petri dishes containing 10 mL freshly prepared potato dextrose agar medium (potato, 200 g; dextrose, 20 g; agar, 18 g; and distilled water, 1000 mL). Five plates were used for each dry fruit sample. Plates were incubated at 27 ± 2 °C for 7 days and examined daily, and fungal colony counts were recorded only after 3–4 days. After incubation, the plates were examined visually and also with the help of a compound microscope. Morphologically different mould colonies were individually sub cultured on PDA medium. Fungal species were identified by cultural and morphological characteristics following (Gilman and Joseph, 1998). Per cent relative density of different fungi on dry fruit samples was calculated following Varma and Dubey (2001). For direct plating method, each sample was first surface sterilized with the help of 1% sodium hypochlorite for 45 s and then washed three times with distilled water and then inoculated on Petri plates containing 10 ml PDA and incubated at 27 ± 2 °C for 7 days. Further analysis was done similar to serial dilution method.

2.5. Detection of the most toxigenic strain from the isolated *Aspergillus flavus* isolates

Aflatoxin-producing potential of randomly selected *A. flavus*

isolates from dry fruits during mycoflora analysis was assessed in SMKY medium (sucrose, 200 g; $MgSO_47H_2O$, 0.5 g; KNO_3, 0.3 g; yeast extract, 7.0 g; distilled water, 1000 mL) following Kumar et al. (2010) with slight modification. SMKY medium (25 mL) was taken in 100- mL Erlenmeyer flask and inoculated separately with 50 μL spore suspension (10^6spores per mL) of 7-day old culture of the *A. flavus* isolated from selected dry fruits. The flasks were incubated for 10 days at 27 ± 2 °C. Thereafter, the content of each flask was filtered (Whatman no. 1), and mycelium was oven-dried at 100 °C to determine their biomass. The filtrate was extracted with 20 mL chloroform in a separating funnel. The separated chloroform extract was evaporated till dryness on water bath at 70 °C. The residue left after evaporation was dissolved in 1 mL chloroform and 50 μL of it, was spotted on TLC plate (20 × 20 cm^2 of silica gel). The plate was developed in toluene/isoamyl alcohol/methanol (90:32:2; v/v/v) solvent system (Reddy et al., 1970). The intensity of aflatoxin B_1 (AFB_1) was observed in ultraviolet fluorescence analysis cabinet at an excitation wavelength of 360 nm (AOAC, 1984). The blue spots of AFB_1 on TLC plate were scraped out and dissolved in 5 mL cold methanol, shaken and centrifuged at 3000g for 5 min. The optical density of supernatant was recorded at 360 nm, and the amount of AFB_1 was calculated following Kumar et al. (2010).

$$\text{AF B1 content } (\mu g/L) = \frac{D \times M}{E \times L} \times 1000$$

where D, absorbance; M, molecular weight of AFB_1 (312); E, molar extinction coefficient of AFB_1 (21 800)L, path length (1-cm cell was used).

2.6. Selection of mold strains

The isolate of *A. flavus* LHP-PV-1 the most toxigenic isolate with highest Aflatoxin B_1 content, isolated from *Pistacia vera* samples, was selected as test fungus. Some other molds (*Aspergillus sydowi, Aspergillus terreus, Aspergillus fumigatus, Aspergillus sulphureous, Aspergilus fumigatus, Penicillium purpurogenum* and *Rhizopus stolonifer*) procured during mycoflora analysis were used for determination of fungitoxic spectrum. Culture of the fungi was maintained on Potato Dextrose Agar (PDA) slants at 4 °C. Spore suspension of most toxigenic strain of *A. flavus* LHP-PV-1 was prepared by using a seven days old culture. From that upper layer of fungal mat was scrapped and then sieved in 0.1% Tween-20 solution in distilled water. Spore density was adjusted to 10^6 spores per mL using hemocytometer. The suspension was kept at 4 °C (Dwivedy et al., 2016).

2.7. Plant material and extraction of the essential oil

Aerial parts of *M. cardiaca* var. *Mukta* were procured from CSIR CIMAP., Lucknow, India. The species was identified by Prof. N K Dubey at Dept. of Botany, Banaras Hindu University, Varanasi and voucher specimen (voucher specimen no. Lam 2014/02) was kept in herbarium of Department of Botany. Five hundred grams thoroughly washed aerial part of *M. cardiaca* was subjected to Clevenger hydro distillation apparatus for 4 h (Clevenger, 1928). The volatile fraction (EO) was separated in which a pinch of sodium sulphate was added to absorb water droplets present as impurity. The EO was stored in dry vial at 4 °C in dark condition.

2.8. Chemical characterization of M. cardiaca EO

GC analysis of *M. cardiaca* EO was done on a PerkinElmer AutoSystem XL GC fitted with Equity-5 column (60 m × 0.32 mm i.d., film thickness 0.25 μm). The column oven was set from 70 °C to 250 °C at the rate of 3 °C/min with initial and final hold time of 2 min and programmed to 290 °C at 6 °C/min, with final hold time of 5 min, using H_2 as carrier gas at constant pressure of 10 psi, a split ratio of 1:35, injector and detector (FID) temperatures were 290 °C and 280 °C, respectively. For GC-MS, an Elite-5 MS fused silica capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm) was used in a PerkinElmer Clarus® 680 GC interfaced with a Clarus® SQ 8 C Quadrupole mass spectrometer. Programmed Split/Splitless (PSS) injector temperature was 250 °C with a split ratio of 1:50. Column oven was adjusted from 60 °C to 240 °C at the rate of 3 °C/min and increased to 270 °C at 5 °C/min. Helium was used as carrier with constant flow of 1 ml/min. Transfer line and source temperatures were 220 °C; ionization energy 70 eV; and mass scan range 40–450 amu. Characterization was achieved on the basis of retention time, elution order, relative retention index using a homologous series of *n*-alkanes (C_9-C_{28} hydrocarbons, Polyscience Corp. Niles IL), co-injection with standards in the GC-FID and GC-MS (Aldrich and Fluka), TurboMass NIST 2011 libraries version 2.3.0, Wiley registry of mass spectral data 9th edition and by comparing with the mass spectral literature data (Adams, 2007).

