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RESEARCH ARTICLE

Assessment of *Ocimum canum* Sims. essential oil as phyto-preservative against fungal deterioration of vasicine of stored herbal raw materials of *Justicia adhatoda* L.

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ABSTRACT

The stored herbal raw materials of *Justicia adhatoda* were found contaminated with various storage fungi in which *Aspergillus flavus* exhibited the maximum relative density (26.41%). The chemical profile of *Ocimum canum* essential oil (OcEO) showed 31 considerable peaks. E-Citral (30.31%) was found as major component followed by Z-Citral (18.23%) and Epoxy ocimene (5.77%). OcEO exhibited broad spectrum fungitoxicity and MIC against *A. flavus* LHPJA-09 was recorded at 0.5 mg/ml while aflatoxin B_1 production was completely checked at 0.3 mg/ml. The detectable reduction in ergosterol amount as well as increased leakage of cellular cations (K^+, Ca^{2+} and Mg^{2+}) specify plasma membrane as the possible target site of antifungal action. OcEO also exhibited significant antioxidant potential with IC_{50} value (4.3 µg/ml) comparable to synthetic antioxidants would be helpful to minimize lipid peroxidation. The high performance thin layer chromatography (HPTLC) analysis of fumigated stored raw materials reveals that OcEO was found efficacious to control the degradation of medicinal component, vasicine of *J. adhatoda.* The LD_{50} of OcEO against mice was recorded as 6050.36 mg/kg body weight indicating its non-mammalian toxicity. Hence, OcEO exhibited special virtues possessing antifungal, antiaflatoxigenic, antioxidant and non-mammalian toxicity strengthening its safe exploitation as green preservative.

Keywords: *Ocimum canum*; Essential oil; *Aspergillus flavus*; Aflatoxin B_1; Antifungal; *Justicia adhatoda*, Biodegradation, HPTLC

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INTRODUCTION

Malabar nut, *Justicia adhatoda* L. (Syn.-*Adhatoda vasica, A. zeylanica*; Family- Acanthaceae) is one of the admired medicinal plants widely used in India as traditional medicine for the treatment of various ailments (Das et al., 2009; Dhankhar et al., 2011). The use of herbal drugs having long history and are popularly used in most parts of the world in cure of various human diseases. Recently in allopathic medicine, nearly a quarter of the drugs prescribed to patients are of plant origin (Newman and Cragg, 2020). Traditional methods of collection, storage and marketing in developing countries make the raw materials of herbal drugs prone to fungal and mycotoxin contamination (Ałtyn and Twarużek, 2020; Chen et al., 2020). The fungal and

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mycotoxin contamination adversely affects the medicinal components of herbal raw materials (Pallares et al., 2022) and also harmfully affect the health of patients rather than cure (Omotayo et al. 2019; de Sousa Lima et al., 2020).

Among storage moulds, different species of *Aspergillus* potentially contaminate stored herbal raw materials and adversely affect their medicinal potency by biotransformation of active components (Mishra, et al., 2015; Çorbaci, 2020). Toxigenic Aspergilli secrete aflatoxins in stored products which cause aflatoxicosis in human as well as in animals (Benkerroum, 2020; Kumar et al., 2022). Aflatoxin B_1 (AFB_1) is classified as group-1 human carcinogen due to its potent hepatocarcinogenic and immunosuppressive nature (Marches et al., 2018).

The application of synthetic fungitoxicants is not desirable for the protection of herbal raw materials due to their harmful health effects, resistance development in treated microorganisms and residual toxicity (Rajkumar et al., 2019). Now days, plant based formulations are promoted as safe alternatives of synthetic preservatives, and US-FDA has tagged these green preservatives under GRAS (generally recognized as safe) category. The bioactive components viz. phenolics and terpenoids of a number of essential oils (EOs) have been documented as antibacterial (Pandey et al., 2017), fungitoxicant (Basak, 2018), mycotoxin suppressor (Lasram et al., 2019), insecticidal (Pavela et al., 2019) and effective antioxidants (Maggi et al., 2019). TALENT, EcoSMART and EcoPCOR are some essential oil based formulations largely used in food and agriculture industries (Singh et al., 2019). Therefore, the present investigation explores the possibility of exploitation of *Ocimum canum* L. leaf essential oil (OcEO) as phytopreservative in control of fungal deterioration of *J. adhatoda* raw materials during storage. In addition, safety limit of OcEO was also assessed by acute oral toxicity on mice for possible recommendation as a green preservative.

MATERIALS AND METHODS

Collection and preparation of herbal raw materials

Stored herbal raw materials of *J. adhatoda* were locally procured from herbal drug markets of Varanasi districts of Uttar Pradesh, India, during August, 2021. The procured raw materials were collected in disinfected polythene pockets to prevent new contamination. The raw herbal drugs were grinded into fine powder using pre-sterilized pulverizer. The powdered materials were kept at 5±2ºC for pH, moisture content and mycological analysis (Kumar et al., 2013).

pH and moisture content determination

Aqueous suspensions of powdered drug samples (1:10; w/v) were formed and stirred for 5 h, and the pH was determined using digital pH meter. To determine moisture content, weighed amount of samples were dried at 100ºC until their weights remained constant and per cent moisture content was calculated as following Kedia et al. (2015)

$$\% \text{ moisture content} = \frac{\text{Undried sample wt. - Dried sample wt.}}{\text{Undried sample wt.}} \times 100$$

