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Isolation of *Aspergillus flavus* from stored food commodities and *Thymus vulgaris* (L.) essential oil used as a safe plant based preservative

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ABSTRACT

Grain samples of *Cicer arietinum* (Chickpea), *Zea mays* (Maize), *Cajanus cajan* (Pigeon pea), *Hordeum vulgare* (Barley), *Oryza sativa* (Rice) and *Sorghum vulgare* (Millet) were procured from various retailers of market were subjected to their mould profile. During mycoflora analysis, 1297 fungal isolates were recorded from the food commodities. The least number of fungal isolates (189) were detected from *H. vulgare* while highest (244) from *Z. mays*. The genus *Aspergillus* was found to be most dominant encountered in all the samples, followed by *Cladosporium cladosporoides, Alternaria alternata* and *Penicillium species*. The highest percent relative density was recorded in case of *Aspergillus flavus* (36.24) followed by *A. niger* (28.45) and *C. cladosporoides* (10.95) while the lowest was found in case of *Trichoderma viride* (1.16). Some of the *A. flavus* isolates were toxigenic secreting aflatoxin B_1. The survey reveals that the contamination of food commodities with storage fungi and mycotoxin is alarming and appropriate quality control measures should be taken urgently. The essential oil of *Thymus vulgaris* L. showed highest antifungal efficacy. The thyme oil absolutely inhibited the mycelial growth of A. flavus at 0.7 µl ml^{-1}. The oil also showed significant antiaflatoxigenic efficacy as it completely arrested the aflatoxin B_1 production at 0.6 µl ml^{-1}. Thyme oil as fungitoxicant was also found superior over most of the prevalent synthetic fungicides. The findings recommend the thyme oil as potential botanical preservative in eco-friendly control of biodeterioration of food commodities during storage.

KEYWORDS: Aflatoxin B_1, *Aspergillus flavus*, Food commodities, Mycoflora, *Thymus vulgaris*

INTRODUCTION

Experts believe that 20–60 percent of stored food commodities are lost by stored grain pests viz. insects, fungi, bacteria, rodents etc. (Raja et al., 2001). Post harvest deterioration causes economic losses due to obvious decay and adverse changes in the odour, taste, appearance and nutritive values. In case of severe infection the quality of the commodity gets deteriorated and some times the seeds lose their viability (Jilani et al., 1989). High temperature and relative humidity as well as moisture contents of the stored products are favorable to the development of the pest organisms.

The decomposers of the food grains i.e. fungi and bacteria are always present on the food grains in dormant conditions (usually spores/conidia) and grow under

favorable climatic conditions (Girish, 1986). The fungal growth may cause decrease in germinability (Sinha and Sinha, 1993), decolouration of grains, heating and mustiness, loss of weight, biochemical changes and production of toxins. Climatic conditions in India are most conducive for mould invasion and elaboration of mycotoxins.

Mycotoxins are a group of highly toxic secondary metabolites of fungi capable of causing disease and death in humans and other animals. Thus mycotoxins are insidious poisons (Pitt, 2004). Cereals and grains are major mycotoxin vectors because they are consumed both by humans and animals (Pfohl-Leszkowicz, 2000). Approximately 25–40% of cereals world wide are contaminated with mycotoxins (Pittet, 1998).

Among the mycotoxins, aflatoxins chiefly produced by strains of Aspergillus flavus are the most dangerous and about 4.5 billion people in underdeveloped countries are exposed to aflatoxicoses (Williams et al., 2004). Aflatoxicosis is the poisoning that results from ingesting aflatoxins. Aflatoxins are hepatotoxic, hepatocarcinogenic, mutagenic agents causing immunologic consequences, lipid peroxidation and oxidative damage to DNA (Williams et al., 2004). Synthetic chemicals such as fungicides/ preservatives have been used for a long time and have greatly contributed in management of such losses. The application of such chemicals has led to a number of environmental and health problems due to their residual toxicity, carcinogenicity, hormonal imbalance and spermatotoxicity (Kumar et al, 2007). Because of indiscriminate use, some microorganisms have developed resistance to most widely used synthetic fungitoxicants rendering them out of date (Wilson et al, 1997). Hence, there is a need to develop new fungicides/preservatives with improved performance as well as ecofriendly in nature.

