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Full Length Article

Isolation of Stored Maize Mycoflora, Identification of Aflatoxigenic Fungi and its Inhibition using Medicinal Plant Extracts

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Abstract

Aflatoxigenic fungi are well known and most hazardous contaminants of perishables. This study was designed to screen aflatoxigenic fungi among all isolated mycoflora from stored maize samples and their growth inhibition by using herbal means. The isolated fungi belong to six fungal genera among which *Aspergillus* stood as the most dominant genera. The detection of aflatoxigenic fungi was carried out by direct visual cultural method. *A. flavus* showed very strong florescence response on coconut cream agar (CCA) media which indicates its aflatoxigenic behavior. Further, 45 *A. flavus* isolates were subjected to molecular characterization by using RAPD-PCR techniques. Molecular studies showed diversity among different isolates. These isolates were further checked for their potential to produce aflatoxins using HPLC. Quantitative analyses indicated that all isolates of *A. flavus* can potentially produce AFB1 and AFB2 while none of the isolate was found capable of producing AFG1 and AFG2. Isolates 24, 31, 37, 42 and 44 were ranked as hyper-producing isolates because of which they were further used in inhibition studies. Aqueous extracts from ten medicinal plants were analyzed to evaluate their potential to restrict the mycelial growth of aflatoxigenic *A. flavus* isolates. Leaf extract of *Eucalyptus citriodora* showed highest growth inhibition (100 – 98%) which was followed by *Trachyspermum ammi* seeds and leaves extract of *Ocimum basilicum* i.e., 90.8–91.8% and 82.8– 87.7%, respectively. Hence these plant extracts can be a convenient, cost effective and biologically safe approach to prevent fungal contamination in stored agricultural commodities. © 2018 Friends Science Publishers

Keywords: Mycotoxins; RAPD-PCR; *Eucalyptus citriodora*; *Aspergillus flavus*; HPLC

Introduction

Pakistan is an agricultural country where cereals are the major crops. After wheat and rice, Maize (*Zea mays* L.) is ranked as the most significant cereal crop. It is also known as "the king of cereals" (Bukhsh *et al*., 2010). In last few years the consumption of maize as food, feed and in wet milling industry has increased many folds. The nutritional value of maize grains considerably decreases during subsequent storage and processing. Hence it becomes unfit for both human and animal ingesting as well as for industrial use. The losses arise mostly due to high moisture conditions, inappropriate drying, temperature fluxes, improper harvesting, intercropping with contaminated grains and poor storage conditions (Folayan, 2013). Maize predisposed these factors has high fungal contamination that causes the production of hazardous mycotoxins eventually resulting in the complete deterioration of grain (Dubale *et al*., 2014).

Worldwide more than 1/4th of the cereals are known to be contaminated with various mycotoxins. The source of these toxins are a variety of toxigenic fungi as *Aspergillus flavus* Link, *A. ochraceus* Wilhelm, *A. parasiticus* Speare, *Fusarium verticilliodes* (Sacc.) Nirenberg, *F. proliferatum* (Matsushima) Nirenberg and *Penicillium verrucosum* Dierckx. The spoilage percentage is maximum in subtropical and tropical regions of the world where 80% of the crops are ruined due to the presence of significant quantities of mycotoxins (Cardwell *et al*., 2000; Abbas *et al*., 2006). Among various fungi producing toxins, aflatoxins have been also appeared as an inquisitive option for chemical warfare due to their injurious effects on human, livestock and poultry. Eighteen different kinds of aflatoxins have been discovered in which most frequently reported are aflatoxin B1, B2 followed by G1, G2, M1 and M2 that are synthesized by *A. flavus* and *A. parasiticus* (Creppy, 2002).

Earlier workers from different parts of the world have documented higher quantities of aflatoxins in several perishables and food products that is a major concern for both consumers and food traders. Maize is one of the most vulnerable cereal crops in this context. Approximately 30% of maize samples collected from southeastern Romania were found contaminated with aflatoxin B1 (Tabuc *et al*., 2009). In another recent study Ghiasian *et al*. (2011) found that 58 to 80% of maize samples obtained from Mazandaran and

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Kermanshah provinces of Iran were contaminated with aflatoxins. From several maize fields in Hungary Dobolyi *et al*., 2011 isolated *A. flavus* as the most occurring fungi that resulted in production of aflatoxins.

Over the past decade, use of botanicals has come up as the most potential and safe approach for restricting the growth of these toxigenic fungi as compared to the synthetic fungicides that holds health hazards. Therefore antimicrobial potential of many plant extracts and their constituents have being investigated in keeping perishables safe from post-harvest storage fungi (Centeno *et al*., 2010). Herbal extracts are safe, easy to prepare and apply, biodegradable and lack residual effect. In the recent past several workers have documented antifungal potential of various plants. In Satish *et al*., 2007, analyzed 52 water based extracts of plants for their antimicrobial properties to restrict the growth of eight commonly occurring *Aspergillus* species. The study demonstrated that amongst selected plants, water based extracts of *Eucalyptus globules* Labill, *Achraszapota* (Linn.), *Lawsonia inermis* L. (henna), *Acacia nilotica* (Linn.), *Emblicao fficinalis* (*Gaertn.*), *Datura stramonium* (Linn.), *Peltophorum pterocarpum* (DC.) Baker ex. K. Heyne, *Mimusops elengi* (Linn.), *Prosopis juliflora* (Sw.) DC, *Polyalthia longifolia* Benth. and Hook, *Punica granatum* (Linn.) and *Syzigium cumini* (Linn.) showed extensive antifungal activity towards *Aspergillus* species. Similarly Reddy *et al*., 2009 documented that extracts of *Curcuma longa* (L.), *Syzigium aromaticum* (L.) Merr. Et Perry, *Ocimum sanctum* (Linn.) and *Allium sativum* L. can efficiently suppress the growth of *A. flavus*. In another study by Al-Rahmah *et al*. (2011) methanolic extracts from *Olea europaea* (Linn.), *Eucalyptus globolus, Salvadora persica* (Linn.), *Thymus vulgaris* (Linn.) and *Ziziphusspina-christi* (L.) Desf. Were scrutinized for antifungal and anti aflatoxigenic efficiency. According to their findings the *T. vulgaris* extract showed the highest antifungal and anti aflatoxigenic potential against *A. flavus*. Correspondingly Rajarajan *et al*. (2014) working on antimicrobial effect of *Hyptis suaveolens* (L.) Poit leaf extracts against *A. parasiticus* and *A. flavus* revealed that in a preliminary phytochemical screening, presence of various phytochemicals like tannins, volatile oil, saponins, starch, proteins fats, alkaloids and glycosides is responsible for inhibitory action against tested fungal species.