Mycological analysis of raw materials of J. adhatoda

Fungal spores/conidia associated with stored raw materials of *J. adhatoda* were assessed by serial dilution technique (Mishra et al., 2015). The isolated mycobiota were identified on the basis of colony shape, colony colour (front and reverse), thallus

and conidial characteristics (Ravimannan et al., 2016). The identified fungal species were sub-cultured on potato dextrose agar (PDA) at 4ºC. The relative densities of mycobiota occurring on stored herbal raw materials of *J. adhatoda* were calculated following Kedia et al. (2015).

$$\text{Relative density of fungus (\%)} = \frac{\text{No. of isolates of a fungus}}{\text{Total no. of isolates of all fungi}} \times 100$$

Isolation of AFB_1 producing strain

In order to access AFB_1 synthesizing property, 20 of the *A. flavus* isolates were randomly chosen following TLC procedure of Mishra et al. (2015). Fifty µl conidial suspension (≈10^6 conidia/ml) of selected *A. flavus* isolates were separately inoculated in 49.5 ml SMKY (Sucrose, $MgSO_4$, KNO_3, and Yeast extract in 200g, 0.5g, 0.3g, and 7.0g, respectively in 1L distilled water) broth medium in conical flask (150 ml) and mixed appropriately followed by incubation at 27±2 °C for 10 days. After incubation, content of each conical flask (≈50 ml) was filtered and separated filtrate was extracted with chloroform (40 ml) in a separating funnel to dissolve AFB_1 in chloroform. The separated chloroform extract was evaporated near dryness on water bath at 60-70°C. After evaporation, chloroform extract residue was re-dissolved in 1 ml chloroform and 50 µl of it was spotted on TLC plate (20×20 cm^2 of silica gel). The TLC plates were developed in toluene: isoamyl alcohol: methanol (TIM) mobile phase (90:32:2; v/v/v) and intensities of AFB_1 in the form of fluorescent blue spots were observed under ultra-violet fluorescence analysis cabinet at 360 nm (Dwivedy et al., 2018). The fluorescent AFB_1 spots were scrapped and dissolved in cold MeOH (5 ml), and centrifuged for 5 min at 3000 rpm. The optical density of supernatant was recorded at wavelength of 360 nm. (Dwivedy et al. (2018) was followed to calculate AFB_1 content.

$$\text{AFB1 (µg/L)} = \frac{\text{Absorbance x molecular weight of AFB1 (312)}}{\text{Molar extinction coefficient of AFB1 (21800) x path length (1 cm)}} \text{ x } 1000$$

Extraction of O. canum leaf essential oil (OcEO)

The leaves of *O. canum* were collected from Dafi, Varanasi for essential oil (EO) extraction. The Urban Flora of Varanasi (Dubey and Kishore, 2021) was used to identify the collected plant and its voucher specimen (Lam/O-117/2021) was deposited in the herbarium of botany department, Banaras Hindu University. The collected leaves were washed in tap water followed by hydro-distillation using Clevenger's apparatus for EO extraction. The EO was passed through anhydrous sodium sulphate to eliminate water traces, and kept at 4ºC (Dwivedy et al., 2018).

GC-MS analysis of OcEO

Chemical profile of OcEO was performed at Central Institute of Medicinal and Aromatic Plants, Lucknow, India. To determine chemical profile, OcEO was subjected to GC-MS (Perkin Elmer Turbomass Gold MA, USA) using 60m × 0.32mm × 0.25mm capillary column. The GC was performed with injection temperature 250ºC; detector temperature 270ºC; column temperature program isotherm at 70ºC for 2 min, 3ºC/min gradient to 250ºC, isotherm duration was 10 min and flow rate of carrier gas (He) was 1 ml/min. The components of OcEO were identified by comparing their retention times and mass spectra with authentic reference compounds in the literature available in mass spectral libraries of Wiley, NIST and NBS (Adams, 2007).

Fungitoxic spectrum of OcEO against some storage fungi

Fungitoxic efficacy of OcEO was also recorded against 15 storage moulds viz. *Alternaria alternata*, *Aspergillus candidus, A. flavus, A. fumigatus*, *A. nidulans, A. niger*, *A.paradoxus, A. terreus*, *A. versicolor, Cladosporium cladosporioides, Curvularia lunata*, *Fusarium nivale, F. oxysporum*, *Penicillium italicum* and *Trichoderma viride* recovered from *J.adhatoda* herbal raw materials through poisoned food assay following Prakash et al. (2012). Requisite amount of OcEO dissolved separately in 0.5 ml of 5% tween-20 mixed with 9.5 ml PDA medium in different presterilized Petri dishes to attain final concentrations i.e. 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.5 and 2.0 mg/ml. The negative control sets without OcEO were kept parallel to the treatment sets along with positive controls with prevalent synthetic fungicides, SAAF (Carbendazim 12% + Mancozeb 63%) and Bavistin (Carbendazim 50% WP). A fungal disc (5 mm diameter) of each test fungus was inoculated on PDA in Petri dishes (15 cm diameter) separately followed by incubation at 27±2 °C for 10 days. After incubation, minimum inhibitory concentration (MIC) was recorded (Prakash et al., 2012).

