INTERNATIONAL JOURNAL OF ADVANCES IN PHARMACY, BIOLOGY AND CHEMISTRY

Research Article

Materials and Biocontrol of Mycoflora by Medicinal Plant Extracts

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ABSTRACT

Mycotoxins are known to produce adverse effects in poultry. The presence of mycotoxins in poultry feeds may decrease feed intake and affect animal performance. The present investigation was undertaken to find the fungal population and mycotoxin level in different samples of poultry raw feed materials such as, sorghum, maize, layer mash, soyabean and rape seed meal samples followed by inhibition of the isolated fungal flora using two plant extracts viz., *Acalypha indica, Tridax procumbens* by well diffusion method. From 20 poultry raw feed samples, the isolated species of fungi belong to the genus, *Aspergillus* sp, *Penicillium* sp, *Rhizopus* sp, *Fusarium* sp, *Mucor* sp, *Cladosporium* sp, *Curvularia* sp and *Trichoderma viride*. Aflatoxins B_1 and B_2 were detected in 12 samples and their levels ranged between 6.3±0.34 and 199.3±0.66. Cold water extracts of *Acalypha indica* showed the efficient inhibition (100%) against *Trichoderma viride*, *Aspergillus niger* whereas *Tridax procumbens* plant extracts showed highest zone of inhibition (37.8 mm) against *Fusarium sp* while the other isolated fungal isolats showed varied zones of inhibition.

Keywords: poultry raw ingredients, mycotoxin, fungal flora.

INTRODUCTION

Agricultural products including cereals and oilseed meals constitute a major component of poultry feed ingredients. Mold contamination is wide spread in tropical countries where poultry production and processing are expanding rapidly (Van den Bergh *et al*., 1990; Okoli *et al.,* 2006). Mycotoxins have attracted worldwide attention due to significant loss associated with their impact on human and animal health, and consequenational economic implications (Bhat and Vashanti, 1999). Mycotoxin contaminations of crops are now-a-days a world wide problem. According to a survey, more than 50% of the world crop is inflicted with different kinds of mycotoxins which is a serious threat to the health of humans and animals (Manoj Bharti, 2008).Poultry are highly susceptible to mycotoxicoses caused by aflatoxins, trichothecenes, ochratoxin and some fusariotoxins (Mabbett, 2004; Okoli, 2005). Mycotoxins are secondary metabolites produced by filamentous fungi that cause a toxic response (Mycotoxicosis) when ingested by poultry or livestock. Mycotoxins are mainly produced by *Aspergillus*, *Penicillium* and *Fusarium* genera (Akande *et al*.,2006; Manoj Bharti, 2008).The production of mycotoxins is often species specific; therefore, identification of fungi is of prime importance. Among different mycotoxins, aflatoxins (AF) and ochratoxin A [OTA] are the most important contaminants of poultry feeds. The presence of OTA in feeds raises concern in livestock industry due to subclinical intoxications and poor growth in animals. (Gentles *et al*., 1999).In farm animals, mycotoxins have negative effects on feed intake, animal performance, reproductive rate, growth efficiency, immunological defence as

well as on carcinogenic, mutagenic, tetragenic, tremorgenic causing tumor (or) damage the central nervous system, hemorrhagic and also causing damage to the liver and kidney. (Ratcliff, 2002).Many of the plants used in traditional medicine are readily available in rural areas at relatively cheaper than modern medicine.(Mann et.al.,2008). *Acalypha indica* Linn is a family of Euphorbiaceae found in all parts of the tropics. This herbs found in fields and waste places through out the hotter parts of the world. This plant is used for treating various disease for centuries including antifungal and antibacterial activity..(Azamahani *et al.,* 2002). *Tridax procumbens* L. is a small herb of family Asteraceae. *Tridax procumbens* Linn. is one of the medicinally important plants commonly found in subtropical countries. The leaves of the plant are known to be used for the treatment of wound in traditional medicine. (Chatterjee,2001; Ali *et al.,* 2001).Based on the above fact a study was carried out to isolate mycotoxin producing fungi and inhibition of the isolated fungi by plant extracts.

MATERIALS AND METHODS

Collection of sample

Five different types of poultry feed ingredients viz., sorghum (4 samples), maize(2 samples), layer mash (5 samples), soya bean (5 samples), rape seed meal (4 samples) were collected from different poultry farms in Namakkal District, Tamil Nadu.

