

STUDY OF PREVALENCE OF DIFFERENT FUNGAL SPECIES IN CALF FEED AND COMPARATIVE ANTIFUNGAL EFFICACY OF METHANOLIC EXTRACTS OF INDIGENOUS PLANTS AGAINST *ASPERGILLUS* SPECIES

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ABSTRACT

Azadirachta indica

Mycotoxins are secondary metabolites produced by different fungus species which are harmful both for the animals and human beings. In this study, feed samples were taken from different livestock farms and commercial feed mills and levels of aflatoxin B1 and prevalence of *Aspergillus* species in each sample was recorded. The anti-fungal potential of different plant extracts was also evaluated. The aflatoxin B1 was measured by HPLC. Results showed Aflatoxin B1 was most prevalent in feed collected from the farms as compared to commercial feed mills. Six genera of fungus were found and 14 species of these six genera were identified. Comparative efficacy of methanolic extracts of garlic (*Allium sativum* L.), clove (*Syzygium aromaticum*) and neem (*Azadirachta indica*) were determined by agar well diffusion method. Garlic (*Allium sativum* L.) showed significantly higher antifungal activity followed by clove (*Syzygium aromaticum*) and neem (*Azadirachta indica*) at (P<0.05).

Keywords: aflatoxin B1, HPLC, *Aspergillus* spp., *Allium sativum* L., *Syzygium aromaticum*,

INTRODUCTION

In most developing countries like Pakistan, livestock production is an important part of the national economy. Subsistence and semi-commercial small holder farming system is dominated by resource poor farm households (Lanyasunya *et al.*, 2005). Variable environmental conditions and agricultural practices pose a serious threat to of mycotoxins contamination of feedstuff (Wu, 2006).

Contamination of feed with mycotoxins is responsible for disease and loss of livestock (Okoli *et al.*, 2007). Molds are capable of reducing the nutritional value of feedstuff as well as elaborating several mycotoxins. Also, mycotoxins may be carried over into meat and eggs when animals are fed with contaminated feed (Lynch *et al.*, 1972). Various *Aspergillus* species (*A. parasiticus*, *A. flavus*, and *A. nomius*) produce aflatoxins as secondary metabolites (Creppy, 2002). The aflatoxins may present in the feed (B1, B2, G1, and G2) and milk (M1 and M2) as reported by

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Allcroft and Carnaghan (1963). High intake of aflatoxins in feed may adversely affect animal health, performance and reproduction (Whitlow and Hagler, 2002). Chronic aflatoxin intoxication in calves is responsible for anorexia, weight loss, feed efficiency and liver damage (Keyl and Booth, 1971; Lynch, 1972).

Medicinal plants are used to cure different diseases in Asia, Europe, Africa and other countries centuries ago (Anjum *et al.*, 2014). Plants are potential sources of antimicrobial drugs (Mahesh and Satish, 2008). Fresh garlic (*Allium sativum*) is reported to have antimicrobial, anti-inflammatory, antithrombotic, anti-atherosclerotic, serum lipid lowering and anticancer activities due to presence of an active compound Allicin (diallylthiosulphinate) in it (Yunus *et al.*, 2015).

Clove oil and many other plants possess a phenolic compound called eugenol (4-allyl-2-methoxyphenol) which produce fragrance and give flavor to food (Ghosh *et al.*, 2005). Eugenol has been reported to inhibit the growth of microorganisms including fungi of different species (Wang *et al.*, 2010).

Neem (*Azadirachta indica*) is a widely distributed tree, present in Pakistan, India, Nepal and Sri Lanka etc. (Keller *et al.*, 2007). is used as traditional Ayurvedic medicine (Okoli *et al.*, 2007). Neem has more than 2000 year's history of usage (Brahmachari, 2004). Various routs of application of neem are reported for different ailments. Aggarwal and Dhawan(1995) reported that in constipation and deworming, it is used orally while in rheumatism, ulcer and skin infections, preferably it is applied topically. Many researchers have reported acaricidal, antibacterial, antifungal, antimalarial, antiparasitic, anti-inflammatory and immunomodulatory properties of neem oil in different animals (Biswas *et al.*, 2002; Brahmachari,

2004; Gossé *et al.*, 2005; Du *et al.*, 2007;). Due to high efficacy, biodegradability and minimum side effects of Azadirachtin, it has emerged as a natural antimicrobial agent (Keller *et al.*, 2007).