2.9. Determination of total phenolic content of M. cardiaca EO

Total phenolic content of *M. cardiaca* EO was determined spectrophotometrically using the Folin–Ciocalteu reagent following Gholivand et al. (2010). A solution (0.1 ml DMSO) containing 1000 μg oil was prepared and mixed with 46 ml of distilled water in a volumetric flask. 1 ml Folin–Ciocalteu reagent was added and the mixture was thoroughly shaken using an electronic shaker and was left for 3 min to allow complete reaction. Three milliliters aqueous solution of 2% Na_2CO_3 was then added. The solution was incubated for 4 h at room temperature (25 ± 2 °C). At the end of the incubation period, the absorbance of each mixture was measured at 760 nm. The same procedure was also applied to standard solutions of gallic acid (0–1000 μg/0.1 mL) and a standard curve was obtained. Total phenolic content of the oil was obtained by comparing the absorbance value of the oil at 760 nm with the standard curve and expressed as μg gallic acid equivalent/mg of oil.

$$\text{Absorbance} = 0.0012 \times \text{gallic acid } (\mu g) + 0.024$$

2.10. Fungitoxic evaluation of M. cardiaca EO against toxigenic strain A. flavus LHP-PV-1

The fungitoxicity of *M. cardiaca* EO was assessed in terms of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) against the toxigenic strain of *A. flavus* LHP-PV-1 by food poisoning method in a PDA medium. Requisite amounts of the EO were first homogenized in 0.5 ml 5% Tween- 20 solution in distilled water and were then mixed in PDA (potato dextrose agar) so as obtain different concentrations (0.25–5.0 μl/ml) The medium was then inoculated with 10 μl *A. flavus*-LHP-PV-1 (Density = 10^6spores/ml). Inoculated PDA plates containing 5% Tween- 20 (0.5 ml) and 9.5 ml PDA served as control. Streptomycin (300 mg/l) was added to the medium for controlling bacterial growth in both treatment and control sets. The plates were incubated at 27 ± 2 °C for one week. The lowest concentration of the essential oil resulting in complete suppression of growth of the toxigenic isolate *A. flavus*-LHP-PV-1 was recorded as the MIC. For determination of MFC of the essential oil, discs of the PDA medium from Petri plates showing no fungal growth were cut and were re inoculated onto EO free PDA plates and monitored for fungal growth. The lowest concentration preventing revival of fungal

growth was taken as the MFC.

2.11. M. cardiaca EO in suppression of aflatoxin B_1 production by the toxigenic isolate of A. flavus LHP-PV-1

Requisite amounts of *M. cardiaca* EO were dissolved separately in 0.5 mL Tween-20 (5%) and added to 24.5 ml SMKY to achieve concentrations ranging from 0.25 to 1.25 μL/mL, in an Erlenmeyer flask (100 mL). For preventing bacterial growth streptomycin (300 mg/L) was added to the medium. Each set was then inoculated with 25 μl *A. flavus* LHP-PV-1 (10^6spores/mL) and was incubated for 10 day at (27 ± 2 °C). After incubation, the content of each flask was filtered by Whatman No. 1 filter paper and the filtered mycelium was dried at 80 °C (12 h) and weighed. The AFB_1 in each set was quantified by the method explained earlier in section 2.5.

2.12. Fungitoxic spectrum of M. cardiaca EO

The fungitoxic spectrum of *M. cardiaca* essential oil was evaluated against nine common storage molds (*Aspergillus flavus*, *Aspergillus sydowi, Aspergillus terreus, Aspergillus fumigatus, Aspergillus sulphureous, Aspergilus fumigatus, Penicillium purpurogenum* and *Rhizopus stolonifer*) using the poisoned food technique. Requisite amount of essential oil was added separately to plates containing 0.5 ml 5% Tween- 20 and homogenized thoroughly and then 9.5 ml molten PDA was mixed to obtain the final concentrations of 1.25 μl/ml. A 5 mm disc from a seven day old colony of each fungus was separately placed upside down on the center of the plates. Plates containing only 5% Tween- 20 (0.5 ml) and 9.5 ml PDA inoculated with the test fungi served as control. The plates of both treatment and control sets were incubated at 27 ± 2 °C for one week. The percent inhibition of fungal growth was calculated by the following formula:

$$\%\ \text{mycelial inhibition} = \frac{dc - dt}{dc} \times 100$$

where, dc is average diameter of fungal colony in control sets and dt is average diameter of fungal colony in treatment sets.