The main objective of this study was to explore the extent of mycoflora associated with stored maize in Punjab, Pakistan and to identify the toxicogenic species among them. The investigation was extended to find the genetic diversity among the various isolates of top most occurring toxicogenic fungi and to find an environment safe strategy to control their growth. Up to our knowledge such a study is not reported from this area before. Previous literature from Pakistan showed that Saleemi *et al*. (2012) isolated, identified, and checked the AFB_1synthesis potential of *A. flavus* isolated from maize samples but no inhibition study was performed on these isolates neither their genetic diversity was explored. Whereas, (Niaz and Dawar, 2009; Saleem *et al*., 2012; Hussain *et al*., 2013; Sultana *et al*., 2017), only isolated and identified maize mycoflora but these studies did not check their toxin production potential, molecular characterization and growth inhibition. While our study covered different ecological zones of Punjab, Pakistan via extensive sampling, hence it compares variations in different zones with variable environmental conditions. Ultimately, aqueous extracts of different parts of ten medicinal plants has been analyzed for their anti aflatoxigenic potential.

Materials and Methods

Following methods are described in step wise procedure.

Sampling

A total of seventy five stored maize samples were taken through extensive sampling from fifteen major maize growing districts of Punjab, Pakistan belonging to three agro-ecological zones.

Zone-1 (Southern irrigated zone)	Zone-2 (Northern irrigated zone)	Zone-3 (Arid rain fed zone)
i. Bahawalpur	vi. Lahore	xi. D.G Khan
ii. Bahawalnagar	vii. Faisalabad	xii. Bhakkar
iii. Multan	viii. Chiniot	xiii. Khushab
iv. Rahim yar khan	ix. Sialkot	xiv. Chakwal
v. Lodhra	x. Sahiwal	xv. Attock

These store houses were traditional large sheds commonly called house type godowns constructed with some variations to suit climatic and other conditions of the area. Varietal detail of maize sampled from these zones has been mentioned in Table 1.

Media Preparation

Following two media were used for fungal isolation from sampled maize

1. Malt Extract Agar (MEA) was prepared by adding 2% (w/v) malt extract agar obtained from Merck, UK and 1.5% agar in 1 L of distilled water.
2. Potato Dextrose Agar (PDA) was prepared by adding 20 g potato starch, 20 g dextrose and 15 g agar in 1 L of distilled water.

For the identification of toxicogenic fungi from the isolated mycoflora three coconut based media were prepared including coconut milk agar (CMA) medium, coconut agar medium (CAM) and coconut cream agar (CCA) medium (Davis *et al*., 1987; Dyer and McCammon, 1994).

Coconut agar medium (CAM): A readily accessible brand of desiccated coconut (carbohydrate 22.5 g, protein 2 g, fat 17.5 g/100 g as per company report) was acquired from local market. 100 g Dehydrated coconut powder was poured in a separating funnel containing 600 mL of hot water. Aclear layer of extract (350 mL) was gently separated from the

bottom of the funnel and then 1.5% agar was added. The pH of the medium was adjusted to 6.8 using 2N solution of NaOH.

Coconut cream agar (CCA): CCA medium was made using 50% (w/v) coconut cream (protein 2.13 g, carbohydrate 5.20 g/100 g, total fat 20.35 g, saturated fat 18.86 g) and adding it to 50% distilled water. Media was solidified by adding agar 1.5% (w/v).

Coconut milk agar (CMA): Coconut milk (protein 1.84 g, carbohydrate 3.01 g/100 g, total fat 17.84 g, saturated fat 15.68 g) was obtained locally. Its pH was adjusted to 6.8 using 2N solution of NaOH and then solidified using agar @ 1.5%.

The media was autoclaved (121ºC at 15Ibs/Inch^2 pressure) for 15–20 min and then was allowed to cool to 40–45ºC. Then streptomycin capsules were added to avoid any bacterial growth.

Isolation and Microscopic Identification of Mycoflora from Maize Samples

Maize storage fungi was isolated from collected samples through direct plating method. The collected maize samples were dipped in 1% sodium hypochlorite solution for 2 min to surface sterilize followed by several washings with sterile distilled water. Ten seeds from each sample were placed on PDA and MEA plates using sterilized forceps. The plates were then incubated at 25ºC for 7 days. The fungal colonies emerged were counted to calculate percentage frequency of occurrence by the given formulae (Ghiasian *et al*., 2011).

$$\text{Percentage frequency (\%)} = \frac{\text{No. of samples from which fungal species isolated}}{\text{Total number of samples}} \times 100$$

The fungal species which were isolated from tested samples were identified taxonomically according to their macroscopic features (colony color, diameter, texture) as well as microscopic characteristics (shape of conidia and conidiophore).

Identification of Aflatoxigenic Fungi

All isolated fungal species were inoculated on coconut agar medium (CAM), coconut cream agar (CCA) and coconut milk agar (CMA) media and then incubated in dark for one week at 28°C. The colonies were checked for aflatoxin production (Heenan *et al*., 1998) by examining reverse side of media plate under long wave ultraviolet (UV) light (365 nm) to check the blue fluorescence characteristic of aflatoxins.

Finding Genetic Diversity among Fungal Isolates

As *A. flavus* showed highest percentage of occurrence and showed strongest florescence response under UV radiation hence was choosen for further studies. The genetic diversity among geographically distant isolates of *A. flavus* showing morphological similarity was done using Random Amplified Polymorphic DNA (RAPD) technique. First total genomic DNA from all the isolates of *A. flavus* was extracted using CTAB method (Doyle and Doyle, 1990). DNA bands were visualized by using agarose gel electrophoresis.

Random Primer Screening

Random Amplified Polymorphic DNA (RAPD) analysis was done following the method of Ranganath *et al*. (2002). DNA extracted from *A. flavus* isolates were amplified through polymerase chain reaction using random primers. For the initial screening a set of 10 random decamers were obtained from SBS Genetech Co. Ltd-Beijing, China. Each primer was diluted up to 100 picomole before its use in RAPD analysis. Among these some primers successfully generated clear, differentiable and reproducible bands in various isolates of *A. flavus.*

List of primers used in RAPD analysis.

Name	Sequence (5'– 3')	MW	Nmol	GC (%)
SBSB01	GTT TCG CTC C	2970	111.1	60%
SBSB02	TGA TCC CTG G	3019	109.3	60%
SBSB03	CAT CCC CCT G	2923.9	112.9	70%
SBSB04	GGA CTG GAG T	3108.1	106.2	60%
SBSB05	TGC GCC CTT C	2955	111.7	70%
SBSB06	TGC TCT GCC C	2955	111.7	70%
SBSB07	GGT GAC GCA G	3093	106.7	70%
SBSB08	GTC CAC ACG G	3013	109.5	70%
SBSB09	TGG GGG ACT C	3084	107	70%
SBSB10	CTG CTG GGA C	3044	108.4	70%

Reaction Mixture for RAPD

Amplification reactions of RAPD were carried out in volumes of 25 µL comprising following components. Reaction mixture was prepared of on ice under sterile conditions.