In present study, prevalence of toxigenic *Aspergilli* was determined in concentrate feed samples collected from commercial dairy farms and feed mills by HPLC and antifungal activity of different plant extracts was assessed against these *Aspergilla*. Further, the study was aimed to investigate the antifungal activity of garlic, clove and neem methanolic extracts against toxigenic *Aspergilli* isolated from concentrate feed.

MATERIALS AND METHODS

The study was conducted in Gujranwala and Kasur Districts of Punjab, Pakistan. A total of 74 concentrate feed samples (n = 43 from Gujranwala *i.e.* farms = 27, Feed mills = 16 and n = 31 from Kasur *i.e.* farms = 20, feed mills = 11) were collected (50 g from each farm) from livestock farms (Containing ≥ 10 calves/farm). The samples were screened for the presence of aflatoxins using HPLC following Masoero *et al.* (2007). Briefly, ten grams of dried feed was mixed with methanol and water solution (80:20 V/V), shacked for 45 minutes at 150 revolutions per minute. Five mL from the filtrate was eluted with 45 mL of bi-distilled water through an immune-affinity / mycosep column. 5 mL distilled water was used to wash the column then eluted with 2.5 mL of methanol. The elution was air dried, dissolved again in one mL acetonitrile: water (25:75 V/V) solution and filtered. The Aflatoxin B1 (AFB1) was separated with reverse phase chromatography and was detected by fluorescence after post-column dramatization with pyridinium hydrobromide

perbromide (PBPB). The excitation and emission wave lengths for fluorescence detector were 365 nm and 440 nm, respectively. The samples positive for AFB₁ were processed for isolation of fungi following Pitt and Hocking (1997). One gram of the sample was diluted in 9 mL of sterile distilled water followed by ten folds serial dilution. One mL of each dilution was spread on Sabouraud's dextrose agar (SDA) and incubated for 24 h. Purified cultures were identified based on macroscopic (Obverse and reverse) and microscopic (type and shape of hyphae and spores) as reported by Wijedasa and Liyanapathirana, (2012).

Plant parts of clove, garlic and neem were dried, powered grind and then extracted three times with boiled methanol. The extracts were concentrated using rotary evaporator (Buchi, Switzerland) following Shahidi Bonjar *et al.* (2004). Antifungal activity of methanolic extracts of afore mentioned plants was evaluated against *Aspergillus* species using well diffusion method (Santoyo *et al.*, 2009).

Statistical analysis

Kolmogorov Smirnov's test was used to measure the normal distribution of data. Data are represented as mean±S.D. One way analysis of variance followed by Dunnett's comparison tests was used to test the hypothesis. Results with P<0.05 were significant. Data analysis was performed using statistical package SPSS (Version 13.0 SPSS Inc., Chicago, IL, USA). Minimum Inhibitory Concentration (MIC) was analyzed following Wiegand *et al.*, (2008).

RESULTS AND DISCUSSION

Out of total 74 concentrate feed samples

collected, 67 samples were found positive for aflatoxin B₁. Out of these 67 samples, 36 were from Gujranwala (27 and 9 from farms and feed mills, respectively) and 31 from Kasur (20 and 11 from farms and feed mills, respectively). Aflatoxin B₁ level was higher in commercial feed (Gujranwala = 40.33±2.21 ppb; Kasur = 49.0±1.95 ppb) as shown in Table 1, than farms feed (Gujranwala = 34.96±2.65 ppb; Kasur = 44.95±2.41 ppb) in both districts (Figure 1, P<0.05).

Out of mycotoxins contaminated concentrate feed samples, the highest frequency of *Aspergillus* (43.3%) was observed followed by *Fusarium* (38.8%), *Mucor* (8.9%), *Penicillium* (5.9%), *Rhizopus* (1.5%) and *Alternaria species* (1.5%). Out of 29 *Aspergilli*, maximum frequency (72.4%) of *A. flavus* was recorded followed by *A. parasiticus* (13.7%), *A. fumigates* (6.8%) and *A. niger* (6.8%) as shown in Table 2. Out of 26 *Fusarium* species, maximum frequency (23.8%) of *F. chlamydosporum* was recorded followed by *F. oxysporum* (10.4%) and *F. solani* (4.4%), respectively (Figure 2). Methanolic extracts of garlic, clove and neem showed antifungal activity. Maximum zone of inhibition against *A. flavus* and *A. parasiticus* was shown by garlic extract (16.5 mm) followed by clove (13.04 mm) and neem (9.06 mm), respectively as shown in Figure 3 (P<0.05).