2.13. Effect of M. cardiaca EO on ergosterol content in plasma membrane of A. flavus LHP-PV-1

Method of Tian et al. (2012) was used for the estimation of ergosterol content in the fungal plasma membrane. For this 100 μl of spore suspension of *A. flavus* (10^6 spores/ml) prepared in 0.1% Tween-20 was inoculated in SMKY medium having 0, 0.25, 0.50, 0.75, 1.0 and 1.25 μL/mL EO homogenised in 5% Tween-20 and then incubated at 27 ± 2 °C for 4 days. The fungal biomass was filtered, washed twice with distilled water and the net wet weight of each cell pellet was measured. 5 mL of 25% alcoholic potassium hydroxide solution was added to each cell pellet, and vortex-mixed for 2 min, followed by incubation at 85 °C in a water bath for 4 h. Sterols were extracted from each sample, by adding a mixture of 2 ml sterile distilled water and 5 ml *n*-heptane followed by sufficient vortex mixing for 2 min. After 1 h, the separated *n*-heptane layer was examined by scanning between 230 and 300 nm through spectrophotometer. A characteristic four peaked curve denotes the presence of ergosterol and the late sterol intermediate 24 (28) dehydroergosterol in the *n*-heptane layer. A flat line indicated the absence of ergosterol. Method of Tian et al. (2012) was used to calculate the amount of ergosterol as a percentage of the wet weight of the cells by following formula.

% ergosterol + % 24(28) dehydroergosterol = (A282/290)/pellet weight; % 24(28) dehydroergosterol = (A230/518)/pellet weight; and % ergosterol = (% ergosterol + % 24(28) dehydroergosterol) − % 24(28) dehydroergosterol.

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where 290 and 518 are the E values (in percentages per cm) determined for crystalline ergosterol and 24 (28) dehydroergosterol, respectively, and pellet weight is the net wet weight (g).

2.14. Determination of leakage

Effect of *M. cardiaca* EO on leakage of vital cellular ion content was determined following Helal et al. (2007) with slight modification. Five day old mycelia of *A. flavus* LHP-PV-1 grown on SMKY medium was harvested and washed thrice with sterile distilled water. Thereafter, the filtrate was suspended in 20 ml 0.85% saline solution and fumigated with 1.25 and 2.50 μl/ml *M. cardiaca* EO for 12 h. Control sets did not contain EO. The biomass was again filtered and the supernatant was analyzed using atomic absorption spectrometry for Ca^{2+}, K^{+} and Mg^{2+} ions.

2.15. Antioxidant activity of M. cardiaca EO

Free radical scavenging activity of the *M. cardiaca* EO was measured by recording the level of bleaching of a purple color DPPH solution to yellow following Prakash et al. (2011). During the assay, DPPH solution is mixed with a substance having capacity to donate a hydrogen atom, leading to the reduced form 1, 1 diphenyl-2-picrylhydrazine (non radical). Different concentrations (5.0–25.0 μL/mL) of the essential oil were added to 5 mL of 0.004% DPPH solution in methanol. The mixture was incubated at room temperature (25 ± 2 °C) for 30 min and then, the absorbance was measured against a blank at 517 nm using spectrophotometer. Butylated hydroxytoluene (BHT) (2.0–14 μg/mL) was used as positive control. Antioxidant activity of the sample was measured in terms of reduction in absorbance of DPPH free radical. The IC_{50} value of the test compounds represents the concentration that caused 50% neutralization of DPPH radicals was calculated from the graph plotted between percentage inhibition and concentration of sample in DPPH solution. Per cent free radical scavenging activity (I %) was calculated by following formula.

I % = (A_{blank}–A_{sample}/A_{blank}) x 100.where, A_{blank} is the absorbance of the control (without test compound), and A_{sample} is the absorbance of the test compound.

2.16. Safety profile of the M. cardiaca EO

The safety profile of the *M. cardiaca* EO was recorded by acute oral toxicity on mice (*Mus musculus* L.; average weight 30 g and age 3 months) in terms of LD_{50} value. Mice were procured from the Institute of Medical Sciences, Banaras Hindu University, Varanasi,

and were given normal animal feed. They were acclimatized in the laboratory under controlled environmental conditions (30 ± 2 °C) for a week before performing the LD_{50} experiments. A stock solution of Tween-80 and distilled water (1:1) was prepared. Different doses of essential oil (50–300 μL) along with 0.5 mL stock solution were orally administered separately through a micropipette attached with a catheter to each treatment set containing 10 mice. Single dose of the EO was given to each mice of the treatment set. In control sets, equal doses of Tween- 80 and distilled water (1:1) were administered. The mortality of the test animals was observed from 4 to 24 h and LD_{50} of EO was calculated by Probit analysis following Finney (1971).

2.17. Statistical analysis

All the experiments were performed in triplicate and data analysis was done on mean ± SE subjected to one way ANOVA. Means are separated by the Tukey's multiple range test when ANOVA was significant (pb0.05) (SPSS 16.0; IBM Corporation, IL, USA).

3. Results and discussion

3.1. Mycoflora analysis of selected dry fruits and selection of test fungus

The level of fungal contamination of dry fruits samples is mainly governed by pH and moisture content. However, the mycoflora analysis (Table 1) clearly indicates that, for tested samples, pH plays an important role in deciding the extent of fungal contamination. pH of *Foenix dactilifera, Ficus carica, Prunus amygdalus* and *Pinus gerardiana* was in the range 4–5 and in these samples fungal contamination was minimum while heavy fungal contamination as well as diversity were observed in *Citrulus lanatus, Buchnania lanzan, Cucumis melo, Nelumbo nucifera, Juglans regia, Pistachia vera* and *Anacardium occidentale* having pH in range 5.31–6.90. Except *Buchnania lanzan* (pH = 5.31), rest of the heavily contaminated dry fruits have pH range between 6 and 7 which is conducive for growth of storage pests including molds (FAO, 1980). However, other than pH and moisture content, chemical profile of the substrate (dry fruits) may also be a determinant for fungal contamination and aflatoxin B_1 production as has been reported by Singh et al. (2008). In addition, high average temperature of Varanasi region, the collection site of the dry fruits samples, may also be a reason for heavy fungal infestation (Velluti et al., 2000; Peterson et al., 2001). Quantification of Aflatoxin B_1 content of different isolates of *Aspergillus flavus* obtained from selected dry fruits depicted *A. flavus* LHP-PV-1 as most toxigenic isolate having Aflatoxin B_1 secretion equal to 2026.4 μg/L (Table 2). Thus, selected as test fungus for further studies.