Enumeration of Fungal Colonies In Different Poultry Raw Samples (Basalan *et al.*, 2004)

Sabouraud's Dextrose agar (SDA Hi-media) was prepared and sterilized by autoclaving at 121^{o}C for 15 minutes and allowed to cool to 50^{o}C poured in to sterile Petri dishes. For culturing, 10g of powdered sample was added to 90ml of sterilized 0.1% peptone water, allowed to stand for 15 minutes, and shaken well for 15 minutes. From this diluted sample, 0.1ml was pipetted on to the surface of petri plates containing the sabouraud's dextrose agar and spreaded using sterilized L-rod. The Petri plates were incubated at 25^{o}C for 5 to 7 days. All colonies were counted and multiplied by the dilution factor to calculate colony forming unit (CFU) for per gram of animal feed. The different fungi isolated from poultry samples were identified based on the colony morphology and microscopic observation (Udaya prakash, 2004).

Detection of Multitoxin by Thin Layer Chromatography

The presence of multitoxin in the poultry samples was detected as per the standard procedure (A.O.A.C., 1984.)

Separation of Extract: About 10g of the ground sample was taken and blended at high speed for 3 min with 36 ml of Acetonitrile, 4 ml of 4% KCL and 0.8ml of 5N HCl. The extract was filtered using Whatmann No.1 filter paper. 20ml filtrate was transferred in to 250ml separating funnel and added 20ml of water and 20ml of hexane and shake well. The hexane layer is discarded. The lower layer was collected and again added 20ml of hexane and shakes well and collected lower layer. Then 10ml of chloroform was added into acetonitrile phase and collected chloroform layer and evaporated to dryness. The residue is dissolved in 0.2 ml chloroform.

Development of TLC Plates

For examination of extracts, aluminium – packed silica gel 60F $_{254}$ 20 × 20 Cm^2 plates (Merck, type 1.05554-0007) was used as per the standard procedure (A.O.A.C 1984). Plates were spotted along 1.5 cm from the bottom with 40µl aliquots of extract and different volume (2,5,8 and 10 µl) of aflatoxin B_1 and B_2 standard (4 µg / µl) were spotted on different TLC plates. The plate was developed in chloroform : acetone (CA) (9 : 1) in one direction and toluene : ethyl acetate : Formic acid (TEF) (5 : 4 : 1) in the second direction perpendicular to the first direction in equilibrated, TLC chromo tanks at room temperature until the solvent front had reached a line marked 2cm from the top of the plate. After development, plates were removed and air-dried in a fume-cabinet and then examined in a UV light. Aflatoxins B_1 and B_2 visualized blue fluorescence.

Calculation

$$\text{Multitoxin (ppb)} = \frac{S \times C \times D \times 1000}{T \times E}$$

S = Standard volume which matches with test volume in fluorescence intensity
C = Concentration of standard
D = Dilution factor
T = Test volume which matches with standard volume in fluorescence intensity
E = Effective weight of the sample = 4.9

Effect of Medicinal Plants on The Growth of Mycoflora

Collection of Plants

The fresh leaves of *Tridax procumbens*, *Acalypha indica* were collected from Pachal village, Namkkal District, Tamil Nadu. The identification of the plants was confirmed by referring to the books "Indian Medicinal plants" (1996), Medicinal plants of India (Rasheeduz zafar ,1994),.

Preparation of Plant Extracts

Cold Water Extraction

Cold water extract (1:3 w/v) was prepared by homogenizing 25g of sample with 75ml of distilled water followed by filtering through muslin cloth. Filtrate was then centrifuged at 5000rpm for 10 minutes and the supernatant collected was made up to a final volume of 100ml. (Girija Shankar and Thaumanaavn, 2005)

Well Diffusion Assay

Antifungal activity of the plant extracts was carried out by well diffusion method (Perez *et al.*, 1990). A sterile swab was used to evenly distribute fungal culture over the appropriate medium. The plates were allowed to dry for 15 minutes before use for the test. Wells were created using sterile cork borer and with sterile micropipette different volumes (25µl, 50µl, 75µl, 100µl) of the plant extracts were transferred to each well. The plates were incubated at 28°C for 24 hours, after which the plates were examined for inhibition zones.