Ailment due to aflatoxin exposure was first recognized in livestock, few decades ago with similar patterns of disease apparent in different species (Samuel *et al.*, 2009). Susceptibility to aflatoxins has been noted to vary considerably between species and individuals including human beings. Susceptibility to aflatoxin toxicity is higher in young ones as compared to elders. In developing countries, most of the time people are exposed to aflatoxin because they do not properly follow the standards of Food and Drug Administration (FDA)

set for the manufacturing technology (Ariyo *et al.*, 2013).

Out of total 74 concentrate feed samples collected, 67 samples had >20 ppb of most toxic aflatoxin B₁. Lanyasunya *et al.* (2005) also stated that aflatoxin B₁ have toxic effects when it contaminates food grains. Its potent toxic effect was illustrated by an outbreak of lethal aflatoxicoses that took lives in past. Similar findings are reported by Yunus *et al.* (2015) and Malachová *et al.* (2014).

Aflatoxin B₁ level was higher at feed mills than farms feed in Gujranwala and Kasur (Figure 1). Our results of presence of aflatoxins B₁ in the calf feed is in agreement with the finding of Martins *et al.* (2007). Higher level of Aflatoxins in concentrate feed samples of feed mills may be due to inadequate storage facilities for ingredients.

Out of mycotoxin contaminated concentrate feed samples, the highest frequency of *Aspergillus* (43.3%) was observed followed by *Fusarium* (38.8%), *Mucor* (8.9%), *Penicillium* (5.9%), *Rhizopus* (1.5%) and *Alternaria species* (1.5%). Several researchers have isolated *Aspergillus flavus*, *Alternaria* and *Rhizopus* from the concentrate feed samples (Ariyo *et al.*, 2013; Dalcerro *et al.*, 1997; dos Santos *et al.*, 2003; Keller *et al.*, 2007; Pereyra *et al.*, 2008; Rosa *et al.*, 2006; Schneewis *et al.*, 2000). *Aspergillus* species are more prevalent fungi isolated from feed samples (Figueroa *et al.*, 2009; Rosa *et al.*, 2006).

Maximum frequency (72.4%) of *A. flavus* was recorded followed by *A. parasiticus* (13.7%), *A. fumigatus* (6.8%) and *A. niger* (6.8%). Out of 26 *Fusarium* species, maximum frequency (23.8%) of *F. chlamydosporum* was recorded followed by *F. oxysporum* (10.4%) and *F. solani* (4.4%), respectively. *Aspergillus flavus* more prevalent species in concentrate feed samples as reported by Figueroa *et al.* (2009); Rosa *et al.* (2006). Ariyo *et*

al. (2013) also reported *A. fumigatus* from calf feed which is in line with our findings.

Aspergillus, *Penicillium* and *Fusarium* are toxigenic fungi; they produce very important mycotoxins including ochratoxin, aflatoxin, fumonism and trichothecene. High level of *Mucor spp.* and *Rhizopus spp.* was a result of the feed ingredients higher in carbohydrate content. The low incidence of *Mucor spp.* and *Rhizopus spp.* in our study was in contrast with the Copetti *et al.* (2012) which showed a high frequency of these fungi in animal feed ingredients, this may be due to the alteration in the environment and the season in which ingredients were purchased.

Garlic showed statistically significant higher zone of inhibition against *A. flavus* followed by clove and neem, respectively. Shadkchan *et al.* (2004) reported that allicin (garlic) can significantly reduce mortality, prolong survival and reduce fungal load in mice infected with *A. fumigatus*. Chalfoun *et al.* (2004) found that clove causes inhibition of *A. niger* mycelial development. Many reports are available on the antiviral, antibacterial, antifungal, anthelmintic, anti-molluscal and anti-inflammatory properties of plants (Behera and Misra, 2005; Govindarajan *et al.*, 2006; Kumarasamy *et al.*, 2002; Palombo and Semple, 2001; Samy and Ignacimuthu, 2000). It was concluded that methanolic extracts of garlic, clove and neem may be used for control of mycotoxin intoxication of concentrate feed used for calf rearing.