Hence, a perusal of the results on mycoflora analysis and the quantitative estimation of aflatoxin B_1 (Table 2) emphasize that the dry fruits were biodeteriorated quantitatively by the saprophytic fungi as well as qualitatively by the aflatoxin B_1 secreted by different toxigenic isolates of *A. flavus*. Therefore, a preservative for dry fruits should have efficacy to inhibit growth of the associated fungi as well as to suppress the aflatoxin B_1 secretion by the toxigenic strains of *A. flavus*.

3.2. Isolation and chemical characterization of M. cardiaca EO

Isolation of EO was done from aerial parts of the plant and the EO yield was 12 ml/kg biomass. Phenolic content in the oil was recorded to be 7.1 μg/mg. It has been observed that chemical profile of essential oils varies with seasonal and geographical

Table 1
Mycoflora analysis of dry fruits.

Dry fruits	Fungal species																		Total sp.	% occurrence of fungus on dry fruit	% Moisture content	pH
	A.f	*A.n*	*A.r*	*A.m*	*A.h*	*P.pu*	*Cla*	*Rhi*	*M.b*	*P.sp*	*A.l*	*A.sy*	*A.fu*	*Mu*	*A.u*	*A.su*	*A.a*	*C.l*	*A. t*	*P.c*				
Anacardium occidentale	1	25	–	–	–	1	–	–	–	2	–	–	–	–	–	–	–	–	–	–	4	3.52	5.41	6.27
Pistachia vera	10	5	–	–	–	–	1	–	1	5	3	–	–	–	–	5	–	1	–	–	8	3.77	12.76	6.90
Nelumbo nucifera	81	7	–	–	–	–	–	–	–	–	18	18	–	1	–	–	–	–	–	–	5	15.20	10.66	6.12
Cucumis melo	12	2	21	26	–	2	–	–	–	–	1	–	74	5	–	13	–	–	1		10	19.09	5.28	6.12
Juglans regia	5	19	6	3	–	–	1	1	–	–	11	–	8	–	–	–	–	–	–	–	8	6.56	4.00	6.08
Foenix dactilifera	2	–	–	–	2	3	–	–	–	–	–	–	–	–	–	7	1	–	–	1	6	1.94	13.85	4.70
Ficus carica	–	–	–	–	1	–	–	–	–	–	5	–	–	–	1	–	–	–	–	–	3	0.85	18.24	4.76
Prunus amygdalus	1	–	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	2	0.24	5.16	4.88
Citrulus lanatus	212	–	–	–	–	–	–	–	–	–	–	–	–	4	–	–	–	–	–	–	2	26.27	4.93	6.26
Pinus gerardiana	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	0.00	7.29	4.56
Buchnania lanzan	4	4		–	–	–	–	–	–	116	34	–	–	2	–	25	–	–	–		7	22.50	5.02	5.31
Total colony of each fungus	328	62	27	29	3	6	2	1	1	124	72	18	82	12	1	50	1	1	1	1				
% Relative density	39.90	7.54	3.28	3.52	0.36	0.72	0.24	0.12	0.12	15.08	8.75	2.18	9.97	1.45	0.12	6.08	0.12	0.12	0.12	0.12				

A.f = *Aspergillus flavus*, A. n = *Aspergillus niger*, A.r = *Aspergillus repens*, A. m = *Aspergillus minutus*, A. h = *Alternaria humicola*, P. p = *Penicillium purpurogenum*, Cla = *Cladosporium* sp., Note: Rhi = *Rhizopus stolonifer*, M. b = *Monolia brunia*, P.sp = *Penicillium* sp., A. l = *Aspergillus luchuensis*, A. sy = *Aspergillus sydowii*, A. fu = *Aspergillus fumigatus*, Mu = *Mucor* sp., A. u = *Aspergillus unguis*, A. su = *Aspergillus sulphureus*, A. a = *Alternaria alternata* C. l = *Curvularia lunata*, A. t = *Aspergillus terreus*, A. s = *Aspergillus sulphureus*, and P. c = *Penicillium citrinum*.

Table 2
Aflatoxin content of different isolates of *Aspergillus flavus* obtained from selected dry fruits.