RESULTS AND DISCUSSION

Among the different poultry raw materials analyzed, sorghum sample – 2 (76×10^2), maize sample – 1 (43×10^2), layer mash sample – 1 (32×10^2), Soya bean sample – 4 (41×10^2) and rape seed meal sample – 3 (24×10^2) recorded highest fungal colonies when compared to other samples. (Table 1 and 2). In all the samples, the fungal population belongs to following, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus terreus*, *Fusarium sp*, *Rhizopus sp*, *Mucor sp*, *Penicillium sp*, *Curvularia sp*, *Cladosporium sp* and yeast. The highest percentage of relative density was shown by *Aspergillus flavus* (57.14%), *Penicillium sp* (16.38%) and *Aspergillus niger* (15.12%) in sorghum samples. *Aspergillus flavus* (57.53%), *Aspergillus niger* (15.06%) were predominant fungi in maize samples, *Aspergillus flavus* (44.64%), *Aspergillus niger* (16.07%) and *Penicillium sp* (11.60%) in layer mash samples, *Aspergillus flavus* (56.83%), *Aspergillus niger* (12.23%) and *Penicillium sp* (7.194 %) in soya bean samples and in rape seed meal samples, the highest percentage of relative density was shown by *Aspergillus flavus* (55.42%) followed by *Aspergillus niger* (7.22%) whereas others had low relative density. The predominance of *Aspergillus flavus* could be due to physiological characteristics, which enable it to survive adverse conditions. It is also a rapidly growing fungus. Silva *et al.*, (2000) reported the predominance of the genera *Phoma* (57%), *Aspergillus* (43%), *Fusarium* (25%), *Rhizopus* (21%), along with the presence of nine other filamentous fungi. The most frequently found fungi were *Aspergillus flavus* and *Fusarium moniliforme*. Asevedo *et al.*, (1994) analysed 90 samples

of corn from various regions of Brazil and reported that *Aspergillus, Penicillium, Fusarium, Rhizopus, Acremonium, Cladosporium Neurospora,* and *Pacillomyees* were isolated. Eighteen isolates of *Aspergillus* and Eighteen isolates of *Penicillium* and six isolates of *Fusarium* proved to be toxic and produced mycotoxin which was isolated from corn grains and sunflower seeds. (Abdel Mallek *et al.* 1993). Osho et al., (2007) reported that *Aspergillus flavus, Fusarium species, Rhizopus species, Aspergillus niger* were found in layer mash samples collected from south west of Nigeria. Kady and Youssef (1993) analyzed 100 soya bean samples from different places of Eqypt. The common species found in soya bean samples were *Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger, Mucor, Rhizopus stolonifer, Penicillium chrysogenum.*

Generally poultry raw materials are contaminated with multitoxin due to various fungal growth. Multitoxin levels were analyzed in different poultry raw materials which were compared with that of standards. A total of 20 samples were tested for multitoxin, sorghum sample – 1 showed the highest concentration of aflatoxin B_1 (199.3ppb), and aflatoxin B_2 (89.67 ppb) followed by sorghum sample – 4 showed the concentration of aflatoxin B_1 (120.23 ppb), aflatoxin B_2 (59.33 ppb). (Table -3). This is in accordance with the work of Silva *et al.*, (2004) who analyzed 10 harvested and 130 stored Brazilian sorghum samples in which 59 *Aspergillus flavu* and *Fusarium proliferatum*, 35 *Fusarium verticillioides* were isolated. Thirty eight (64.4%) isolates of *Aspergillus flavus* strains produced detectable levels of aflatoxin B_1 and aflatoxin B_2 ranging from 12.00 to 3282.50 mg/kg.

In the present study, Cold water extraction of *Acalypha indica* provided efficient inhibition (100%) on the growth of *Aspergillus niger* and *Trichoderma viride.* Whereas Cold water extraction of *Tridax procumbens* at 100 µl provide efficient inhibition zone (37.8 mm) in *Fusarium sp* followed by *Aspergillus niger* (30.6mm), *Mucor sp* (30.5mm), *Penicillium sp* (27.8mm), *Rhizopus sp* (27.0mm), *Trichoderma viride* (26.2mm), *Aspergillus ochraceus* (25.0mm), *Aspergillus flavus* (24.6mm), *Aspergillus terrus* (24.0mm) and *Aspergillus fumigatus* (22.5mm). (Table-4).