Therefore, in the present piece of investigation essential oils (EOs) of some angiosperms have been investigated for their fungitoxicity against the toxigenic strain of *A. flavus*, a potent post harvest storage fungus of deteriorating cereals and pulses. In addition, the essential oil of Thymus vulgaris L. has been investigated regarding its potential to inhibit fungal growth, aflatoxin production and also found superior over most of the prevalent synthetic fungicides.

MATERIALS AND METHODS

Collection of stored grain samples and preparation

Stored grain samples of *Cicer arietinum* (Chickpea), *Zea mays* (Maize), *Cajanus cajan* (Pigeon pea), *Hordeum vulgare* (Barley), *Oryza sativa* (Rice) and *Sorghum vulgare* (Millet) were collected from various retailers of market of Gwalior, India. These grain samples were chosen on the basis of their availability in the market and popularity of uses. The grain samples were collected in sterilized polythene bags to avoid further contamination. In the laboratory, grains were finely ground in a common household blender. Before using the blender's cup was rinsed in 90 percent alcohol. The powder was sieved through No. 50 mesh sieve, kept tightly packed in a new paper bag and stored at 5°C for further analysis (Mandeel, 2005).

Mycological analysis of food commodities

Ten gram of powdered sample of each food commodities was added separately to 90 ml sterile 0.85 percent saline solution in 250 ml Erlenmeyer flask and thoroughly homogenized on electric shaker with constant speed for 15 min. Fivefold serial dilutions were then prepared following Aziz *et al.* (1998). One ml of suitable dilution (10^{-4}) of each powdered grain suspension was used separately to inoculate Petri dishes containing 10 ml freshly prepared Potato Dextrose Agar medium (Potato, 200 g; Dextrose, 20 g; Agar, 18 g; Distilled water 1000 ml; pH, 5.6±0.2), then plates were incubated at 27±2 °C for 7 days and examined daily but counts were recorded only after 3–4 days. After incubation, the plates were examined visually and with the help of compound microscope. Morphologically different mould colonies were individually subculture on PDA medium. Identification of fungal species was done by culture and morphological characteristics (Moubasher, 1993).

Description of test fungus

Aspergillus flavus has a world-wide distribution and normally occurs as a saprophyte in soil and on many kinds of decaying organic matter. Colonies are granular, flat, often with radial grooves, yellow at first but quickly becoming bright to dark yellow-green with age. Conidial heads are typically radiate, mostly 300–400 um in diameter, later splitting to form loose columns, biseriate but having some heads with phialides borne directly on the vesicle.

Evaluation for Antifungal screening of essential oils from higher plants and Selection of test plant

Different parts of some aromatic angiospermic taxa of the locality were collected for the extraction of essential oils. The oils were extracted from the highly aromatic parts of the plants. The extraction of essential oils was performed by hydrodistillation method using Clevenger's apparatus. This apparatus offers several advantages viz. Compactness, complete distillation and separation of the

essential oil and an accurate determination of the recovery of the essential oil content using small quantities of plant material. This method does not involve any risk of loss of the active constituents of the samples. Therefore, isolation of essential oil plants was done through hydrodistillation by Clevenger's apparatus in the present investigation. The isolated fractions of plant parts exhibited two distinct layers an upper oily layer and the lower aqueous layer. Both the layers were separated and the essential oils were stored in clean glass vials after removing water traces with the help of capillary tubes and anhydrous sodium sulphate.

The fungitoxicity of isolated essential oils was tested against toxigenic strain of *A. flavus* at 1.0 $\mu l ml^{-1}$ following poisoned food technique (Pandey and Dubey, 1994) using PDA as nutrient medium.