Reagent	Final concentration	Amount per reaction (µL)
PCR Buffer {10X}	10X	2.5
$MgCl_2$ (25 mM)	1.5–3.0 mM	1.5
dNTPs (2.0 mM)	0.2 mM	2
RAPD Primer	100pMole⁄µL	1
Taq Polymerase (2.5U⁄µL)	1U	1
Double distilled deionized water		15
Genomic DNA	50ng	2
Final volume		25

RAPD Cycling Conditions and Analysis of Amplified DNA Fragments

RAPD amplification was done using PCR machine (Eppendorf) in which initial denaturation was done at 94ºC for 5 min. This was followed by 40 cycles of denaturation at 94ºC for 1 min, annealing (35ºC for 1 min), primer extension (72ºC for 1 min) and finally extension (72ºC for 15 min). The reaction was finally held at 4ºC. Amplification products were then analyzed through electrophoresis in 1% agarose. The amplified products were carried out in two replications with a negative control in each reaction.

Data Analysis

All RAPD profiles were recorded in a binary matrix using two discrete characters i.e., 0 for absence and 1 for presence of RAPD markers. The bands were calculated in agarose gels beginning from top to bottom of the lanes. The amplification profiles of all the isolates of *A. flavus* were then compared with one other. In order to evaluate the overall distribution of genetic diversity, data was analyzed through Multivariate Statistical Package software (MVSP).

Extraction of Aflatoxins from Isolates of *A. flavus*

Extraction of aflatoxins was carried out by solvent extraction method (Yazdani *et al*., 2010). For this allisolates of *A. flavus* were grown on coconut cream agar medium. Colony margins were scraped into a test tube (32 x 200 mm) having 10 mL chloroform: acetone in a ratio of 85:15 v/v. This suspension was again incubated at 25ºC for 30 min while agitating after every 5 min on a vortex stirrer. The extract was then filtered through Whatman No. 1 filter paper and the filtrate was evaporated to dryness under vacuum. The residue was re-suspended in methanol (1 mL) and filtered aseptically using syringe filter assembly of 0.2µm. The filtrate was kept at 4ºC and later was analyzed by HPLC system (Agilent 1100 series, Agilent Technologies, Santa Clara, CA, USA) equipped with a reversed phase C18 column (Merck, Darmstadt, Germany) and a fluorescence detector for quantification.

To analyze aflatoxins, a mobile phase consisting of water: methanol: acetonitrile (60:20:20) at a flow rate of 1 mL min^{-1} was applied, and aflatoxins were detected at excitation and emission wavelengths of 360 nm and 440 nm, respectively.

Selection of Medicinal Plants

Ten medicinally important plant species from eight different families were selected based on their well-documented antifungal components and easy availability for antifungal and detoxification trials

Sr. No	Botanical name	English name	Family	Plant part used
1	*Eucalyptus citriodora* H.	Eucalyptus	Myrtaceae	Leaves & Branch
2	*Trachyspermum ammi* L.	Ajwain	Umbelliferae	Leaves & seeds
3	*Foeniculum vulgare* M.	Fennel	Umbelliferae	Leaves & Stem
4	*Ocimum basilicum* L.	Sweet basil	Lamiaceae	Leaves & Branch
5	*Mentha arvensis* L.	Mint	Lamiaceae	Leaves & Stem
6	*Acacia nilotica* L.	Kikar	Mimosaceae	Leaves & Branch
7	*Cassia fistula* L.	Amaltas	Fabaceae	Leaves & Branch
8	*Nigella sativa* L.	Kalonji	Ranunculaceae	Leaves & seeds
9	*Azadirachtaindica* A.	Neem	Meliaceae	Leaves & Branch
10	*Allium sativum* L.	Garlic	Amaryllidaceae	Leaves & Bulb

Extract Preparation

The selected plant materials were collected from University of the Punjab, Lahore, Pakistan. The aqueous extract of medicinal plants was made by homogenizing the plant tissues (10 g leaves, branches, bulbs etc.) with sterile distilled water (10 mL) using an electric blender. Muslin cloth was used to filter the homogenate and centrifuged (14,000 rpm for 20 min). Finally, syringe filter assembly was used to sterilize the supernatant for downstream analysis.

Antifungal Assay

Antifungal potential of aqueous plant extracts to control aflatoxigenic *A. flavus* isolates was analyzed through fungal growth inhibition assay following Fiori *et al*. (2000). The filter-sterilized plant extracts were mixed with molten, autoclaved MEA medium presenting10% concentration. Aflatoxigenic isolates of *A. flavus* from 5 days old culture were inoculated centrally into the prepared plant extract ME agar plates. These plates were then placed at room temperature (28 ± 2˚C) and consequently scrutinized for growth of the inoculated fungi. After 4 days of incubation the colony diameter was measured by measuring the average radial growth. The percentage inhibition of fungal growth was calculated in contrast to the control using following equation. Each treatment was replicated thrice. In control plates fungal isolates were inoculated in MEA plates without any plant extract.

$$\text{Percentage of growth inhibition} = [(a-b)/a]\times 100$$

Where, a = mean diameter of fungal colony in control
b = mean diameter of fungal colony cultured in plates with plant extract

Statistical Analysis

Data acquired in all above trials were subjected to statistical analysis using DSSTAT software. Analysis of Variance (ANOVA) and differences among the means were calculated for significance at $P \leq 0.05$ using Tukey's test.

Results

Mycoflora Isolated from Aflatoxin Contaminated Maize Samples

According to the fungal isolation data 18 fungal species belonging to 6 different genera were isolated from samples of stored maize that were later characterized based on cultural and morphological characters observed under binocular microscope (Table 2 and 3). On the basis of frequency of occurrence Aspergillus was reported as the most dominating genera (57.65%) in stored maize samples (Table 2).

Seven different species of this genus were isolated among which *A. flavus* possessed highest frequency of occurrence of 60.0% hence stood as the most prevalent fungal species. Rest of the isolated species of Aspergillus followed this with 45.3% frequency of occurrence by *A. niger*, 41.3% by *A. terreus*, 32% by *A. japonicas*, 26.7% by *A. fumigatus*, 25.3% by *A. versicolar* and 5.3% by *A. oryzae* (Table 3).

Genus Penicillium followed this with a frequency of occurrence 24.10%. Among the isolated species of Penicillium the most common species were *P. herquei* and *P. claviforme* with frequency of occurrence 34.6 and 26.7% respectively. Whereas *P. aurantiogriseum* and *P. oxalicum* depicted 24.0 and 13.3% occurrence amongst the isolates of Penicillium genera (Table 3).

In addition, four different Fusarium species including *F. solani*, *F. guttiforme*, *F. subglutinans* and *F. globosum* were also identified. Highest percentage frequency of occurrence was shown by *F. guttiforme* (25.3%) followed by *F. solani* (18.7%), *F. globosum* (10.7%) and *F. subglutinans* (9.3%) respectively (Table 3). While, Alternaria, Curvularia and Cladosporium stood as the least occurring genera with percentages of occurrence 0.98, 0.98 and 0.65% respectively (Table 2).

Isolation of Aflatoxigenic Fungi by Direct Visual Method

Mycoflora producing aflatoxins was screened amongst isolated fungi from stored maize samples based on the florescence produced by toxigenic fungi visualized upon exposure to UV radiations at 365nm. For elimination of non-aflatoxigenic mycoflora through simple and reliable visual method, all the isolated fungi were grown on three different coconut media (coconut milk agar, coconut agar medium and coconut cream agar) to record florescence response of tested fungi under UV radiation.