The results of the present study reveal that the poultry feeds and its raw materials should be inspected routinely for fungal growth and mycotoxins. It is critically important to monitor poultry feeds for the presence of mycotoxins and fungi. This study confirmed that *Acalypha indica, Tridax procumbens* may provide good inhibitory effects against fungal growth due to their fungi toxicity activity.

Table 1: Incidence of mycoflora in sorghum, maize and layer mash samples

S. No	Fungal species	CFU of fungi in sorghum sample ($\times 10^2$)				Total isolates	Relative density (%)	CFU of fungi in maize samples ($\times 10^2$)		Total isolates	Relative density (%)	CFU of fungi in layer mash samples ($\times 10^2$)					Total isolates	Relative density (%)
		*S-1	S-2	S-3	S-4			*M-1	M-2			*LM-1	LM-2	LM-3	LM-4	LM-5		
1	*Aspergillus flavus*	22	40	32	42	136	57.14	25	17	42	57.53	15	8	10	12	5	50	44.64
2	*Aspergilus niger*	9	12	8	7	86	15.12	5	6	11	15.06	7	5	2	3	1	18	16.07
3	*Aspergillus terrus*	1	-	1	-	2	0.840	-	2	2	2.739	-	1	-	-	1	2	1.785
4	*Aspergillus fumigatus*	-	-	-	-	-	-	-	-	-	-	-	1	-	-	1	2	1.785
5	*Fusarium sp*	2	-	-	1	3	1.260	2	1	3	4.109	1	-	-	1	-	2	1.785
6	*Rhizopus sp*	-	1	-	1	2	0.840	1	-	1	1.369	-	-	1	-	-	1	0.892
7	*Mucor sp*	1	-	1	-	2	0.840	1	-	1	1.369	-	1	-	-	-	1	0.892
8	*Penicillium sp*	10	20	9	-	39	16.38	1	-	1	1.369	3	5	1	-	4	13	11.60
9	*Cladosporium sp*	1	-	-	1	2	0.840	-	-	-	-	-	-	-	-	-	-	-
10	*Curvularia sp*	-	1	-	-	1	0.420	1	-	1	1.369	-	-	-	-	-	-	-
11	yeast	25	16	8	3	-	-	5	2	7	-	23	15	12	9	7	-	-
	Unidentified	4	2	1	8	15	6.302	7	4	11	15.06	6	10	4	2	1	23	20.53
	Total Isolates	50	76	52	60	238		43	30	73		32	31	18	18	13	112	
	Total Species	7	5	4	5			7	4	8		4	6	4	3	5		

* S- Sorghum, M- Maize, LM- Layer Mash

Table 2: Incidence of mycoflora in soyabean and rape seed meal samples

S.No	Fungal species	CFU of fungi in Soya bean samples (× 10^2)					Total isolates	Relative density (%)	CFU of fungi in Rape seed meal samples (× 10^2)				Total isolates	Relative density (%)
		*SB-1	SB-2	SB-3	SB-4	SB-5			*RSM-1	RSM-2	RSM-3	RSM-4		
1	*Aspergillus flavus*	10	22	25	16	6	79	56.83	8	15	10	13	46	55.42
2	*Aspergilus niger*	-	2	5	9	1	17	12.230	1	3	-	2	6	7.228
3	*Aspergillus fumigatus*	1	-	-	-	-	1	0.7194	-	-	-	-	-	-
4	*Aspergillus ochraceus*	-	-	-	-	-	-	-	2	-	-	1	3	3.614
5	*Fusarium sp*	-	-	-	1	2	3	2.158	-	1	-	1	2	2.409
6	*Rhizopus sp*	1	1	-	-	-	2	1.438	1	2	-	-	3	3.614
7	*Mucor sp*	2	-	-	-	-	2	1.438	-	1	-	2	3	3.614
8	*Penicillium sp*	-	2	2	5	3	10	7.194	-	-	-	1	1	1.204
9	*Cladosporium sp*	-	1	-	1	1	3	2.158	-	-	-	-	-	-
10	*Tirchoderma sp*	-	-	-	1	1	2	1.438	-	-	1	1	2	2.409
11	Yeast	16	22	8	14	3	-	-	19	14	28	16	-	-
12	Unidentified	3	7	2	8	-	20	14.38	5	1	8	3	17	20.48
	Total Isolates	15	35	34	41	14	139		19	17	24	23	83	
	Total Species	4	5	3	6	5			2	4	7	5		