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Table 1. Table shows the estimation of Aflatoxin B₁ in feed determined by HPLC (ppb). Feed samples were collected from Gujranwala and Kasur districts from the commercial and private feed. Means in the same rows having different superscripts show significant differences from each other (P<0.05).

Area	Commercial feed (Mills)	Private feed (Farmers)
Gujranwala	40.33±2.21 ^a	34.96±2.65 ^b
Kasur	49.0±1.95 ^a	44.95±2.41 ^b

Table 2. Table shows the frequency of distribution of different fungus genera and species of different genera isolated from calf feed. 14 species of fungus was observed belonging to six genera.

Fungus genus	Species	Frequency (%)
Aspergillus	<i>A. flavus</i>	72.4
	<i>A. paraciticus</i>	13.7
	<i>A. niger</i>	6.8
	<i>A. fumigatus</i>	6.8
Fusarium	<i>F. chlamydosporum</i>	23.8
	<i>F. oxysporum</i>	10.4
	<i>F. solani</i>	4.4
Rhizopus	<i>R. arrhizus</i>	1.5
Mucor	<i>M. amphibiorum</i>	33.3
	<i>M. circinelloides</i>	50
	<i>M. indicus</i>	16.6
Penicillium	<i>P. crustosum</i>	75
	<i>P. palmae</i>	25
Alternaria	<i>A. solani</i>	1.5

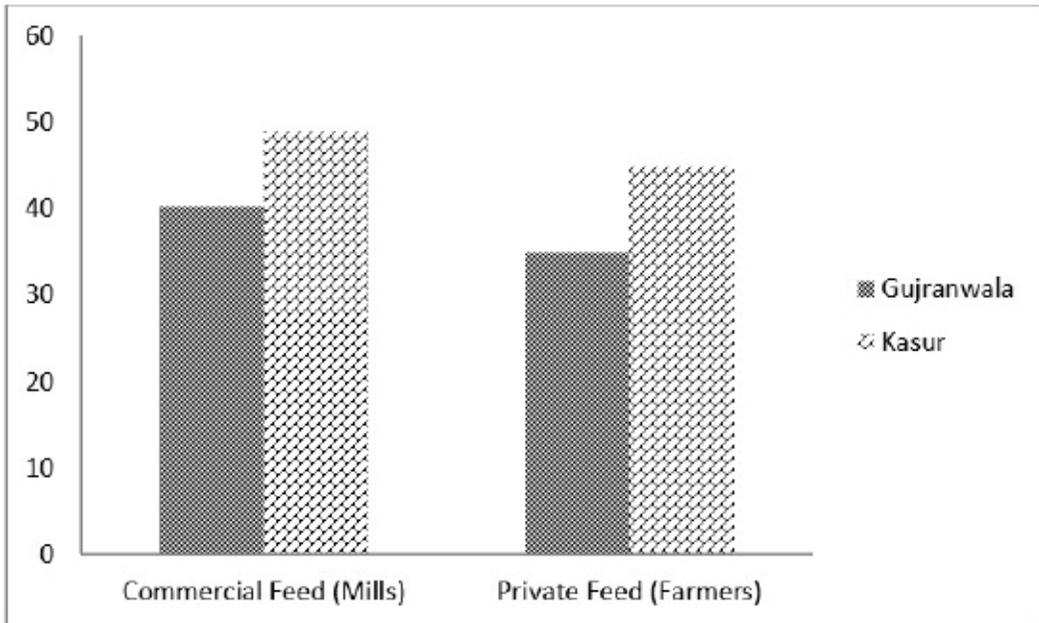


Figure 1. Graphical presentation of analysis of feed samples by HPLC (ppb) indicating the Aflatoxin B₁ level in feed from commercial and private feed.