Antifungal and aflatoxin inhibitory efficacy of OcEO

Fungitoxic and aflatoxin inhibitory efficacy of isolated OcEO was tested against the toxigenic strain of *A. flavus* LHPJA-09 using SMKY broth as nutrient medium following Mishra et al. (2015). Requisite amount of the OcEO dissolved separately in 0.5 ml of 5% tween-20 were pipetted aseptically to different pre-sterilised Erlenmeyer flasks (150 ml) containing 49.5 ml of SMKY broth to procure the final concentrations viz. 0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml. The flasks without OcEO were treated as control sets. Then, flasks were inoculated aseptically with 50 µl conidial suspension (≈10^6 conidia/ml) of toxigenic strain *A. flavus* LHPJA-09 prepared in 0.1 % Tween-80 (Rosengaus et al., 2000) and incubated for 8 days at 28 ± 1°C. The content of flask was filtered and mycelia were oven dried at 100°C untill their weight remained constant for biomass determination. Mycelial biomass of treatment as well as control sets was measured and per cent inhibition in mycelial biomass was determined as follows-

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

Where,

dc = Average dry weight (g) of fungal mycelium in control sets

dt = Average dry weight (g) of fungal mycelium in treatment sets

The filtrates of control and treated sets were extracted separately with 40 ml chloroform in a separating funnel to quantify the AFB_1 production. AFB_1 production in each set was estimated by aforementioned technique of Dwivedy et al. (2015).

Effect of OcEO on ergosterol content and leakage of cellular cations of A. flavus LHPJA-09

The impact of OcEO on ergosterol in the plasmalemma of *A. flavus* LHPJA-09 was assessed following Tian et al. (2012). Required amount of OcEO was dissolved separately in 0.5 ml tween-20 (5%) was mixed with SMKY broth (49.5 ml) in Erlenmeyer flasks (150 ml) to procure the final concentrations i.e. 0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml. The control flask had no

OcEO. Conidial suspension (100 µl) of *A. flavus* LHPJA-09 was inoculated separately in each flask which was incubated for 1 week at room temperature. The content of treated and control sets were filtered and obtained mycelia were washed using distilled water. The fungal biomass (fresh) of each set was determined and mashed separately using mortar and pestle. Five ml alcoholic KOH (25%) was added to individually mashed biomass and subjected to vortex mixing for 2 min followed by water bath incubation at 80ºC for 4 hrs. To extract sterols, a combination of n-heptane (5 ml) and distilled water (2 ml) was supplemented to each incubated sample and vortex mixed for 2 min and kept to settle down for 1 h. After settlement, the collected n-heptane layer was analysed between 240 to 300 nm wavelengths by UV-Vis scanning spectrophotometry. The presence of ergosterol is indicated by characterized by four peaked curve.

The leakage of cellular cations from *A. flavus* LHPJA-09 cells fumigated with OcEO was determined by method adopted by Helal et al. (2007). Predetermined biomass of *A. flavus* LHPJA-09 was transferred into 20 ml 0.85% saline solution and fumigated with OcEO of requisite concentrations viz. 0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml for 24 h incubation at 25±2 °C temperature. Thereafter, fumigated biomass was filtered and filtrate was analysed for leakage of cations (Ca^{2+}, K^{+} and Mg^{2+}) through atomic absorption spectrophotometry.

Free radical scavenging activity of OcEO

The antioxidant activity of the OcEO was determined by DPPH radical scavenging assay on TLC as well as its free radical scavenging activity was measured through spectrophotometery following Tepe et al. (2005). The extent of colour change of the purple-coloured DPPH solution to yellow colour clearly indicated that OcEO exhibited free radical scavenging activity. Different graded concentrations (1.0 to 10.00 µg/ml) of OcEO were added separately to 4% methanolic solution of DPPH (5 ml) and kept at 27 ºC. After 30 min, the absorbance was recorded at 517 nm. DPPH free radical inhibition with decline in absorbance was used to evaluate their antioxidant activity following Sharififar et al., (2007). The free radical scavenging activity was also compared with synthetic antioxidants used as positive control viz. Butylated hydroxyanisole (BHA), Butylated hydroxytoluene (BHT) and ascorbic acid. Per cent free radical scavenging activity (FRSA) was calculated using the following formula, where A is the absorbance

$$\% \text{ FRSA} = (A_{control} - A_{sample}/ A_{control}) \times 100$$

In situ antifungal activity

To determine the antifungal efficacy of OcEO during storage, 500 g of surface sterilized raw materials of *J. adhatoda* were kept separately in five different plastic containers having aerial volume 2.0 litres. Each container was inoculated with 1 ml spore suspension (≈10^6 spores/ml) of *A. flavus* LHPJA-09. Four containers were fumigated with 0.5 mg/ml w/v (MIC against *A. flavus*) OcEO while one container run parallel as control without OcEO treatment. All the containers were sealed and stored for six months at room temperature i.e. 27±2ºC (Bajpai and Kang, 2012; Kumar et al., 2013). After storage, mycological analysis of raw materials for *A. flavus* colonization was performed Mishra et al. (2015).

HPTLC analysis of raw materials for vasicine

The OcEO treated and untreated *J. adhatoda* raw materials of *in situ* study were subjected for HPTLC (high performance thin layer chromatography) analysis following Srivastava et al. (2009). Raw materials were powdered (100 mesh) separately and

5.0 g of each were soaked in 20 ml acetone (AR) for 60 min. After saturation, raw materials filtered and filtrates were evaporated to dryness by rotary evaporation. Dried acetone extract (10 mg) was dissolved in acetone (10 ml) to prepare a standard solution (1 mg/ml). Whereas, procured vasicine (1,2,3,9-Tetrahydropyrrolo[2,1-b]quinazolin-3-ol) from Sigma-Aldrich (Germany) was used as reference by preparing standard solution in acetone (1 mg/ml).