Antifungal and Antiaflatoxigenic property of Thyme essential oil

Antifungal as well as antiaflatoxigenic efficacy of thyme essential oil was determined by culture of toxigenic *A. flavus* in SMKY broth medium separately (Diener and Davis, 1966). The method followed by Sinha *et al.* (1993) was adopted for the estimation of aflatoxin B_1. Different concentrations of the oil viz. 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 ppm were prepared by dissolving separately their requisite amount in 0.5 ml acetone and then mixing it with 24.5 ml of SMKY medium in 100 ml Erlenmeyer flask. For control set requisite amount of sterilized distilled water in place of oil was added to the medium. After pouring flasks were aseptically inoculated separately with 5 mm diameter disc of seven days old culture of the toxigenic *Aspergillus flavus* isolated from selected food commodities samples. The flasks were incubated for 10 days at 27±2 °C in incubation chamber.

Chemical characterization of thyme oil through GC-MS analysis

GC-MS analysis of oil samples was done at Central Institute of Medicinal and Aromatic Plants, Lucknow, India. The analysis was carried out on Perkin-Elmer Turbomass/Auto XL system using a PE-5 (50 · 0.32 M, 0.25 l film thickness) capillary column with oven temperature programmed from 100 to 28 °C at 3 °C/ min initial temperature holder of 20 min. Helium was employed as carrier gas at 10 psi inlet pressure and spectra generated at 70 eV. Identification of compounds was carried out by comparing the MS of each peak with those of authentic reference compounds shown in the literature (Adams, 1995).

The GC-MS analysis of thyme oil showed that oil contained many compounds among them thymol was identified as major component and its antifungal property was also investigated.

Detection of toxigenic strains of A. Flavus and estimation of aflatoxin B_1

Aflatoxin B_1 producing potential of different cultures of *A. flavus* isolates during mycoflora analysis was tested in SMKY medium (Sucrose, 200g; $MgSO_4.7H_2O$, 0.5g; KNO_3, 0.3g; Yeast extract, 7.0g; Distilled water, 1000ml) (Diener and Davis 1966). The method followed by Sinha et al. (1993) was adopted for the estimation of aflatoxin B_1. 25 ml of medium was taken in 100 ml Erlenmeyer flask and inoculated separately with 5 mm diameter disc of seven days old culture of the *A. flavus* isolated from selected raw herbal drug samples. The flasks were incubated for 10 days at 27±2 °C. After incubation content of each flask was filtered through Whatman filter paper no. 1. The filtered mycelium was dried at 100 °C for 24 h and their biomass was determined.

The filterate was extracted with 20 ml chloroform in a separating funnel. After separation chloroform extract was passed through anhydrous Sodium sulphate kept in Whatman filter paper no. 42. The extract was evaporated till dryness on water bath at 70 °C. The amount of aflatoxin B_1 was determined by TLC technique. The residue left after evaporation was dissolved in 1 ml chloroform and 50 µl of chloroform extract spotted on TLC plate (20×20 cm^2 of silica gel) then developed in Toluene:Isoamyl alcohol: Methanol; (90:32:2;v/v/v) solvent system proposed by Reddy et al. (1970). The intensity of aflatoxin B_1 was observed in Ultra Violate Fluorescence Analysis Cabinet at an excitation wavelength of 360 nm (AOAC 1984). The presence of aflatoxin B_1 was confirmed chemically by spraying trifluoroacetic acid. For quantitative estimation, spots of aflatoxin B_1 on TLC were scraped out and dissolved in 5 ml cold methanol, shake and centrifuge at 3000 rpm for 5 min. Optical density of supernatant was recorded at the wavelength of 360 nm and the amount of aflatoxin B_1 was calculated following Sinha et al. (1993).

$$\text{Aflatoxin B1 content } (\mu g/kg) = \frac{D \times M}{E \times l} \times 1000$$

Where,

D absorbance

M molecular weight of aflatoxin B_1 (312)

E molar extinction coefficient of aflatoxin B_1 (21,800) and

l path length (1 cm cell was used)

Comparative efficacy of the fungitoxicity of thyme essential oil with prevalent synthetic fungicides

The fungitoxic potential of thyme EO was compared with of some prevalent synthetic fungicides/preservatives

viz. Benzimidazole (Benomyl) (United Phosphorus Limited, India), Zinc dimethyl dithiocarbamate (Ziram), Diphenylamine (DPA) and Phenyl mercuric acetate (Ceresan) (BASF India Limited, India) by recording the MIC following aforementioned poisoned food technique.

