Biological approach to aflatoxin control in stored poultry feed

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Abstract

The anti-aflatoxigenic potentials of the leaves of *Psidium guajava*, *Ficus benghalensis*, *Gardenia radicans*, *Punica granatum* and *Ziziphus jujuba* Mill. were investigated. Poultry feed inoculated with *Aspergillus flavus* was treated with plant leaves powder and stored for the period of six months at 28 °C and 16% moisture level. At the end of each month, aflatoxin (AFT) concentrations were determined by high performance liquid chromatography. The proximate and mineral analysis was performed at the end of the storage period. Plant leaves control the AFTs (AFB1 and AFB2) efficiently without compromising the feed quality. *Punica granatum* leaves (5%) completely inhibited the AFTs in the stored feed over the period of six months. *Ziziphus jujube* Mill., *P. guajava*, *F. benghalensis*, and *G. radicans* also showed promising anti-aflatoxigenic activities. Results revealed that the AFT production by *A. flavus* in a stored poultry feed can be inhibited using the plant leaves under investigation.

Anti-aflatoxigenic activity, medicinal plant, storage conditions, nutritional quality, high performance liquid chromatography

Poultry farming has gained importance throughout the world and has become a dynamic industry in South Asian countries since protein demand is increasing day by day (Mahesar et al. 2010). At present, more than 140 poultry feed mills are operating with an annual capacity of ~ 4 million tonnes of feed to meet the feed demand of poultry farms in Pakistan (MLD 2009). Poultry feed comprises peanuts, cotton seeds, cereal grains and proteins (soya been, corn gluten, copra and sunflower) (Ahmad et al. 2012; Anjum et al. 2012). Since the feed is used throughout the year, it is stored for months. Factors such as prolonged storage, variation in temperatures, feed composition and storage conditions play an important role in the fungal growth which contaminates the feed (Stack and Carlson 2003; Iqbal et al. 2015b). Aflatoxins (AFTs) not only decrease the quality of food and feed, but also adversely affect the health of organisms. Moreover, AFTs then also appear in meat and milk, affecting human beings (Anjum et al. 2012).

Aspergillus flavus is one of the dominant microbes responsible for contamination of feed and other agricultural commodities. Aflatoxins are reported to be genotoxic, carcinogenic, teratogenic, and hepatotoxic in nature (Stack and Carlson 2003; Iqbal et al. 2015b). The tropical regions are facing difficulties controlling AFT contamination. In spite of precautionary measures, absolute safety has never been achieved. Although certain fungicides have been known to inhibit the *A. flavus* growth during storage, consumer apprehension about possible risks linked with fungicides resulted in extensive search for more effective and safer control strategies (Chow 1980). Chemical detoxification involves the use of chemical agents (Shi et al. 2006), whereas physical methods comprise radiation and microwave heating (Inan et al. 2007) On the other hand, biological approaches are more effective and safer for AFTs and in this regard, plants are the rich sources of

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phytochemicals which have promising fungicidal activities (Saxena et al. 2013). The use of medicinal plants to control AFTs is of great importance since these are safer in comparison to chemical or physical treatments (Volcani Center 2004; Shi et al. 2006; Inan et al. 2007; Asif 2015a,b,c,d,e,f; Adaramola et al. 2016; Hamid et al. 2016; Adaramola and Onigbinde 2017). Moreover, in view of the current scenario of environmental pollution (Iqbal and Bhatti 2015; Iqbal et al. 2015a; Iqbal and Nisar 2015; Qureshi et al. 2015; Sayed 2015; Iqbal 2016; Mushtaq et al. 2016; Nadeem et al. 2016; Peter and Chinedu 2016; Tahir et al. 2016a,b; Ukpaka 2016a,b,c; Iqbal et al. 2017; Ngobiri and Okorosaye-Orubite 2017; Shoukat et al. 2017), there is a need to adopt safe and eco-friendly methods.

Medicinal plants (*Psidium guajava*, *Ficus benghalensis*, *Gardenia radicans*, *Punica granatum* and *Ziziphus jujuba* Mill.) were investigated for inhibition of AFTs in stored broiler feed. The aim of this research was to evaluate their potential to inhibit AFTs produced by *A. flavus* during storage. The treatment efficiency was evaluated on the basis of AFT inhibition and nutritional value of the stored feed over the period of six months of storage.

Materials and Methods

Chemicals and reagents

The culture media and standard antibiotic discs were purchased from Oxoid Ltd., (Hampshire, UK). Aflatoxin standards were purchased from Supelco (Bellefonte, PA, USA). All other reagents and chemicals, i.e., methanol, acetonitrile, and *n*-hexane used were of analytical grade (Merck, Darmstadt, Germany). Trifluoroacetic acid (TFA) of Riedel-de Haen was used as the derivatizing agent to enhance fluorescence. The immunoaffinity column was purchased from AflaTest® WB VICAM, USA.