Chromatography was performed on glass backed HPTLC plates (20 cm × 10 cm) coated with 0.25 mm layer of silica gel Si60 F_{254} (Merck, Germany). Standard solution (vasicine) and acetone extracts of treated and untreated samples of known concentrations were applied to plate as bands (6 mm width) with the help of automatic TLC applicator (Camag Linomat 5), with the nitrogen flow of delivery speed of 150nL/s from the application syringe. The plates had been developed to a height of 80 mm using mobile phase of chloroform: ethanol: acetic acid (95: 4: 5; v/v/v) in a glass twin furrow chamber (20 cm × 10 cm). After removing plates from the chambers, completely air dried at room temperature (27°C) and peak areas for samples and standard were recorded by densitometry in absorbance/ reflectance mode with slit dimensions 6 mm × 0.45 mm at 260 nm, using TLC scanner 3 (Camag) equipped with Camag Wincats software version 4.06.

Safety limit determination of OcEO

The safety limit of OcEO was determined by acute oral toxicity recording LD_{50} value (where 50% test animals were killed) on mice (*Mus musculus* L.) with an average weight ≈35 g and age 3-4 months. Requisite amounts of OcEO was mixed separately with tween-80 and distilled water (2:1) to prepare different solutions containing desired dose of OcEo viz. 50, 100, 150, 200, 300, 400, 450 and 500 mg/ml for each set of mice (12 mice per set). The different solution of OcEO (500 µl) was orally administered to each set of mice separately. In control sets, 500 µl stock of tween-80 and distilled water (2:1) without OcEO was given to mice. The percent mortality of mice in each set was recorded after 24 h and LD_{50} was recorded following Ogbuehi et al. (2015).

Statistical analysis

The experiments were performed in triplicate and data expressed as Mean ± SE. One way ANOVA ($P < 0.05$) and Tukey's multiple range tests were analysed using SPSS (version 16.0).

RESULTS

The collected raw materials of *J. adhatoda* were found associated with spores of various storage moulds due to appropriate pH (6.78±0.11) and relatively higher moisture content (20.66±1.82 %). During mycological analysis, total 1296 fungal isolates were recovered and among them *A. flavus* was found to be the most dominant fungus exhibited highest relative density (26.01%) followed by *Cladosporium cladosporioides* (24.61%) and *A. niger* (21.76%) while *A. paradoxus* exhibited the lowest (0.62%) relative density (Table 1). Thirty five percent isolates of randomly selected *A. flavus* were found toxigenic and *A. flavus* LHPJA-09 was selected as test fungus due to its higher potential of afltoxin B_1 production (2267.009 µg/l) presented in Table 2.

The OcEO was extracted through hydrodistillation and characterized with its pungent smell, yellow green colour and 0.96 % yield (w/w). The GC/GC-MS analysis of OcEO showed 31 considerable peaks. The GC-MS analysis exhibited E-Citral (30.31%) as major component followed by Z-Citral (18.23%), Epoxy ocimene (5.77%), Trans carvone oxide (4.01%), 3-

Heptadecen-5-yne (3.90%), β-Pinene (3.40%), β-Costol (2.59%), Chrysanthenone (2.22%), Tetrahydroionone (2.11%), Caryophyllene oxide (2.04%) and 2,3,4,5-Tetramethyl cyclopent-2-en-1-ol (2.03%) were recorded as major components. Rest other identified components were found in trace amount (Table 3; Fig. 1).

Table 1: Mycological screening of *J. adhatoda* herbal raw materials

Isolated Fungi	No. of isolates	Relative density (%)
Alternaria alternata	16	1.25
Aspergillus candidus	14	1.09
A. flavus	337	26.41
A. fumigatus	91	7.13
A. nidulans	17	1.33
A. niger	282	22.10
A.paradoxus	08	0.63
A. terreus	23	1.80
A. versicolor	14	1.09
Cladosporium cladosporioides	319	25.0
Curvularia lunata	56	4.39
Fusarium nivale	09	0.71
F. oxysporum	21	1.64
Penicillium italicum	23	1.80
Trichoderma viride	07	0.54
Mycelia sterilia (Unidentified)	39	3.05
Mucorales Genera	(5) Genera	
Total isolates	**1276**	

Table 2: Detection of aflatoxigenic potential of *A. flavus* isolates

Strain	Toxigenicity	AFB_1 content (µg/l)
A. flavus LHPJA-01	Non-toxigenic	-
A. flavus LHPJA-02	Non-toxigenic	-
A. flavus LHPJA-03	Toxigenic	1156.403
A. flavus LHPJA-04	Toxigenic	1511.339
A. flavus LHPJA-05	Non-toxigenic	-
A. flavus LHPJA-06	Non-toxigenic	-
A. flavus LHPJA-07	Non-toxigenic	-
A. flavus LHPJA-08	Toxigenic	1671.633
A. flavus* LHPJA-09	**Toxigenic**	**2267.009**
A. flavus LHPJA-10	Non-toxigenic	-
A. flavus LHPJA-11	Non-toxigenic	-
A. flavus LHPJA-12	Toxigenic	1064.807
A. flavus LHPJA-13	Non-toxigenic	-
A. flavus LHPJA-14	Non-toxigenic	-
A. flavus LHPJA-15	Toxigenic	1797.578
A. flavus LHPJA-16	Non-toxigenic	-
A. flavus LHPJA-17	Non-toxigenic	-
A. flavus LHPJA-18	Non-toxigenic	-
A. flavus LHPJA-19	Toxigenic	904.514
A. flavus LHPJA-20	Non-toxigenic	-

*The strain in bold is most toxigenic

OcEO exhibited a broad spectrum antifungal activity against storage fungi isolated from *J. adhatoda* raw material. The MICs of OcEO against tested storage moulds ranges between 0.4 to 0.7 mg/ml. The fungitoxic efficacy of OcEO was also comparable to tested prevalent synthetic fungicides i.e. SAAF and Bavistin (Fig. 2). The OcEO completely checked the biomass production of *A. flavus* LHPJA-09 at 0.5 mg/ml as well as absolutely inhibited the AFB_1 production at 0.3 mg/ml concentration (Fig. 3).