Data showed variable levels of florescence on different coconut media by the toxigenic fungi. Three Aspergillus species produced clear blue florescence demonstrating significant levels of aflatoxin production when grown on coconut media. Amongst these, *A. flavus* presented strongest blue florescence in coconut cream agar media though its florescence progressively reduced in CMA and CAM respectively (Fig. 1). *A. terreus* and *A. japonicas* displayed no florescence when grown in CAM whereas weak florescence response was recorded in CMA and CCA media (Table 4). All the remaining isolated species from five fungal generas including Alternaria, Curvularia, Penicillium, Fusarium and Cladosporium originated as the non-synthesizers of aflatoxins because they failed to produce the characteristic blue florescence in all the three tested media. Recorded results revealed CCA as the most effective medium for the characterization of aflatoxigenic mycoflora in comparison to the CMA and CAM media.

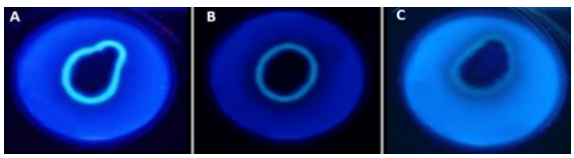

Fig. 1: Comparison of florescence by aflatoxigenic *Aspergillus flavus* grown on three coconut based media under UV radiation. Whereas, A: coconut cream agar medium (CCA); B: coconut milk agar medium (CMA); C: coconut agar medium (CAM)

Table 1: Varietal detail of maize sampled from three ecological zones of Pakistan

	Zone 1	Zone 2	Zone 3
Agatii 2002	-	+	+
Agaitii- 85	-	-	+
Afgoye	+	+	-
Cargle-D-MS- 3937	+	-	+
Desi	+	-	-
FH-810 hybrid	+	-	+
FH-963 hybrid	+	-	-
FH- 793 hybrid	+	-	-
Golden 85	-	-	+
Hybrid 80y80	-	-	+
HY-1898 hybrid	+	-	-
HY-1921 hybrid	+	-	-
ICI 8464	-	+	-
ICI 984	+	-	-
Moncento 6525	-	+	-
Moncento 919	-	+	-
Moncento 6142	+	-	-
Moncento 979	+	-	+
MMRI yellow	-	+	+
Neelum	+	-	+
NK- 278	+	-	-
Pioneer 30y87	+	+	-
Pioneer 8711	-	+	-
Pioneer 32B41	+	+	+
Pioneer 32B33	-	+	+
Pioneer 6339	-	+	-
Pioneer 8441	-	+	-
Pioneer 31R88	+	-	-
R-886	+	-	+
Sahiwal 2002	+	+	+
Sanchanta NK- 6621	+	-	+
Sanchanta NK 6615	-	-	-
Supra	-	+	+
Sadaf	-	-	-
2166	-	+	-
2278 AS	+	-	-
6789	+	-	+
4881	-	+	+

Whereas, + shows presence and – shows absence of that variety in the mentioned zone

Table 2: Frequency of occurrence of different fungal genera isolated from maize grains

Fungal Genera isolated	Occurrence frequency of maize samples (%)
Aspergillus	57.65
Penicillium	24.10
Fusarium	15.64
Alternaria	0.98
Curvularia	0.98
Cladosporium	0.65

After the primary screening of aflatoxigenic fungi, *A. flavus* was chosen for next investigations firstly because it presented a strong blue florescence under ultraviolet radiation on CCA media and secondly because of its highest frequency of occurrence amongst all isolated mycoflora from stored maize samples.

Genetic Variation among Isolates of *A. flavus*

Molecular characterization of 45 isolated strains of *A. flavus*

Table 3: Morphological characterization of isolated mycoflora and their percentage frequency in maize samples collected from three zones of Punjab

Fungi	Macroscopic characters			Microscopic characters		No. of samples contain isolated fungi			% F
	Color	texture	Diameter (cm)	Conidiophore	Conidia	Zone 1	Zon 2	Zon 3	
Aspergillus flavus	Yellow green	powdery	6-7	Hyaline, long, rough, matulae and phailides covered with entire vesicle.	Sub-spherical, pale green, echinulate, 3.5 to 6µm in diameter	16	7	22	60.0
Aspergillus Niger	Black	powdery	5-6	Hyaline, thick walled, brownish, matulae and phailides covered with entire vesicle.	Globose, roughened, brown black, 6-7µm in diameter.	12	12	10	45.3
Aspergillus terreus	Orange brown	floccose	6-7	long, smooth, thick walled, hyaline	Globose to slightly ellipsoidal, smooth walled, 1.6-2.5µm in diameter.	11	11	9	41.3
Aspergillus fumigatus	Bluish green	velvety	7	Clavate vesicle. Thick walled	Globose to subglobose, smooth, echinulate, green 2.5-3µm in diameter	6	3	11	26.7
Aspergillus Oryzae	Grayish yellow to dull brown	floccose	5-6	hyaline, up to 4-5 mm in length, warty or rarely smooth	Globose to sub globose, 5-6 µm in diameter, green, smooth to finely rough-walled.	0	4	0	5.3
Aspergillus versicolar	Yellow green	velutinous	4-5	Smooth walled and brittle, hyaline to yellowish, length 500 – 700 µm long.	Globose, echinulate, 2-3µm in diameter.	4	5	10	25.3
Aspergillus japonicas	Dark brown to black	floccose	6-7	500-1000 µm tall, globose or echinulate, pigmented vesicle.	Globose, 3-3.5 µm in diameter, strongly echinulate	5	7	12	32.0
Penicillium aurantiogriseum	Greyish green	velvety	1.7-2.4	Biverticillate, Slender, ampulli form, smooth to roughen	Spherical to subspheroiddal, 3.6-4.0 µm, smooth.	6	1	11	24.0
Penicillium herquei	Yellow green to light olivaceous green	velvety	6-7	smooth, roughened to spinose, thin walled, biverticillate	Ellipsoidal, 3.5-4.0 x 2.2-3 µm in diameter, roughened, thin walled.	10	7	9	34.6
Penicillium claviforme	Grey-green	floccose	2.5-3	synnematous, smooth-walled, hyaline, irregularly arranged	Ellipsoidal, smooth walled, hyaline to greenish, 4.0-4.5 x 3.0-3.5 µm	5	5	10	26.7
Penicillium oxalicum	Dull green with pale blue green margins	velvety	3.5-5	Two stage branched phialides, cylindrical, tip distinctly tapering	elliptical, smooth, 4.5-6.5x 3-4µm.	4	1	5	13.3
Fusarium Solani	White to cream	floccose	4.5-5.5	Short and multi branched form sporodochia, hyaline, globose, smooth to roughened clamydospores present.	Macroconidia fusiform, curved, short, with blunt apical and pedicellate basal cells 28-42µm, Microconidia cylindrical to oval, one to two celled, 8-16µm	0	6	8	18.7
Fusarium Guttiforme	White later become grey or purple	velvety	3.5-4.5	Few or no sporodochia, no clamydospores.	Macroconidia thin walled, slender, straight to slightly curved, usually 3 septate. Microconidia are obovoid, polyphailides	9	7	3	25.3
Fusarium subglutinans	White, violet or pink	cottony	4.5	Mycelium erect strongly branched. Phialides 1-2, associated with sterile coiled hyphae. Sporodochia present verticillately branched. No clamydospores.	Microconidia are oval or elliptical, mono-cellular. Macroconidia are long, thin. 3 to 5 septa conidia with foot-shaped basal cells.	4	1	2	9.3
Fusarium globosum	Initially white, becoming pale to purple	floccose	1.6-2.7	Conidiogenous cells monophialidic & polyphialidic, formed on aerial hyphae on Conidiophores, hyaline.	Microconidia are clavate to ellipsoidal, globose borne on mono and poly phialides. Macroconidia curved, foot shaped.	1	7	0	10.7
Alternaria alternate	Creamy	cottony	6-7	Simple straight or curved, 1-3 septate to 50 µm long. 3-6 µm wide, with one of several apical conidiophores.	Ovoid, obclavate and dark brown in color, 5-9µm	0	1	2	4.0
Curvularia lunata	Brown to black	velvety	5	Geniculate, brown. Conidiogenous cells terminal, sympodial, cylindrical	Solitary, simple, often curved, clavate, obovoid or pyriform with 3 transverse septa, pale or dark brown, 20-30 µm.	1	2	0	4.0
Cladosporium cladosporioides	Olive green	velvety	5	Erect, straight or flexuous, unbranched or branched only in the apical region.	Formed in longbranched chains, mostly 0-1 septate, ellipsoidal to lemon-shaped, smooth walled, olivaceous-brown, 1-celled, 4-8 µm.	1	0	1	2.7