Dry friuts	Toxigenic isolate of *A. flavus*	Aflatoxin B_1 (μg/L)
Anacardium occidentale	-	-
Pistachia vera	AF-LHP-PV-1*	2026.4
	AF-LHP-PV-2	1751.2
	AF-LHP-PV-3	1648.0
	AF-LHP-PV-4	1132.8
	AF-LHP-PV-5	1579.2
	AF-LHP-PV-6	1328.0
Nelumbo nucifera	AF-LHP-NN-1	308.8
	AF-LHP-NN-2	194.4
	AF-LHP-NN-3	629.6
	AF-LHP-NN-4	1796.8
	AF-LHP-NN-5	1396.0
	AF-LHP-NN-6	136.8
	AF-LHP-NN-7	640.8
Cucumis melo	AF-LHP-CM-1	1740.0
	AF-LHP-CM-2	1923.2
	AF-LHP-CM-3	1281.6
Juglans regia	-	-
Foenix dactilifera	-	-
Ficus carica	-	-
Prunus amygdalus	-	-
Citrulus lanatus	AF-LHP-CL-1	1591.2
	AF-LHP-CL-2	1579.2
	AF-LHP-CL-3	1408.0
	AF-LHP-CL-4	938.4
	AF-LHP-CL-5	1396.0
	AF-LHP-CL-6	1212.8
	AF-LHP-CL-7	1923.2
Pinus gerardiana	AF-LHP-BL-3	308.8
Buchnania lanzan	AF-LHP-BL-4	228.8

- = Not found, * = Most toxigenic isolate.

conditions and such variations would influence their biological activity (Bakkali et al., 2008). Hence, before testing any essential oil, it is desirable to standardise it chemically through GC-MS analysis before recommending for formulation. (Lahlou and Berrada, 2003; Raina et al., 2003; Mishra et al., 2013). The GC-MS analysis of *M. cardiaca* oil revealed 60 compounds comprising 99.0% of EO. The retention index and area percentage of identified compounds are summarized in Table 3 and amongst different identified compounds carvone (59.6%) was the major component followed by Limonene (23.3%), β-Myrcene (2.5%), 1-8-Cineole (2.1%), *Cis*-dihydrocarvone (1.5%), and β-Bourbonene (1.5%). However, in earlier report by Chowdhury et al. (2007) on the chemical profile of *M. cardiaca* oil from Bangladesh, carvone (60.90%) and D-limonene (21.58%) were the major components but, 1,8 cineol was missing and α- Bourbonene was present instead of β- Bourbonene. Such variations in composition of EO are probably due to change in geographical condition. Fig. 1 represents the chemical structures of the major components. Carvone is a frequently used botanical pesticide with trade name "Talent" particularly to protect potatoes during storage from bacterial rottings and in enhancement of their shelf life (Dubey et al., 2010). 1, 8 cineole and DL-limonene have also been reported as antimicrobial agent (Van Vuuren and Viljoen., 2007). Keeping in view the development of resistant races by the microorganisms against the formulations based on single bioactive molecules, the recommendation of essential oil as such containing more than one active principle would be a novel approach. Different components would act synergistically and such preservatives based on such formulations would not be affected by the pathogenic races of storage fungi and would retain their activity for longer periods. Hence, in the present investigation, the essential oil of *M. cardiaca* as such was subjected to antifungal and aflatoxin inhibitory efficacy.

Table 3
Chemical characterization of *M. cardiaca* essential oil through GC- MS.

S. No	Compound	Retention Index	Percentage composition
1	2,5-Diethyl tetrahydrofuran	898	0.1
2	Tricyclene	920	0.1
3	α-Thujene	927	t
4	α-Pinene	935	0.8
5	Camphene	951	t
6	Sabinene	975	0.6
7	β-Pinene	980	0.9
8	**β-Myrcene**	**991**	**2.5**
9	3-Octanol	997	0.2
10	α-Phellandrene	1007	0.1
11	α-Terpinene	1018	0.1
12	*p*-Cymene	1026	0.2
13	**Limonene**	**1031**	**23.3**
14	**1,8-Cineole**	**1034**	**2.1**
15	(*Z*)- β-Ocimene	1036	t
16	(*E*)-β-Ocimene	1047	t
17	γ-Terpinene	1059	0.1
18	*Cis*-sabinene hydrate	1069	0.7
19	Terpinolene	1091	0.1
20	Linalool	1101	t
21	*Trans*-sabinene hydrate	1103	t
22	3-Octanyl acetate	1123	0.1
23	Allo-ocimene	1130	t
24	*Trans*-*p*-menth-2-en-1-ol	1139	nd
25	*Cis*-verbenol	1142	nd
26	*Trans*-verbenol	1148	nd
27	Menthone	1157	nd
28	Isomenthone	1167	t
29	δ-Terpineol	1170	0.1
30	Terpinen-4-ol	1181	0.6
31	α-Terpineol	1194	0.2
32	neo-Dihydrocarveol	1198	1.25
33	***Cis*-dihydrocarvone**	**1201**	**1.5**[c]
34	*Trans*-dihydrocarvone	1208	0.1
35	*Trans*-carveol	1224	0.5
36	*Cis*-carveol	1238	0.1
37	Pulegone	1243	0.8
38	**Carvone**	**1253**	**59.6**
39	Piperitone	1260	0.5
40	*Cis*-carvone oxide	1267	t
41	Carvone isomer	1271	t
42	*Trans*-carvone oxide	1281	0.1
43	Dihydroedulan II	1294	t
44	Dihydroedulan I	1299	0.1
45	Iso-dihydrocarvyl acetate	1331	0.9
46	*Cis*-carvyl acetate	1368	0.3
47	**β-Bourbonene**	**1392**	**1.5**
48	β-Bourbonene isomer	1394	0.1
49	β-Elemene	1397	0.1
50	β-Caryophyllene	1427	1.3
51	β-Cedrene	1427	c
52	β-Gurjunene	1435	0.2
53	$C_{15}H_{24}$	1450	0.2
54	(*E*)- β-Farnesene	1459	0.6
55	α-Humulene	1461	0.1
56	γ-Gurjunene	1473	0.1
57	Germacrene D	1489	1.0
58	δ-Cadinene	1528	nd
59	Caryophyllene oxide	1591	t
60	Viridiflorol	1600	0.2
		Total	99.0

Retention Index on Equity-5 capillary column using a homologous series of *n*-alkane (C_9-C_{28}) hydrocarbons, Polyscience Corp. Niles IL; t: trace (<0.1%), nd: not detected, c: coeluting with previous compound. Compound mentioned in bold letters are major components.