Table 3: Chemical composition of OcEO by GC-MS analysis

RT	Compounds	Percentage
7.201	3-Methyl-2-heptanone	0.18
8.401	2-Methylcyclopentanol acetate	0.35
10.476	2,6-Dimethyl-2,7-octadiene-1,6-diol	0.15
11.177	6-Methyl-5-hepten-2-one	0.22
11.301	4-Methyl-1-buten-1-yl ester pentanoic acid	0.13
12.951	Linalool oxide	0.59
14.826	Caryophyllene diepoxide	0.87
15.501	Linalol oxide	0.97
15.901	β-Pinene	3.40
19.226	Epoxy linalol	0.21
19.401	2-Nonyne	0.36
20.351	D-Nerolidol	1.10
21.751	4-Methyl-4-hepten-3-ol	0.79
21.926	Linalyl propionate	0.50
22.551	**Z-Citral**	**18.23**
23.126	Linalyl acetate	0.40
23.301	3-Methyl-6-(1-methylethyl)-2-cyclohexen-1-one	0.43
23.951	**E-Citral**	**30.31**
24.451	Epoxy ocimene	5.77
24.676	Trans carvone oxide	4.01
25.376	Tetrahydroionone	2.11
26.526	Lancifolol	0.03
27.101	Caryophyllene oxide	2.04
27.926	Neric acid	0.35
28.776	3-Heptadecen-5-yne	3.90
30.326	Chrysanthenone	2.22
31.401	2,3,4,5-Tetramethyl cyclopent-2-en-1-ol	2.03
38.276	β-Costal	2.59
58.202	Citronellyl acetate	0.78
60.652	α-Farnesene	0.98
61.727	3,7-Dimethyl-2,6-octadienal	0.53

RT = Retention Time; Compounds in bold are major components

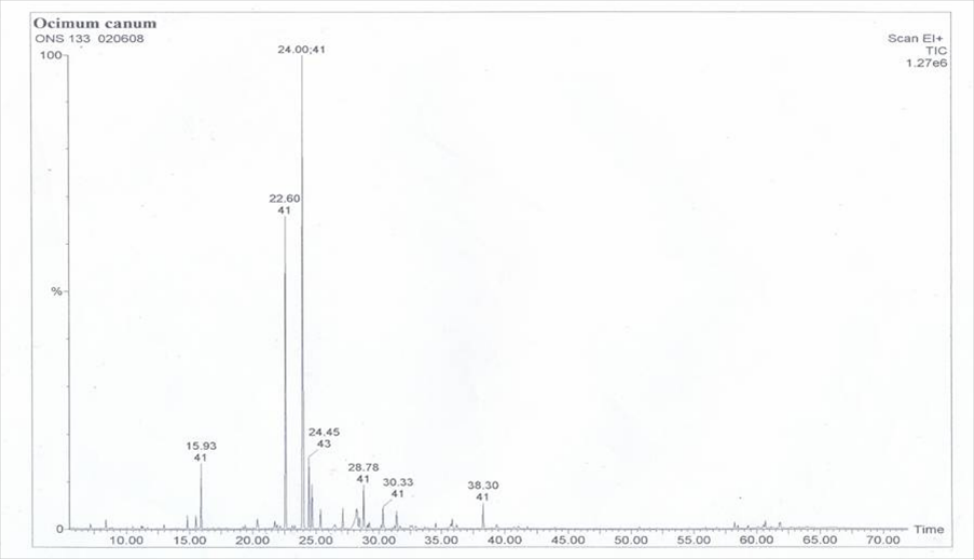

Figure 1: GC-MS Chromatogram of OcEO

Ergosterol content of cell membrane was also determines and found decreasing with increasing concentration of OcEO. The per cent inhibition of ergosterol contents at 0.1, 0.2, 0.3 and 0.4 mg/ml concentration was 38.5%, 73.1%, 84.4% and 95.7% respectively (Table 4, Fig. 4). In addition, leakage of cations (Ca^{2+}, K^{+} and Mg^{2+}) through plasma membrane with increasing OcEO concentration was also observed in contrast to control (Fig. 5).