Whereas, % F = percentage frequency

was carried out through (randomly amplified polymorphic DNA) RAPD-PCR technique. For evaluating the genetic variation amongst the *A. flavus* isolates initially ten RAPD decamers were tested. Out of these ten tested primers, only three (Primer SBSB10, SBSB06 and SBSB01) successfully presented amplification in all of the 45 isolates of *A. flavus*.

DNA bands produced through above mentioned three primers were easily reproducible and scorable and their size ranged approximately from 200 bp to 2000 bp. The Fig. 2, 3 and 4 explains the banding patterns of the forty five isolates obtained through above mentioned three primers. Table 5 shows degree of polymorphism among the isolates. The average number of polymorphic bands per primer was 46.2. The data of the amplified bands was converted into binary form using multivariate statistical package computer software program where 1 represented the presence of amplified DNA band and 0 indicated the absence of band. A dendrogram was then made through UPGMA Euclidean analysis using the above-mentioned software program (Fig. 5).

Although degree of polymorphism recorded was low yet the tested isolates of Aspergillus species presented diversity among them. All the tested fungal isolates depicted more than 30% diversity thus authenticating that they all belongs to a single species. Genetic diversity recorded amongst the isolates from zone 3 was higher than those isolated from other two zones. For example the isolates 24, 31, 37 and 44 that exhibited more than 33% genetic diversity, belonged to the zone 3. This genetic diversity

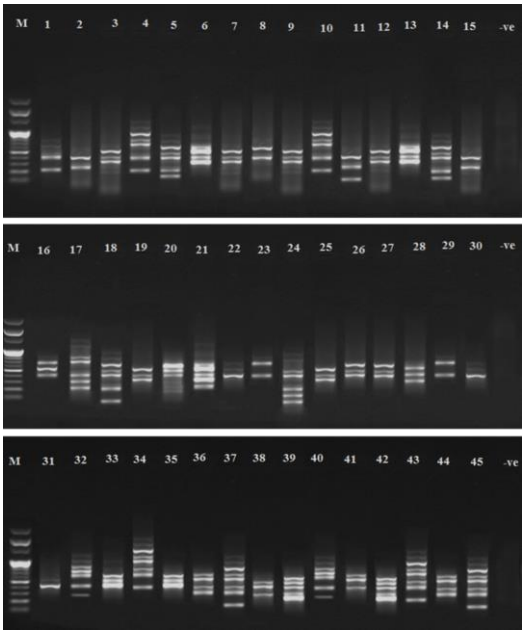

Fig. 2: RAPD amplification profile of 45 isolates of *Aspergillus flavus* by primer SBSB01. Where –ve sign indicates the negative control in RAPD-PCR and M shows 100 bp DNA ladder, (Enzynomics) cat # DM001 100

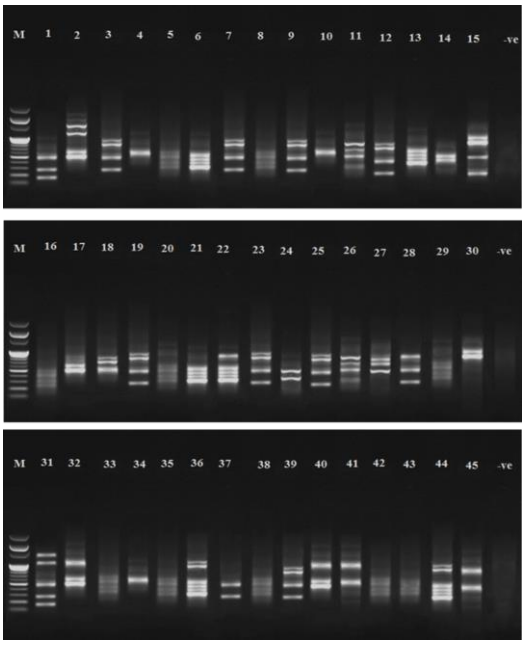

Fig. 3: RAPD amplification profile of 45 isolates of *A. flavus* by primer SBSB06. Where –ve sign indicates the negative control in RAPD-PCR and M shows 100 bp DNA ladder, (Enzynomics) cat # DM001 100

among different isolates prompt to investigate the fluctuating levels of toxin synthesis in them.

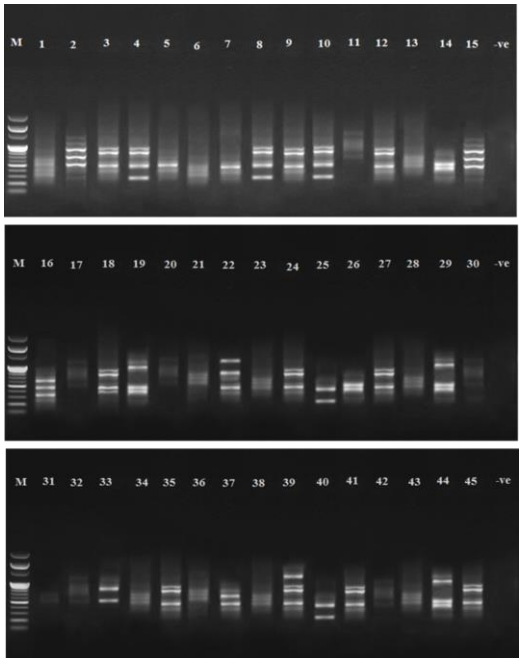

Fig. 4: RAPD amplification profile of 45 isolates of *Aspergillus flavus* by primer SBSB 10. Where –ve sign indicates the negative control in RAPD-PCR and M shows 100 bp DNA ladder, (Enzynomics) cat # DM001 100