RESULTS

During mycoflora analysis, 1297 fungal isolates were recorded from food commodities (Table 1. The least number of fungal isolates (189) were detected from *H. vulgare* while highest (244) from *Z. mays*. The genus *Aspergillus* (with five species) was found to be the most dominant encountered in almost all the samples, followed by *C. cladosporoides, A. alternata* and *T. viride*. Some mucorale were also isolated. The relative density values (%) of each fungal species was calculated and ranged from 1.16% to 36.24% (Table 1. The highest percent relative density was recorded in case of *A. flavus* (36.24), followed by *A. niger* (28.45) and *C. cladosporoides* (10.95). The lowest relative density was found in case of *T. viride* (1.16) followed by *F. nivale* (1.46). The occurrence frequency (%) of recovered mycoflora on each food commodities was calculated and found lowest (14.57%) in case of *H. vulgare* while highest (18.81%) in *Z. mays*. Rest of the samples shared intermediate percent occurrence frequency (Table 1).

During study only *Thymus vulgaris* exhibited absolute fungitoxicity at 1.0 µlml^{-1}. *Ageratum conyzoides* (81.17%), *Hyptis suaveolens* (85.67%), *Lantana indica* (80.20%), and *Ocimum gratissimum* (87.03%) exhibited good fungitoxic activity. *Commiphora mukul* (53.53%), *Eucalyptus citriodora* (62.17%) and *Eupatorium cannabinum* (56.97%) showed moderate fungitoxicity while *Artemisia vulgaris* (25.67%), and *Citrus reticulate* (21.63%) showed poor fungitoxicity (Table 2).

Table 2: Antifungal screening of some higher plant essential oils of different family against *Aspergillus flavus*

Plant Name	Family	Antifungal activity (%) (1.0 µl ml^{-1})
Aegle marmelos	Rutaceae	72.50 ± 3.17
Ageratum conyzoides	Asteraceae	81.17 ± 4.08
Artemisia vulgaris	Asteraceae	25.67 ± 3.37
Callistemon lanceolatus	Myrtaceae	67.83 ± 3.47
Citrus reticulata	Rutaceae	21.63 ± 2.86
Commiphora mukul	Buseraceae	53.53 ± 4.06
Curcuma longa	Zingiberaceae	79.77 ± 3.45
Cymbopogon citratus	Poaceae	62.37 ± 3.08
Eucalyptus citriodora	Myrtaceae	62.17 ± 3.16
Eupatorium cannabinum	Asteraceae	56.97 ± 2.89
Hyptis suaveolens	Lamiaceae	85.67 ± 2.60
Lantana indica	Verbenaceae	80.20 ± 3.49
Murraya koenigii	Rutaceae	37.50 ± 2.49
Ocimum gratissimum	Lamiaceae	87.03 ± 1.88
Thymus vulgaris	Lamiaceae	100.00 ± 0.00
Zingiber officinalis	Zingiberaceae	79.63 ± 3.55

Values are mean (n = 3) ± standard error

The mycelium growth and aflatoxin B_1 production were recorded to decrease on increasing the concentrations of the oil. It is evident from that in case of thyme oil at 0.7 µlml^{-1} mycelium growth was absolutely inhibited while aflatoxin B_1 production was checked even at 0.6 µlml^{-1} (Table 3). While in case of thymol, mycelium growth was completely checked at 0.2 µlml^{-1} but hundred percent inhibition of aflatoxin B1 production was recorded at 0.1 µlml^{-1}. Both mycelial biomass and aflatoxin B1 production exhibited a statistically significant declining trend with increasing concentration of the oil (Table 4). The GC-MS analysis of thyme oil showed that oil contained many compounds among them thymol was identified as major component.