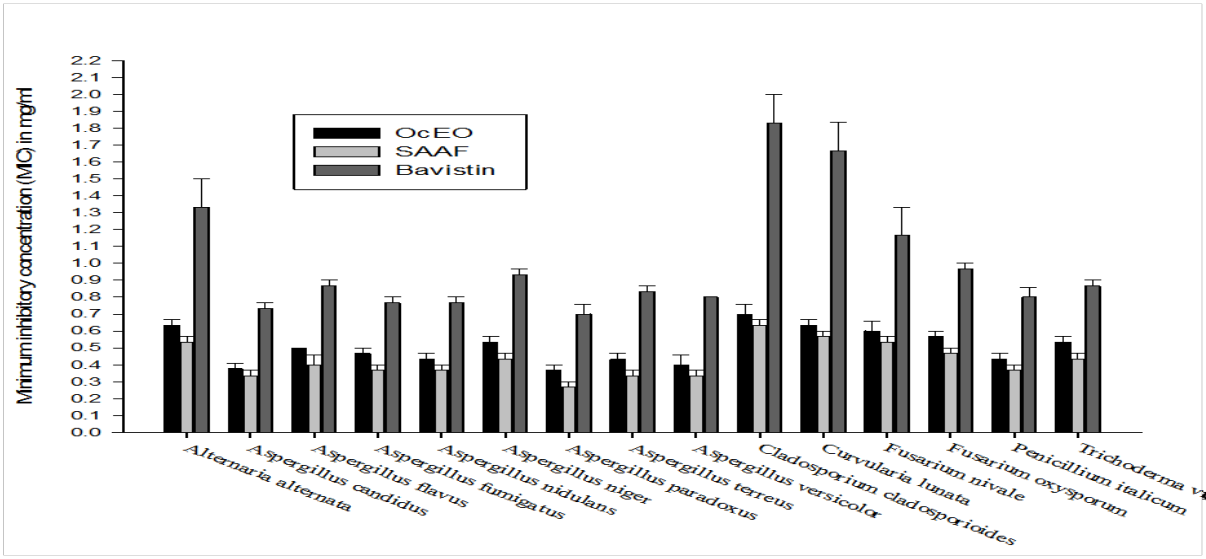

Figure 2: Fungitoxic spectrum of OcEO against some storage moulds and its comparative fungitoxicity with prevalent synthetic fungicides

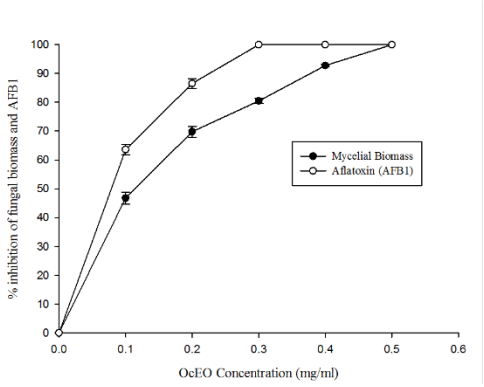

Figure 3: Effect of OcEO concentrations on *A. flavus* LHPJA-09 biomass and AFB_1 production

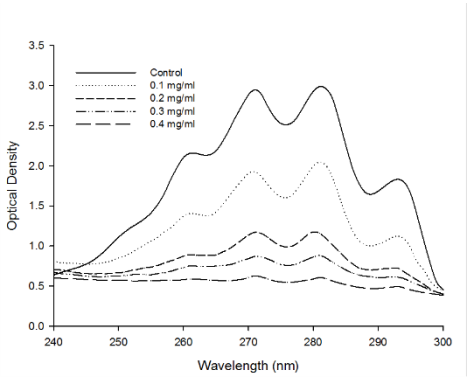

Figure 4: Effect of OcEO concentrations on ergosterol content in cell membrane of *A. flavus* LHPJA-09

The development of yellow spots due to bleaching of purple colour of DPPH confirmed the positive antioxidant activity of OcEO. The IC_{50} values of OcEO and prevalent synthetic antioxidants are summarized in Fig 6. The OcEO exhibited significant free radical scavenging activity as its IC_{50} value (4.3 µg/ml) was found higher than ascorbic acid (2.9 µg/ml) and lower than BHT (6.6 µg/ml) and BHA (6.3 µg/ml). The OcEO drastically reduces the *A. flavus* population in four different fumigated containers i.e. 73.87, 78.40, 80.02 and 77.12% at concentration of 0.5 mg/ml (Table 5). The HPTLC profile (Fig. 7) and densitometric chromatogram (Fig. 8) showed that vasicine content was degraded drastically by fungal invasion. Inoculated raw

materials fumigated with OcEO (sample 1, 2, 3 and 4) contains vasicine content 3.068, 3.370, 4.090 and 3.349 µg/ml respectively while in control, vasicine was not detected. The calibration curve of vasicine was linear and the value of its determination via height was: R_f, 0.29; regression equation, Y = 300.287 + 27.692x; r^2, 0.769 and via area was R_f, 0.30; regression equation, Y =5666.404 + 1263.822x; r^2, 0.907 (Table 6). Furthermore, LD_{50} of OcEO was recorded as 6050.36 mg/kg body weight during safety limit trials on mice indicating a non-mammalian toxic nature of the oil (Fig. 9).

Table 4: Inhibitory effect of OcEO on ergosterol synthesis

S. No.	Conc. of OcEO (mg/ml)	Per cent (%) Inhibition in ergosterol content
1.	Control	-
2.	0.1	38.5
3.	0.2	73.1
4.	0.3	84.4
5.	0.4	95.7
6.	0.5*	No biomass

* = MIC (Minimum inhibitory concentration)

Table 5: Per cent inhibition in *A. flavus* isolates after six months fumigation of OcEO (0.5 mg/ml)

Samples	No. of *A. flavus* isolates	Per cent inhibition
Control	861	-
J. adhatoda raw material-1	225	73.87
J. adhatoda raw material-2	186	78.40
J. adhatoda raw material-3	172	80.02
J. adhatoda raw material-4	197	77.12

Table 6: R_f values and linear regression data of HPTLC determination of vasicine

Compound	Regression via	R_f	Regression equation	r^2
Vasicine	Height	0.29	Y = 300.287 + 27.692x	0.769
	Area	0.30	Y = 5666.404 + 1263.822x	0.907

Y = densitometric response; x = concentration; r^2 = correlation coefficient

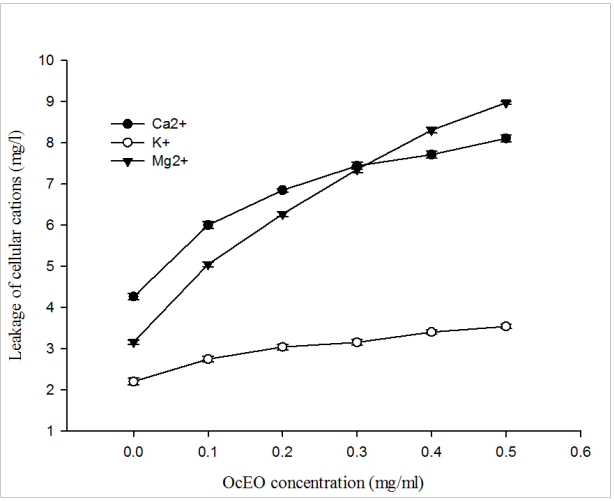

Figure 5: Effect of OcEO concentrations on leakage of cations from *A. flavus* LHPJA-09 hypha