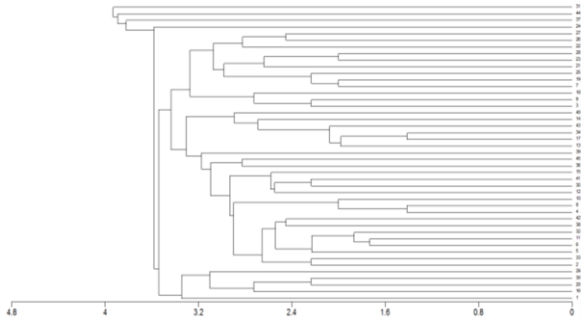

Fig. 5: UPGMA cluster analysis based dendrogram depicting the genetic relationship among different *A. flavus* isolates

Aflatoxigenicity of Isolates of *A. flavus*

Isolated strains of *A. flavus* were checked for their potential to synthesize aflatoxins. This quantitative analysis was carried out by HPLC (Table 6). Results confirmed the status of all the isolates of *A. flavus* as aflatoxigenic fungi as they all showed production of AFB1 and AFB2 in varying levels. However, none of them produced AFG1 and AFG2. Fungal isolates from maize samples of zone 3 showed highest quantities of AFB1 and AFB2 when compared to the other 45 isolates of *A. flavus*. Amongst the isolates of zone 3, *A. flavus* isolates 24, 31, 37, 42 and 44 appeared as the strongest aflatoxin producers with a production level ranging between

2279.1–14200.0 µg L^{-1} of AFB1 and 283.0–7100.0 µg L^{-1} of AFB2.Whereas, isolates 26, 27, 28, 29, 38, 40, and 43 showed a toxin production range between 321011.9–1906.4 µg L^{-1} for AFB1 and 242.3–369.1 µg L^{-1} for AFB2. The rest of the *A. flavus* isolates from zone 3, produced AFB1 between 228.7–950.2 µg L^{-1} and AFB2 between 109.8–135.0 µg L^{-1} (Table 6).

In zone 2, seven isolates of *A. flavus* were identified from stored maize samples. Among these seven isolates, *A. flavus* 18 and 17 stood as the highest toxin producing strains as they exhibited a range of 204.2-290.5 µgL^{-1} and 40.6–48.8 µg L^{-1} for AFB1 and AFB2, respectively. Other isolates of this zone synthesized significantly poor levels of aflatoxins ranging between 22.8–79.5 µg L^{-1} for AFB1 and 7.4–35.4 µg L^{-1} for AFB2. Similarly in zone one, 16 isolates of *A. flavus* were identified and their aflatoxins synthesis level was quantified in a range of 62.8–899.8 µg L^{-1} for AFB1 and 40.9–142.8 µg L^{-1} for AFB2 (Table 6).

Antifungal Activity of Aqueous Plant Extracts against Aflatoxigenic Isolates of *Aspergillus flavus*

Water based extracts of 10 medicinally important plants were checked for their antifungal activity against aflatoxigenic isolates of *A. flavus.* Among these ten plants, *Eucalyptus citriodora* leaves extract appeared as the most potent source for restricting the fungal growth as it completely inhibited the growth of isolates 37, 42 and 44 and 98.8 and 97.5% inhibition was recorded in isolates 24 and 31 respectively (Table 7). This was followed by seeds extract of *Trachyspermum ammi* that significantly restricted the fungal growth of all tested isolates between 90.8–91.8%. *Ocimum basilicum* and *Acacia nilotica* leaves extract was after this with a percentage of inhibition of mycelial growth recorded between 82.8–87.7% and 79.3–83.6%, respectively.

Similarly, when the tested isolates of *A. flavus* were treated with leaves extract of *Mentha arvensis* and branch extract of *Eucalyptus citriodora* the growth inhibition ranged between 74.5–78.5% and 67.3–73.8%, respectively. Branch extract of *O. basilicum*, leaves extract of *T. ammi* and seeds extract of *Nigella sativa* showed almost equally significant antifungal potential as the percentage growth of inhibition in all tested isolates was in the range of 57.0–68.3%, 51.3–58.7% and 45.1–50.8% respectively. Other extracts showed lower antifungal potential as 12.1–49.8% growth inhibition was calculated when fungi was grown on media amended with aqueous extracts of *Cassia fistula, Azadirachta indica, A. nilotica* branch and *M. arvensis* stem (Table 7). Minimum inhibitory activity towards tested isolates was recorded in fungi treated with extracts of *Foeniculum vulgar* and *Allium sativum* exhibiting 7.3–29.9% and 3.6–22.9% growth inhibition respectively.

Discussion

In present investigation, a total of six fungal genera were identified from collected maize samples among which genera *Aspergillus* exhibited highest percentage of frequency of occurrence. This was followed by the genera *Penicillium* and *Fusarium.* Whereas least frequency of occurrence was reported for *Alternaria, Curvularia* and *Cladosporium*. Previously, Saleem *et al*., 2012 reported *Aspergillus* as the predominant genera from maize samples followed by *Penicillium,* and *Fusarium*. Parallel findings have been documented by (Kpodo *et al*., 2000; Gao *et al*., 2007; Youssef, 2009). In a different study by Gonzalez *et al*. (2008) *Aspergillus* species predominated maize silage in Argentina. Similarly a survey by Shah *et al*. (2010) on both stored and field maize in swat region showed the existence of *Aspergillus, Fusarium, Penicillium Rhizopus* and *Mucor* species. (Atehnkeng *et al*., 2008) testified that the leading fungal genra in stored maize are *Aspergillus* and *Fusarium*. In contrast to our data, (Pacin *et al*., 2002; Lamboni and Hell, 2009) found *Fusarium* as the prevailing genus in maize while rendering *Aspergillus* as the least occurring genera. One of the possible reason for this difference may be different sampling strategies, because in the above-mentioned studies freshly harvested maize or silage was used where as in our study samples from stored maize were analyzed. *Aspergillus* and *penicillium* are also reported as most frequently occurring genera in dried and stored food commodities (Amadi and Adeniyi, 2009). Among mycotoxins, the most common are aflatoxins that are synthesized by *A. flavus*, the highest occurring fungal specie in stored maize in this study.

All the isolated fungi were confirmed for aflatoxin synthesis using visual detection of blue florescence when reverse of the colonies are observed under ultraviolet light. Previous workers have successfully used coconut based media for this purpose (Davis *et al*., 1987). Following them three commonly recommended coconut based media were used in this study. Upon comparison between the isolated fungi, *A. flavus* depicted strongest characteristic florescence of blue colour on CCA media. Whereas, *A. terreus* and *A. japonicas* followed these results. The intensity of the florescence also changed with change in media composition. CMA was found less effective in generating the florescence than CCA. Least mycotoxin production was recorded in CMA media. Earlier studies of Dyer and McCammon (1994) are also in accordance with our results as they also reported coconut cream agar media better for visual detection of aflatoxinsin comparison to the medium prepared from dried coconuts. In (Yazdani *et al.*, 2010), evaluated numerous techniques for detection of toxins by *Aspergillus* isolates and reported coconut milk agar media best for the production of characteristic florescence ring around fungal colonies. (Nair *et al*., 2014) also compared coconut media and potato dextrose media for the detection of aflatoxins and found coconut based recipe the most suitable and cost effective for this purpose.