Table 1: Mycoflora analysis of Distribution of fungi isolated from selected food commodities their relative density and frequency of occurrence

Name of food commodities	Fungal isolatesa													Total isolates	Frequency of occurence
	Af	An	Afu	Ac	P	Fn	Fo	Tv	Aa	Cl	Cc	Ms	Mucorales[b]		
C. arietinum (L.)	103	27	8	14	12	5	8	6	7	-	26	3	(4) Genera	219	16.88
Z. mays (L.)	156	32	4	7	-	-	6	4	10	3	21	1	(3) Genera	244	18.81
C. cajan (L.)	48	93	9	3	5	11	14	-	8	4	36	-	(5) Genera	231	17.81
S. vulgare (PERS.)	27	86	18	11	9	2	7	5	12	7	17	3	(2) Genera	204	15.73
H. vulgare (L.)	19	108	13	-	17	-	5	-	6	6	11	4	(3) Genera	189	14.57
O. sativa (L.)	117	23	8	-	11	1	6	-	8	5	31	-	(2) Genera	210	16.19
Total isolates	**470**	**369**	**60**	**35**	**54**	**19**	**46**	**15**	**51**	**25**	**142**	**11**		**1297**	
Relative densities (%)	**36.24**	**28.45**	**4.63**	**2.70**	**4.16**	**1.46**	**3.55**	**1.16**	**3.93**	**1.93**	**10.95**	**0.85**			

[a]Fungal isolates: *A.f.— Aspergillus flavus; A.n.- Aspergillus niger; A.fu.— Aspergillus fumigatus; A.c.- Aspergillus candidus; P.s.— Penicillium sp.; F.n.- Fusarium nivale; F.o.— Fusarium oxysporum; T.v.- Trichoderma viride; A.a.- Alternaria alternate; C.l.- Curvularia lunata; C.c.- Cladosporium cladosporoides, M.s.- Miscellaneous;*

[b]Mucorales including *Rhizopus nodosus, Rhizopus* sp., *Mucor* sp., *Mortierella* sp. and *Absidia corymbifera* were recovered but not included among the total isolates.

(−) Not Detected

Table 3: Antifungal and Antiaflatoxigenic efficacy of Thyme essential oil

Treatment	Biomass (g)	Mean ± SD	Optical density	Aflatoxin content (µg/kg)	Mean ± SD
	0.329		0.029	332.036	
Control	0.291	0.311±0.019	0.031	354.935	343.485±11.449
	0.314		0.030	343.486	
	0.231		0.020	228.990	
100 ppm	0.217	0.224±0.007	0.021	240.440	225.173±17.489
	0.224		0.018	206.091	
	0.183		0.014	160.293	
200 ppm	0.201	0.187±0.012	0.013	148.843	148.843±11.449
	0.178		0.012	137.394	
	0.162		0.008	91.596	
300 ppm	0.134		0.007	80.146	
	0.142	0.146±0.014	0.007	80.146	83.962±6.610
	0.099		0.003	34.348	
400 ppm	0.117	0.109±0.009	0.005	57.247	41.981±13.220
	0.112		0.003	34.348	
	0.017		0.001	11.449	
500 ppm	0.023	0.020±0.003	0.002	22.899	15.265±6.610
	0.020		0.001	11.449	
	0.012		0.000	0.000	
600 ppm	0.009	0.013±0.006	0.000	0.000	0.0 ± 0.0
	0.017		0.000	0.000	
	0.000		0.000	0.000	
700 ppm	0.000	0.0±0.0	0.000	0.000	0.0 ± 0.0
	0.000		0.000	0.000	

Table 4: Antifungal and Antiaflatoxigenic efficacy of Thymol

Treatment	Biomass (g)	Mean ± SD	Optical density	Aflatoxin content (µg/kg)	Mean ± SD
	0.329		0.029	331.992	
Control	0.291	0.311±0.019	0.031	354.888	343.440±11.448
	0.314		0.030	343.440	
	0.042		0.000	0.000	
100 ppm	0.028	0.032±0.007	0.000	0.000	0.000±0.000
	0.026		0.000	0.000	
	0.000		0.000	0.000	
200 ppm	0.000	0.00±0.00	0.000	0.000	0.000±0.000
	0.000		0.000	0.000	