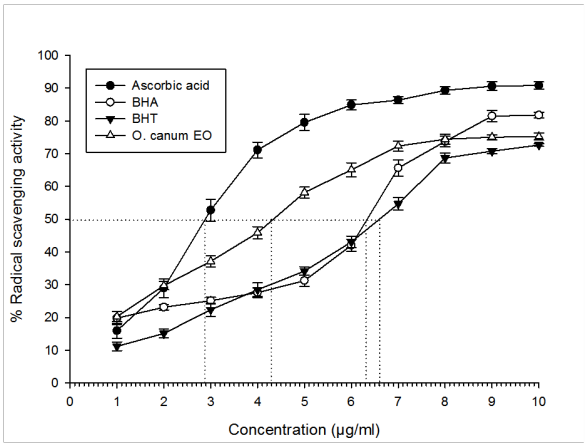

Figure 6: Comparative free radical scavenging activity of OcEO with synthetic antioxidants

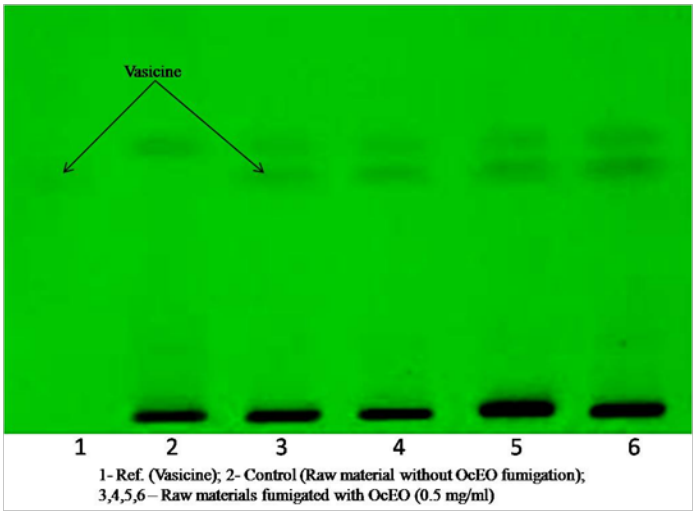

Figure 7: HPTLC profile of fumigated and non-fumigated raw materials of *J. adhatoda*

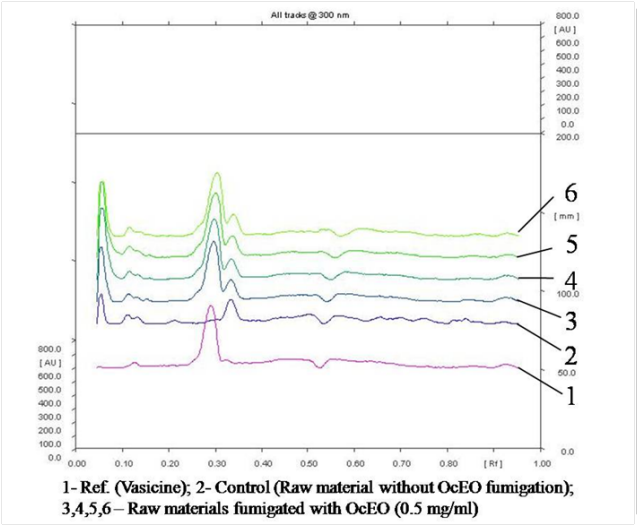

Figure 8: Densitometric scan (at 260 nm) showing vasicine peaks in fumigated samples

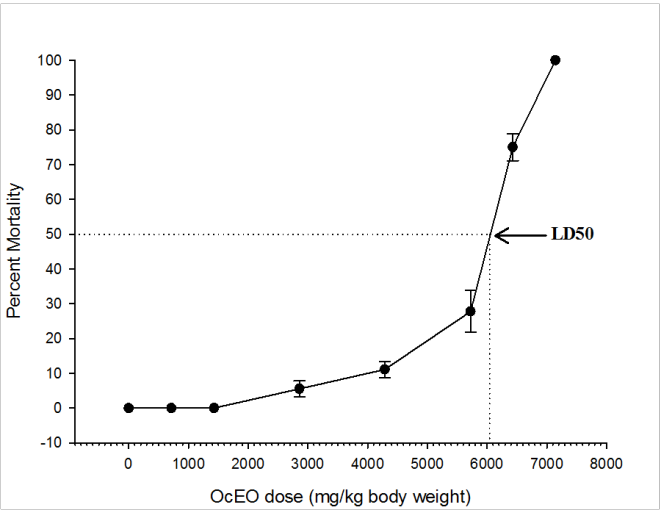

Figure 9: LD_{50} value of OcEO on mice by Acute oral toxicity

DISCUSSION

In Indian subcontinent, stored raw materials of medicinally important plants are usually associated with various moulds and their mycotoxins due hot and humid climatic conditions (Benkerroum, 2020). Fungal contamination degraded the medicinal potency of herbal raw materials (Qin et al., 2020) and rendering them unfit for human consumption. To minimize such storage losses appropriate quality control measures should be searched. Recently, for long term storage of many products various synthetic fungicides are frequently used having residual toxicity, hence, their application on the herbal raw materials would not be desirable (Rajkumar et al., 2019).