*SB - Soya bean , RSM - Rape seed meal

Table 3: Occurrence of multitoxin in different samples of poultry raw materials

S.No	Name of the sample	Aflatoxin B_1(ppb) Mean± S.E	Aflatoxin B_2(ppb) Mean± S.E
1	Sorghum – 1	199.3 ± 0.666	89.67 ± 0.881
2	Sorghum – 2	11.9 ± 0.05	ND
3	Sorghum – 3	12.13 ± 0.09	11.91 ± 0.061
4	Sorghum – 4	120.23 ± 0.379	59.33 ± 1.128
5	Maize – 1	59.33 ± 1.128	24.01 ± 2.081
6	Maize – 2	10.67 ± 1.594	ND
7	Layer Mash – 1	14.9 ± 0.098	ND
8	Layer Mash – 2	7.01 ± 0.006	ND
9	Layer Mash – 3	24.67 ± 0.333	16.33 ± 0.67
10	Layer Mash – 4	6.3 ± 0.34	ND
11	Layer Mash – 5	12.6 ± 0.318	ND
12	Soya – 1	15.6 ± 0.32	11.66 ± 0.285
13	Soya – 2	ND	ND
14	Soya – 3	ND	ND
15	Soya – 4	ND	ND

16	Soya – 5	ND	ND
17	Rape seed Meal – 1	ND	ND
18	Rape seed Meal – 2	ND	ND
19	Rape seed Meal – 3	ND	ND
20	Rape seed Meal – 4	ND	ND

* ND → Not detected, Mean ± S.E = Mean ± Standard error

Table 4: Effect of *Acalypha indica* and *tridax procumbens* - plant extract on the mycelial growth of fungal flora

S.No	Name of the fungus	Cold water extraction of *Acalypha indica*				Cold water extraction of *Tridax procumbens*			
		25µl Mean± S.E	50µl Mean± S.E	75µl Mean± S.E	100µl Mean± S.E	25µl Mean± S.E	50µl Mean± S.E	75µl Mean± S.E	100µl Mean± S.E
1.	*Aspergillus flavus*	12.0 ± 0.009	13.06 ± 0.521	15.8 ± 0.442	19.4 ± 0.809	17.6 ± 0.882	19.5 ± 0.764	22.5 ± 0.736	24.6 ± 0.668
2.	*Aspergillus niger*	NG	NG	NG	NG	26.3± 0.883	27.8 ± 0.168	29.0 ± 0.58	30.6 ± 0.33
3.	*Aspergillus fumigatus*	-	-	-	-	15.8 ± 0.442	17.6 ± 0.882	19.5 ± 0.764	22.5 ± 0.736
4.	*Aspergillus terrus*	13.0 ± 0.096	15.8 ± 0.442	17.6 ± 0.882	20.3 ± 0.185	19.5 ± 0.764	21.2 ± 0.151	22.5 ± 0.736	24.03 ± 0.05
5.	*Aspergillus ochraceus*	14.6 ± 0.670	17.6 ± 0.882	18.3 ± 0.277	21.2 ± 0.151	15.8 ± 0.442	17.6 ± 0.882	19.5 ± 0.764	25.01 ± 0.91
6.	*Penicillium sp*	12.9 ± 0.071	15.8 ± 0.442	17.1 ± 0.057	19.1 ± 0.057	17.67± 0.882	18.6 ± 0.485	19.5 ± 0.764	27.8 ± 0.168
7.	*Fusarium sp*	14.6 ± 0.670	15.8 ± 0.442	16.6 ± 0.579	19.0 ± 0.370	19.5 ± 0.764	22.5 ± 0.736	28.0 ± 0.045	37.8 ± 0.161
8.	*Rhizopus sp*	13.1 ± 0.521	14.6 ± 0.670	15.1 ± 0.098	20.6 ± 0.311	15.8 ± 0.442	17.6 ±0.882	24.6 ± 0.668	27.0 ± 0.410
9.	*Mucor sp*	14.6 ± 0.670	17.6 ± 0.880	19.4 ± 0.809	21.7 ± 0.490	24.6 ± 0.297	26.33± 0.883	27.8 ± 0.168	30.5 ± 0.764
10	*Trichoderma viride*	NG	NG	NG	NG	18.6 ± 0.482	19.5 ± 0.764	22.5 ± 0.736	26.27 ± 0.29

Mean ± S.E = Mean ± Standard error , NG = No growth, - = No inhibition zone

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