3.3. Antifungal and antiaflatoxigenic activity of *M. cardiaca* EO

Antifungal activity of *M. cardiaca* EO was measured in terms of MIC, MFC and fungitoxic spectrum. MIC of the EO was 1.25 μL/mL against *A. flavus* LHP-PV-1 (Table 4) which is lower than some previously reported EO such as *Anethum graveolense* leaf (7.0 μL/

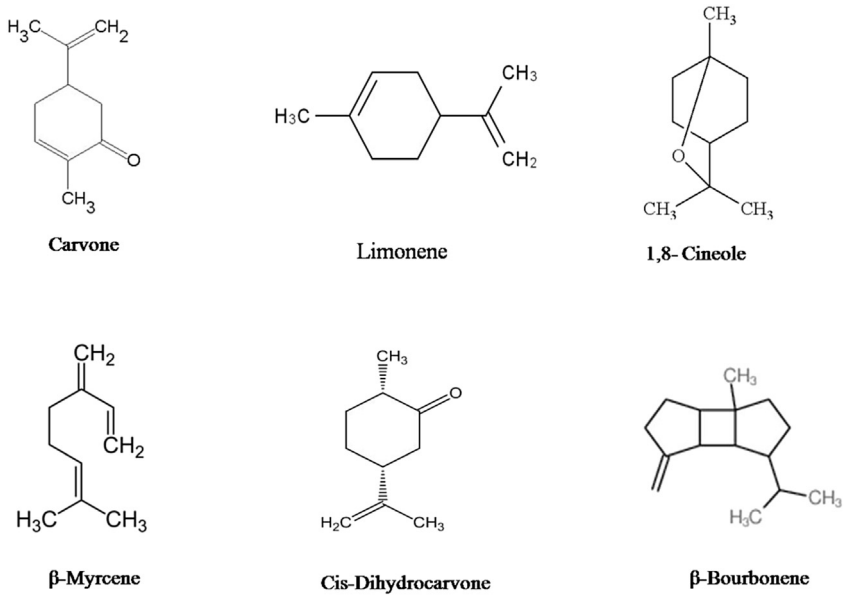

Fig. 1. Structure of major compound present in *M. cardiaca* EO.

Table 4
Effect of different concentrations of *M. cardiaca* EO on mycelial dry weight and Aflatoxin B_1 production in SMKY medium.

Concentration (μl/ml)	AFB_1 content (μg/mL)	Mean Dry Weight (g)
CNT	0.759 ± 0.023^a	0.416 ± 0.016^a
0.25	0.391 ± 0.019^b	0.278 ± 0.018^b
0.50	0.190 ± 0.012^c	0198 ± 0.007^c
0.75	0.047 ± 0.005^d	0.135 ± 0.017^d
1.0	0.000 ± 0.000^d	0.082 ± 0.012^d
1.25	–	0.000 ± 0.000^e

Note: Values are mean (n = 3) ±Standard Error; the means followed by same letter in the same column are not significantly different according to ANOVA and Tukey's multiple comparison tests.

mL) (Prakash et al., 2012a), *Cinnamomum jensenianum* (8.0 μL/mL) (Tian et al., 2012), *Cinnamomum glaucescens* (4.5 μL/mL) (Prakash et al., 2013), *Coriandrum sativum* (2.5 μL/mL), *Commiphora myrrha* (3.0 μL/mL), *Origanum majorana* (3.0 μL/mL), *Hedychium spicatum* (2.5 μL/mL), and *Cananga odorata* (2.0 μL/mL) (Prakash et al., 2012c) as well as some prevalent synthetic fungicides such as Nystatin and Wettasul-80 (Prakash et al., 2010) and synthetic preservatives viz salicylic acid, BHT, ascorbic acid, and gallic acid (Prakash et al., 2012c). The MFC of the EO was 2.25 μL/mL. In addition the *M. cardiaca* EO at 1.25 μL/mL checked the growth of 9 common storage fungi causing biodeterioration of dry fruits. The EO completely inhibited the growth of *Aspergillus flavus*, *Aspergillus sydowii*, *Aspergillus sulphureous*, *Penicillium purpurogenum* and *Fusarium* sp. (Fig. 2). The antifungal efficacy at low doses and against a broad spectrum of food borne moulds strengthens the possible recommendation of *M. cardiaca* EO as plant based preservative.

Minimum antiaflatoxigenic concentration (MAIC) of the EO was 1.0 μl/ml (Table 4), which is lower than that of MIC on which it caused complete inhibition of growth of *A. flavus* LHP-PV-1. Thus, the EO checked aflatoxin secretion by *A. flavus* prior to complete inhibition of biomass suggesting two separate mode of actions for inhibition of fungal growth and aflatoxin secretion as has been earlier reported by Shukla et al. (2009). Inactivation of some key enzymes related with carbohydrate catabolism in *A. flavus* appears to be instrumental in the inhibition of AFB_1 production as has been observed by Tian et al. (2011). Based on the findings of the present investigation, it is advisable to record both antifungal and antiaflatoxigenic activity of any EO in order to prescribe its exact doses to control quantitative as well as qualitative losses of stored food commodities. This is first report on antifungal and antiaflatoxigenic activity of *M. cardiaca* EO.