A number of literatures reveal that some higher plant essential oils can be exploited as plant based fungitoxicant against several moulds and their mycotoxin production (Lasram et al., 2019; Oliveira et al., 2020). An effort was made to evaluate the antifungal efficacy of OcEO and its possible applicability in control of post-collection fungal deterioration of herbal raw materials during storage and also enhancing their market value. Some earlier reports exhibited that the MIC of OcEO (0.5 mg/ml) against *A. flavus* was lower than some earlier reported EOs such as *Cymbopogon flexuosus* (Kumar et al., 2009), *Curcuma longa* (Hu et al., 2017), *Pimenta dioica* (Chaudhari et. al., 2020), *Coriandrum sativum* (Das et al., 2019), *Artemisia nilagirica* (Kumar et al., 2020) etc. The OcEO exhibited remarkable broad spectrum fungitoxicity against all the tested fungal species isolated from *J. adhatoda* raw materials and which is comparable to two prevalent synthetic fungicides viz. SAAF and bavistin. Hence, the OcEO may be recommended for complete protection of stored commodities from the fungal infestation at low concentration.

Furthermore, OcEO also exhibited pronounced efficacy in checking AFB_1 production by *A. flavus* LHPJA-09. The mycelial biomass and AFB_1 production exhibited a significant decreasing trend with increasing OcEO concentration, i.e. reduction of mycelial biomass causes noteworthy reduction in AFB_1 production. Reduction in fungal biomass and AFB_1 production may be due to some phenolics and terpenes present in the OcEO (Josselin et al., 2022). Citral, a well known antifungal agent (Tang et al., 2018; Cai et al., 2019) is major component (E-Citral, 30.31% and Z-Citral, 18.23%) of OcEO, may play a promising role in its fungitoxicity. The major component of OcEO is different from earlier findings where, camphor (Pragadheesh et al., 2013), linalool (Pandey et al., 2014), eucalyptol (Hzounda et al., 2016), thymol (da Silva et al., 2018), etc. were reported as major components. Such variation in composition is mainly due to the age of the plant, season of collection, geographical area and soil characteristics (Heikal , 2017; Rawat et al., 2020).

Among sterols of fungal cells, ergosterol is specific providing membrane integrity, flexibility and stability of membrane linked enzymes (Chellappandian et al., 2018) and significant alteration of its biosynthesis adversely affect fungal growth (Bhattacharya et al., 2020). The decrease in ergosterol level with increasing OcEO concentration clearly denotes that the bioactive components of OcEO targeted the cell membrane. Cations viz. Ca^{2+}, K^{+} and Mg^{2+} present in cytoplasm are essential for metabolic activity of cells (Pilchova et al., 2017; Roy et al., 2020). Therefore, reduction in ergosterol biosynthesis as well as leakage of cations through cell membrane would be the key factor in fungal growth inhibition and thus, suggesting plasma membrane as an imperative site for antifungal action of EOs (Kedia et al., 2015; Dwivedy et al., 2018). The injurious effects on ergosterol biosynthesis as well as enhanced efflux of cations may lead to reduction in nutrient uptake and ATPase dependent metabolic activities which might finally cause cell death (Lambert et al., 2001).

Stored plant parts are also deteriorated by free radical mediated oxidation of unsaturated lipids during storage (Porter et al., 1995; Ahmed et al., 2016). Oxidative stress stimulates *A. flavus* to produce more AFB_1 during storage (Grintzalis et al., 2014) which results quantitative as well as qualitative losses to stored products and reduces shelf life. The OcEO exhibited significant radical scavenging activity in concentration dependent manner. The IC_{50} value (4.3 µg/ml) of OcEO was found quite lower than some earlier reported EOs (Mishra et al., 2015) and also comparable to synthetic antioxidants (Dwivedy et al., 2018). Owing to free radical scavenging activity, the OcEO may serve as a plant based antioxidant in shelf life enhancement as well as protection from oxidative stress by decelerating oxidative rancidity of lipids. The presence of various phenolic compounds and/or synergistic effect among compounds play major role in antioxidant activity of EOs (Fadel et al., 2020; do Nascimento et al., 2020).

To assess the practical applicability of OcEO, the herbal raw materials of *J. adhatoda* were fumigated with OcEO. The substantial reduction in number of *A. flavus* isolates indicates its suitability to control fungal contamination of raw herbal drugs. The protection of medicinal component (vasicine) by OcEO from fungal deterioration strengthens its exploitation as an appropriate fungitoxicant for the protection of herbal raw materials. The OcEO exhibited higher LD_{50} value than some plant based preservatives viz. carvone (1640 mg/kg), pyrethrum (350–500 mg/kg), and organic acids viz. sorbic acid (3200 mg/kg), benzoic acid (2500 mg/kg), and acetic acid (3530 mg/kg) (Isman, 2006; Prakash et al., 2012). The well known importance of OcEO in traditional medicine and high LD_{50} (6050.36 mg/kg) strengthens its exploitation as safe, green preservative to minimize mycological infestation in herbal raw materials during storage (Kumar et al., 2009; Nyarko et al., 2002).

CONCLUSION

The findings of present investigation reveal that, herbal raw materials of *J. adhatoda* get contaminated with various moulds and their mycotoxins. The OcEO exhibited strong antifungal, antiaflatoxigenic and antioxidant activity as well as higher LD_{50} value on test animals strengthen its possible exploitation as an indigenous plant-based green preservative. The fumigation of stored herbal raw materials by essential oils enhances their shelf life by protecting their medicinal value.

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