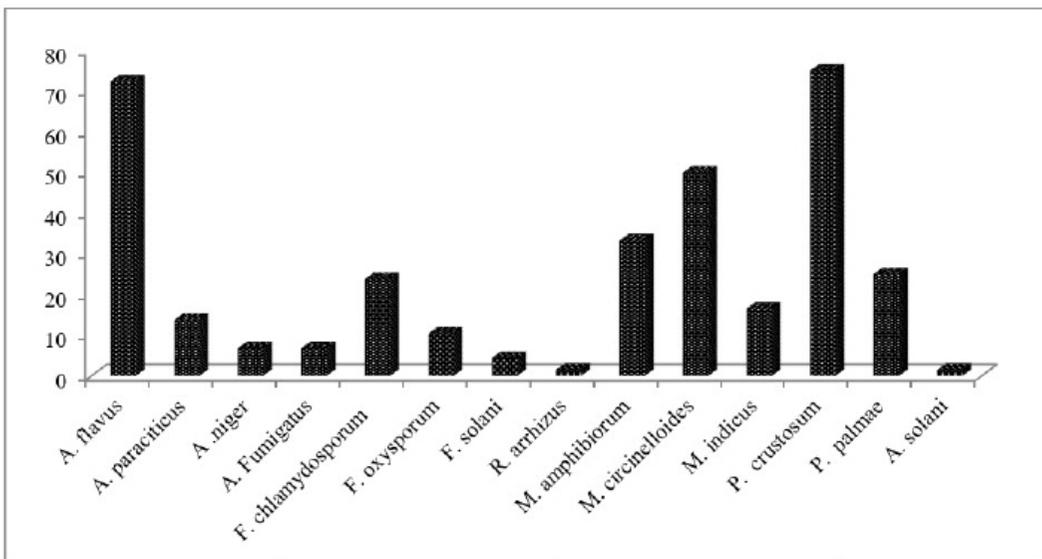


Figure 2. Graphical presentation of the frequency of distribution of different fungal species in calf feed.

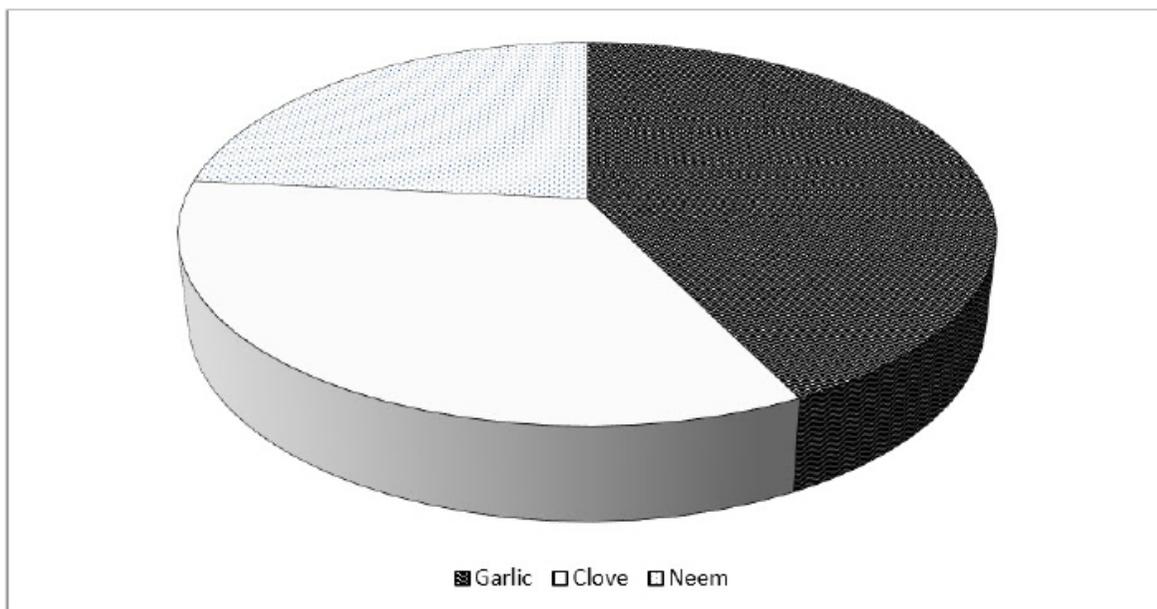


Figure 3. Minimum Inhibitory Concentration (MIC) of different spices used against *Aspergillus* spp. in millimeters (mm). Figure shows that maximum zone of inhibition against *A. flavus* and *A. parasiticus* was shown by garlic extract (16.5 mm) followed by clove (13.04 mm) and neem (9.06 mm), respectively as shown in Figure 3 ($P < 0.05$).

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