3.4. Mode of antifungal action of *M. cardiaca* EO

Antifungal mode of action of *M. cardiaca* EO was assessed by measuring ergosterol content in fungal cell membrane as well as by estimating cellular ion leakage. Ergosterol content was found to decrease on increasing the dose of EO (Table 5; Fig. 3) suggesting the action of *M. cardiaca* EO on ergosterol biosynthesis in fungal plasma membrane. The findings are in accordance with those of Tian et al. (2012) and Kedia et al. (2015) who have reported plasma membrane as site of action of essential oils. The cellular ion leakage test also showed the increased leakage of Ca^{2+}, K^+ and Mg^{2+} ions in treatment sets at 1.25 and 2.50 μl/ml dose as compared to control set (Table 5). Ergosterol has important role in maintaining rigidity of fungal cell membrane (Rodriguez et al., 1985) and the Ca^{2+}, K^+ and Mg^{2+} are required for metabolic activity of cell. Therefore, the inhibition of ergosterol biosynthesis and ion leakage from cell would be the major reason for inhibition of fungal growth and suggest plasma membrane as an important site of antifungal action of *M. cardiaca* EO.

3.5. Antioxidant activity of *M. cardiaca* EO

Antioxidant can also protect stored food materials from free radicals generated due to lipid per oxidation and may be recommended to enhance shelf life of food items. The oil exhibited a dose-dependent free-radical-scavenging activity and its IC_{50} value was recorded as 15.89 μL/mL (Fig. 4). BHT was tested as positive control whose IC_{50} value was recorded as 11.44 μg/mL. Although BHT has higher antioxidant activity than *M. cardiaca* but the reported carcinogenicity of BHT (Ito et al., 1985) compels to search some safer plant based antioxidants *M. cardiaca* EO exhibited better antioxidant activity than *Mentha spicata* (Kedia et al., 2014) *Anethum graveolense* leaf. (Prakash et al., 2012a), *Caesulia axillaris* Roxb

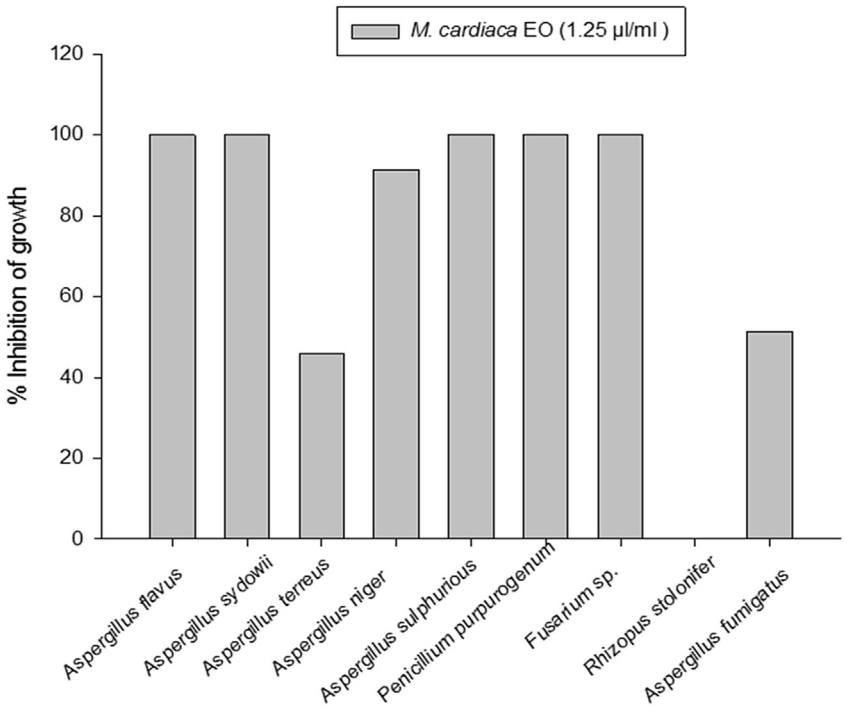

Fig. 2. Fungitoxic spectrum of *M. cardiaca* EO against nine common storage fungi causing biodeterioration of dry fruits.

Table 5
Efficacy of *M. cardiaca* EO on ergosterol synthesis and ion leakages from *A. flavus* cells.

S. No.	Conc. (µL/mL)	% reduction in ergosterol content	Ion content (mg/L)		
			Ca^{2+}	K^{+}	Mg^{2+}
1	CNT	–	2.25 ± 0.23	151.67 ± 1.76	39.60 ± 11.38
2	0.25	04.62	–	–	–
3	0.50	38.88	–	–	–
4	0.75	60.18	–	–	–
5	1.00	100	–	–	–
6	1.25*	100	3.53 ± 0.37	181.00 ± 16.64	47.06 ± 8.49
7	2.50†	–	3.68 ± 0.62	198.33 ± 22.04	73.26 ± 14.38

– = Not determined; * = MIC dose; † = Double MIC dose.