During this study, maximum florescence under UV was observed in *A. flavus* isolates which was the most prevalent specie with highest frequency of occurrence among

Table 4: Isolated mycoflora showing blue florescence in coconut based media

Isolated fungi	Coconut agar media (CAM)	Coconut Milk agar (CMA)	Coconut cream (CCA)
Aspergillus flavus	+/-	+	++
Aspergillus niger	-	-	-
Aspergillus terreus	-	+/-	+
Aspergillus fumigatus	-	-	-
Aspergillus oryzae	-	-	-
Aspergillus versicolar	-	-	-
Aspergillus japonicus	-	+/-	+
Penicillium aurantiogriseum	-	-	-
Penicillium herquei	-	-	-
Penicillium claviforme	-	-	-
Penicillium oxalicum	-	-	-
Fusarium solani	-	-	-
Fusarium guttiforme	-	-	-
Fusarium subglutinans	-	-	-
Fusarium globosum	-	-	-
Alternaria alternata	-	-	-
Curvularia lunata	-	-	-
Cladosporium cladosporioides	-	-	-

Whereas,
+ : medium florescence signal
+/- : weak florescence signal
++ : strong florescence signal
- : no florescence

Table 5: Random three decamers which showed amplification in all isolates, their accessions, sequences, number of polymorphic bands, percentage of polymorphic products and size of bands produced by each primer

Primers	Sequence (5'-3')	Conc. (nmol)	Total no. of bands	No. of polymorphic bands	%age of polymorphic bands	Size of bands (bp)
SBSB01	GTT TCG CTC C	111.1	172	47	27.3%	200-2000
SBSB06	TGC TCT GCC C	111.7	118	42	35.5%	150-2000
SBSB10	CTG CTG GGA T	108.4	94	49	52.1%	200-2000

genus *Aspergillus*. Maximum florescence indicated the concomitant high production of aflatoxins in the initial screening process. Therefore this study was extended to find the genetic variation among different isolates of *A. flavus*.

Advancement in molecular science has provided us more reliable and effective tools to find variations among various microbial isolates (Tiwari *et al*., 2011). A very common method for this is the use of RAPD (random-amplified polymorphic DNA – Williams *et al*., 1990) markers that comprise several advantages including low-cost, being easy to perform, versatile regarding primers, genetic markers evenly dispersed throughout in the genome and particularly requiring no prior knowledge of the genetics of the microorganism (Williams *et al*., 1990; Diaz-Guerra *et al*., 2000). RAPD has also previously been used by Lourenço *et al*., 2007 for finding genetic diversity amongst14 isolates of *A. flavus*. Likewise, in the present study, RAPD confirmed that all the tested fungal isolates are of same species as they showed genetic diversity around 30%. Genetic diversity varied among the three zones, being highest in arid rain fed zone probably because of the warm and humid environmental conditions prevailing there that enhanced the hybridization frequency between the isolates. These environmental conditions also favours the microbial growth with higher probability of crosses among them resulting in increased genetic variation. The genetic variation among the isolated mycoflora was found related to the variability in their mycotoxin synthesis. The strains of *A. flavus* isolated from arid rain fed zone (zone 3) showed highest aflatoxin production showing that faster rate of hybridizations has not only augmented genetic variations but also increased aflatoxin synthesis potential. Fungal isolates of other two zones showed less genetical diversity in comparison to the isolates of zone 3 owning 15 to 25% genetic variation in comparison to 30% variability in isolates of zone 3. In all the three zones no isolate produced AFG1 and AFG2. These results are found in close agreement with Abbas *et al*. (2005) who reported clear differences in levels of aflatoxin production by *A. flavus* isolates. Similarly, (Othman and Delamiy, 2012) screened various *A. flavus* isolates for the synthesis potential of aflatoxin B1 and documented that seven out of eleven isolates produced AFB1 in the range of <10 – 450 ppb. In another study Al-Abdalall (2009) found that all the tested isolates of *A. flavus* were capable of producing AFB1 and AFB2 but with greatly varied levels ranging between 38–496 ppm and 5– 185 ppm, respectively. The aflatoxin production potential of *A. flavus* isolates in the present investigation was greater than that was reported by (Almoammar *et al*., 2013), whose isolates were reported to synthesize AFB1 and AFB2 in the range of 0.9–7.8 µg kg^{-1} and 0.1–6.4 µg kg^{-1}, respectively.

Higher quantities of aflatoxin synthesized by isolated

Table 6: Production of aflatoxins from isolates of *Aspergillus flavus*

Isolation zone	*Aspergillus flavus* isolates	Production of aflatoxins (µg L^{-1})	
		AFB1	AFB2
Zone 1	1	652.5^{p}	78.2^{vw}
	2	899.8^{n}	142.5^{o}
	3	369.7^{r}	101.2^{s}
	4	297.3^{rs}	82.2^{uv}
	5	224.3^{stu}	122.5^{q}
	6	135.0^{u-y}	142.8^{o}
	7	108.9^{v-z}	72.6^{w}
	8	82.1^{xyz}	33.1^{yz}
	9	91.6^{w-z}	78.2^{vw}
	10	162.2^{u-x}	90.1^{tu}
	11	191.3^{tuv}	130.6^{pq}
	12	185.1^{t-w}	101.8^{rs}
	13	161.6^{u-x}	101.3^{s}
	14	145.4^{u-y}	97.5^{st}
	15	62.8^{yz}	40.9^{xy}
	16	293.8^{rs}	181.2^{n}
	17	290.5^{rs}	48.8^{x}
	18	204.2^{stu}	40.6^{xy}
	19	79.5^{xyz}	35.4^{yz}
Zone 2	20	66.9^{xyz}	31.2^{yz}
	21	30.0^{z}	19.4^{z}
	22	22.8^{z}	11.7^{z}
	23	29.8^{z}	7.4^{z}
	24	5885.3^{c}	3827.6^{c}
	25	950.2^{mn}	127.0^{pq}
Zone 3	26	1011.9^{lm}	242.3^{l}
	27	1194.7^{k}	365.5^{gh}
	28	1088.3^{l}	109.8^{r}
	29	1481.1^{i}	291.3^{j}
	30	764.9^{o}	358.1^{h}
	31	14200.0^{a}	7100.0^{a}
	32	1906.4^{g}	369.1^{g}
	33	1594.8^{h}	544.7^{e}
	34	756.6^{o}	324.9^{i}
	35	489.5^{q}	442.3^{f}
	36	297.1^{rs}	373.3^{g}
	37	8879.6^{b}	4439.8^{b}
	38	2011.3^{f}	365.4^{gh}
	39	228.7^{stu}	201.0^{m}
	40	1320.6^{j}	193.2^{m}
	41	263.7^{st}	329.5^{i}
	42	2279.1^{e}	283.0^{jk}
	43	1393.0^{j}	281.5^{k}
	44	5787.3^{d}	662.2^{d}
	45	460.4^{q}	135.0^{op}