The MIC of thyme oil against *A. flavus* was 700 ppm, which is lesser and found economical than tested prevalent synthetic fungicides. In the present investigation MIC of different prevalent synthetic fungicides were compared with MIC of thyme oil. The MIC of Benomyl and Ziram was found to be more than 5000 ppm. DPA completely inhibited the growth of *A. flavus* at 2000 ppm. Ceresan absolutely inhibited the growth of *A. flavus* at 1000 ppm. The observations are presented in (Table 5). As fungitoxicant *Thymus vulgaris* essential was recorded to be better (700 ppm) than most of the synthetic fungicides compared while thymol major constituent of thyme oil showed MIC at 200 ppm.

DISCUSSION

Higher incidence of aspergilli on the food commodities compared to other fungal forms in the present investigation may be due to their saprophytic nature and ability to colonize diverse substrate because secretion of various hydrolytic enzymes by these moulds as has been reported by De Souza (2005). Similarly the higher relative frequency of *A. flavus* and *A. niger* than the remaining fungal species supports the earlier observations made by Roy and Chourasia (1990).

Attempts to control post harvest biodeterioration of various substrates have been carried out by the use of

Table 5: Comparative efficacy of the fungitoxicity of thyme essential oil and thymol with prevalent synthetic fungicides

Fungicides/oil	MIC in ppm against *A. flavus*
Benzimidazole (Benomyl)	>5000
Diphenylamine (DPA)	2000
Phenyl mercuric acetate (Ceresan)	1000
Zinc dimethyl dithiocarbamate (Ziram)	>5000
Thymus vulgaris essential oil	700
Thymol	200

different chemicals. The widespread use of synthetic pesticides have led to the development of resistance, harmful effects on non-target organisms (Coats, 1994) persistence in the environment and bioaccumulation in food web and other side effects on the health of a range of organisms including human and also their cost. Adverse effects of chemical pesticides on environment and human health are burning issues and there is a need to search for a superior and eco-friendly substitute. Therefore, it was thought desirable to find out the efficacy of some higher plant products in control of biodeterioration of some agricultural products. Hence, there is a growing interest to discover safer alternatives for pest control. Because of greater consumer awareness and concern regarding synthetic chemical additives, foods preserved with natural additives have become popular. With the limitations on the use of current pest control methods, there is scope for the discovery of safe, non-polluting, biorational pest management technologies for stored products. Although different fungi have been taken during screening of higher plant products by various workers, in the present study *A. flavus* was selected as test fungus since it causes severe deterioration of several agricultural products and produces aflatoxins which are hazardous to the health of animals and human beings.

Among the 16 essential oils tested during screening program, only *Thymus vulgaris* showed absolute fungitoxicity against *Aspergillus flavus* at 1.0 µl ml^{-1}. Hence, the oil of *Thymus vulgaris* was selected for further investigations. Also in the present investigation *Thymus vulgaris* was selected on the basis of its medicinal importance. Thyme usually used in various disorders as medicine but literatures related to its antifungal activity is still fragmentary. *Thymus vulgaris* (L.) is an aromatic herb belonging to family Lamiaceae. Mostly growing and cultivated in temperate region but poorly distributed in subtropical regions. The plant is characterized with woody, quadrangular stem, leaves simple, exstipulate, inflorescence verticillaster, flower pinkish, bracteate, complete, bisexual, pentamerous, hypogynous and zygomorphic.

The quantitative estimation of aflatoxin B_1 elaboration done in the present study reveals that the samples were highly contaminated by the toxigenic strains of *A. flavus*. The aflatoxin B_1 level recorded in the present study is much higher than the safest limit (20 µg/kg) as recommended by WHO (Mishra and Das 2003).

The thyme essential oil may be recommended for large scale application as a plant based preservative for stored food items because of its strong antifungal as well as antiaflatoxigenic efficacy. Because of broad antimicrobial spectrum, more efficacies over prevalent synthetic preservatives, the thyme essential oil may be formulated as a safe and economical plant based preservative against post harvest fungal infestation and aflatoxin contamination of food commodities.

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