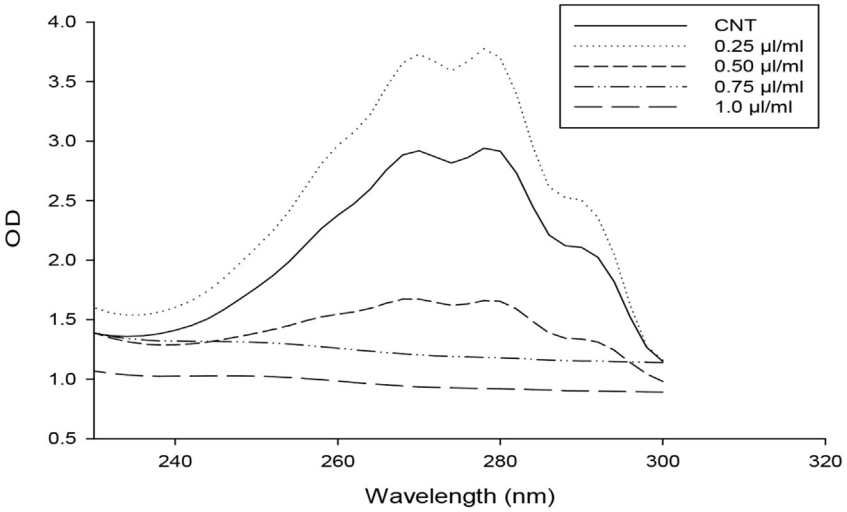

Fig. 3. Effect of different concentration of *M. cardiaca* EO on ergosterol content of *A. flavus* cell membrane.

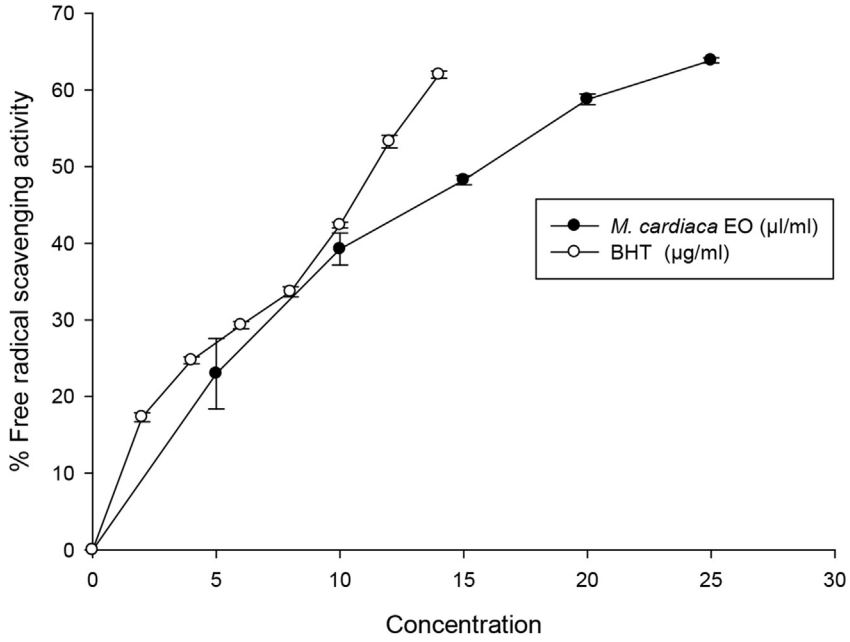

Fig. 4. Free radical scavenging activity of *M. cardiaca* EO.

(Mishra et al., 2012a). and *Jamrosa* (Mishra et al., 2012b). The pronounced antioxidant activity of *M. cardiaca* EO would result into reduction of oxidative stress which would further enforce *A. flavus to* reduce aflatoxin production on food items as has been reported by Jayashree and Subramanyam (2000). Generation of reactive oxygen species has direct correlation with Aflatoxin B_1 secretion leading to quantitative spoilage of food items causing reduction in shelf life and market value. The antioxidant and antiaflatoxin nature of the *M. cardiaca* EO as shown in the present investigation would strengthen its formulation as a novel plant based preservative.

3.6. Safety profile of M. cardiaca EO

Before recommendation formulation for preservation of food commodities it is desirable to assess safety profile for validating compatibility to mammal system. Therefore, safety assessment of *M. cardiaca* EO was done through acute oral toxicity on male mice and LD_{50} value was recorded as 7133.70 mg/kg body weight which was higher than some previously reported plant based preservative viz. pyrethrum (350–500 mg/kg) and carvone (1640 mg/kg) (Isman, 2006), organic acid preservatives viz., acetic acid (3530 mg/kg), sorbic acid (3200 mg/kg), benzoic acid (2000–2500 mg/kg) and formic acid (700 mg/kg) (Prakash et al., 2012b) plant EO viz *Ocimum sanctum* (4571.43 µL/kg) (Kumar et al., 2010) and *Cymbopogon martini* (2569.16 µL/kg) (Mishra et al., 2016). Higher LD_{50} value of the *M. cardiaca* EO strengthens its favourable safety profile as plant based preservative.

Moreover, different components of the oil are widely used as ingredient in food flavouring, toothpaste, mouthwash, chewing gum and candy etc. (Atal and Kapur, 1982; Guenther, 1949). Also strengthens the favourable safety profile of the oil in food industries.

4. Conclusion

In view of strong antifungal activity against moulds causing biodeterioration of dry fruits and ability to suppress aflatoxin secretion and free radical generation as well as favourable safety profile, the *M. cardiaca* EO may be recommended as plant based shelf life enhancer of stored food commodities. Further experiments are, therefore, needed for deciding the dose of *M. cardiaca* EO in food systems for large scale application as preservative.

Acknowledgement

Abhishek Kumar Dwivedy is thankful to Head, CAS in Botany, Banaras Hindu University, Varanasi for providing laboratory facilities; Council of Scientific and Industrial Research (CSIR) New Delhi (09/013(0516)/2013-EMR-I) for research fellowship; Interdisciplinary School of Life Sciences, DST- PURSE and DST-FIST, Banaras Hindu University, Varanasi for financial assistance.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.fct.2017.05.043.

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