Values are mean of three replicates

Values with different alphabetic letters indicate significant differences in aflatoxin

Production level among various isolates as determined by tukey's multiple range test

aflatoxigenic fungi lead us to focus on exploring any eco-safe approach to restrict the growth of these problematic fungi. In last few decades many studies have demonstrated the efficacy of several plant extracts as biocontrol agents to retard the growth of aflatoxigenic fungi and aflatoxin production by them (Gowda *et al*., 2004; Suleiman *et al*., 2008; Reddy *et al*., 2009). Significant emphasis has been given to the medicinal, herbal and aromatic plants for exploring their antifungal potentials against food deteriorating and aflatoxigenic fungi (Maraqa *et al*., 2007; El-Nagerabi *et al*., 2012; Elshafie *et al*., 2013). The above mentioned antifungal activity in plant extracts is due to the presence of several bioactive secondary metabolites including alkaloids, tannins, flavonoids and terpenoids (Nwachukwu and Umchuruba, 2001).

In this study water based extracts of ten selected medicinal plants were checked for their antifungal activity towards aflatoxigenic isolates of *A. flavus*. All the selected plant extracts showed varied degrees of antifungal activity. Highest antifungal activity was recorded in aqueous extract of *E. citriodora* leaves that was followed by seeds of *T. ammi* and leaf extract of *O. basilicum* and *A. nilotica*. Ramezani, 2014 reported that *E. citriodora* extracts contain significant fungitoxic activities. Similarly in another study by Takahashi *et al*. (2004) the extracts of *E. viminalis, E. maculata* and *E. globules* showed significant growth inhibition of *Trichophyton mentagrophytes*. In Saralamp *et al*., 1996, tested various plants against *A. flavus* and founda traditional thai medicinal plant, Betal vine as a most potent source of antifungal activity in comparison to the other tested plants including indian mulberry, false coriander, Chinese radish, chaaphluu and clove. Antifungal activity of above-mentioned plants used in present study has also been described by many scientists (Thanaboripat *et al*., 1997; Thanaboripat *et al*., 2000; Yadav and Majumdar, 2005; Murthy *et al*., 2009; Al-Rahmah *et al*., 2011; Bhalodia and Shukla, 2011; Mickiene *et al*., 2011; Javed *et al*., 2012).

Eucalyptus species are well known for their bioactive phenolics mostly flavonoids. Hyperoside and myricetin has been identified as the antimicrobial compounds in Eucalyptus species (Dezsi *et al*., 2015). Similarly *Trachyspermum ammi* is also a well-known for its antibacterial, antifungal as well as antiviral properties that is because of the presence of bioactive compounds including thymol, carvacrol, pinene, cymene, limonene and terpinene. High quantities of anti-inflammatory compounds (terpenes, glycosides and sterols) have also been reported in this plant (Katasani *et al*., 2011). *Ocimium basilicum* is a rich source of acylated and glycosylated anthocyanins with documented antimicrobial properties (Usman *et al*., 2013). The fungicidal activity recorded in this study is due to the presence of these phytochemicals in selected plant extracts. Hence they can be used as food additives or for manufacturing active packaging to avoid fungal growth and mycotoxin production.

Conclusion

This study highlights the genetic diversity and potential of aflatoxin synthesis by *A. flavus* isolated from Pakistani stored maize samples. Identifying the cause and type of mycotoxin will help us further in developing strategies to improve our storage conditions and management practices. The study was extended to explore some plant sources for their antifungal properties against the most problematic fungi i.e., *Aspergillus flavus* in search of a practical approach to manage aflatoxin production in stored agricultural

Table 7: Effects of aqueous plants extracts on mycelial growth inhibition of *Aspergillus flavus* isolates

Aqueous plant extracts	Percentage of mycelial growth inhibition of *Aspergillus flavus* isolates				
	Isolate 24	Isolate 31	Isolate 37	Isolate 44	Isolate 42
T. ammi leaves	51.3^{f}	58.7^{fg}	58.2^{ef}	55.7^{def}	56.3^{ef}
T. ammi seeds	91.3^{ab}	91.5^{ab}	90.9^{ab}	90.8^{ab}	91.8^{ab}
C. fistula branch	35.4^{g-j}	42.3^{hij}	16.4^{jkl}	32.8^{ghi}	39.3^{ghi}
C. fistula leaves	41.5^{fgh}	49.8^{gh}	23.6^{ij}	37.4^{fgh}	44.8^{fgh}
N. sativa leaves	25.6^{i-m}	29.4^{j-m}	21.8^{ijk}	23.6^{hi}	30.1^{h-k}
N. sativa seeds	45.1^{fg}	49.3^{gh}	50.3^{fg}	46.6^{efg}	50.8^{fg}
*E. citriodora*branch	73.8^{de}	72.1^{de}	67.3^{de}	70.1^{bcd}	72.1^{cd}
E. citriodora leaves	98.8^{a}	97.5^{a}	100.0^{a}	100.0^{a}	100.0^{a}
A. sativum bulb	12.8^{m}	18.9^{m}	3.6^{l}	8.0^{i}	12.6^{k}
*A. sativum*leaves	16.4^{lm}	22.9^{lm}	4.8^{l}	9.2^{i}	16.9^{k}
A. nilotica branch	38.5^{ghi}	43.8^{hi}	40.6^{gh}	36.2^{fgh}	41.5^{f-i}
A. nilotica leaves	83.6^{bcd}	83.1^{bcd}	80.0^{bcd}	79.3^{abc}	81.4^{a-d}
M. arvensis leaves	78.5^{cde}	77.1^{cde}	74.5^{cd}	75.3^{a-d}	78.1^{bcd}
M. arvensis stem	29.7^{h-k}	35.3^{i-l}	32.7^{hi}	27.6^{ghi}	34.4^{hij}
*A. indica*branch	27.7^{i-l}	35.3^{i-l}	12.1^{jkl}	23.0^{hi}	30.6^{h-k}
*A. indica*leaves	30.8^{h-k}	38.8^{h-k}	13.3^{jkl}	27.0^{ghi}	33.3^{hij}
O. basilicum branch	67.7^{e}	65.2^{ef}	57.0^{ef}	64.9^{cde}	68.3^{de}
*O. basilicum*leaves	87.7^{abc}	87.6^{abc}	84.8^{bc}	82.8^{abc}	87.4^{abc}
*F. vulgare*leaves	20.0^{klm}	27.9^{klm}	7.3^{kl}	9.8^{i}	19.7^{jk}
F. vulgare stem	23.1^{j-m}	29.9^{j-m}	9.7^{jkl}	15.5^{hi}	25.1^{ijk}

Values are mean of three replicates
Values with different alphabetic letters indicate significant differences among tested plants extract as determined by Tukey's multiple range test

commodities and their products.

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