Plant collection, extraction and extract preparation

Psidium guajava, *Ficus benghalensis*, *Gardenia radicans*, *Punica granatum* and *Ziziphus jujuba* Mill. plant leaves were collected from the Botanical Garden of the University of Agriculture, Faisalabad, Pakistan. The selected plant leaves were washed, dried under ambient conditions followed by oven drying at 70 °C to constant weight, and then grinded to fine powder.

Antifungal activity

Antifungal extracts were prepared from the leaves by dissolving 10 g powder in 100 ml methanol (80%) and shaken (Gallenkamp, UK) for 24 h at room temperature. The mixture was filtered to separate the extract from residues. The extracts were concentrated under reduced pressure at 45 °C (EYELA, N-N Series, Rikakikai Co. Ltd., Tokyo, Japan) and stored in a refrigerator at 4 °C.

For inoculum preparation, pure fungal strains were obtained from the Department of Clinical Medicine and Surgery, University of Agriculture, Faisalabad, Pakistan. The fungal strains were cultured on the potato dextrose agar (Oxoid, UK) slant and incubated at 28 °C until sporulation (7 days). The spores were harvested in sterilized distilled water containing 0.1% Tween 80 and were counted in the Nebauer chamber with microscopy. The suspension containing 1×10^7 spores/ml was preserved at 4 °C.

For antifungal activity, 20 ml PDA solution containing 1×10^7 spores /ml was spread on sterile Petri dishes. Sterilized discs (6 mm) were impregnated with extract (50 µl) and incubated at 28 °C for 48 h (Wayne 2002). Fluconazole (30 µg/disc) (Oxoid, UK) was used as positive control. The zones of inhibition diameter were measured (mm) by a zone reader.

For MIC estimation, 100 µl of extract (10 mg/ml) was transferred to 96 well plates and 50 µl of Sabouraud dextrose broth (SDB) was added to all wells. Series of dilutions were prepared in a descending concentration order. Finally, 10 µl of fungal suspension (1×10^7 spores/ml) were added to each well. The plates were incubated at 28 °C for 48 h. The absorbance was measured at 620 nm by ELISA reader and MIC was estimated in µg/ml (Sharma and Kumar 2008).

Feed sample treatment

Fresh and toxin free poultry feed samples (4 kg) were obtained from Punjab Feed Corporation, Lahore road, Sheikhupura, Pakistan. The feed sample was dried in an oven at 60 °C and divided into 16 parts (200 g). Each lot was autoclaved, then, moistened (16%) with sterilized distilled water. The feed samples were inoculated by 4 ml of *A. flavus* suspension (1×10^7 spores /ml) under laminar air flow (Dalton, Japan).

Plant leaves powder at three different concentrations (5, 10 and 15% w/w) were added separately in 200 g of inoculated feed. Non-treated feed samples were considered as control. The treated and control samples were

stored at 16% moisture and 28 °C for six months. At the end of each month, feed samples were drawn and AFTs were (AFB1 and AFB2) determined by HPLC.

Aflatoxin extraction and analysis

Two types of AFTs (aflatoxin B1 and aflatoxin B2) were measured as described by Mushtaq et al. (2012). Briefly, the treated feed sample (5 g) was taken in a conical flask and 20 ml of acetonitrile-water (84:16 v/v) was added and shaken for 90 min in an orbital shaker at ambient conditions. The content was filtered and the filtrate was concentrated under reduced pressure to 4–5 ml. Concentrated sample (2 ml) was diluted with 20 ml of deionized water and passed through Vicam (AflaTest WB Columns, Vicam, USA) at the flow rate of 2 ml/min using a suction pump. The column was washed with 20 ml of deionized water, and then dried by air streaming for 1–2 min. The AFTs from the column were eluted using methanol (1 ml, twice). The samples were dried under N_2 blanketing. For derivatization, the dried sample was vortexed with 200 µl of *n*-hexane for 30 s; then 50 µl of trifluoroacetic acid was added and again vortexed for 30 s. Finally, 1.95 µl water : acetonitrile (9:1 v/v) was added and vortexed for 20 s, and the resultant mixture (20 µl) was subjected to HPLC analysis. The LC-system (Shimadzu LC LC-10A Series, Shimadzu, Japan) was used for the estimation of AFTs. Isocratic mobile phase consisting of acetonitrile, methanol and water (22.5:22.5:55) was used at a flow rate of 1 ml/min. Calibration curve was drawn using a series of FATs concentrations (0.05, 0.1, 0.5, 1.0, 5.0, 10 µg/l) in acetonitrile. The AFTs inhibition was calculated using the relation shown in the following equation, where X is the concentration of AFTs in treated samples and Y is the concentration of AFTs in control (un-treated sample):

$$Inhibition\ (\%) = \left[\frac{X-Y}{Y}\right] \times 100$$

Proximate and mineral analysis

The proximate composition (ash, protein, fat, and fiber) and mineral profile of treated and un-treated feed samples were measured at the end of the storage period. For the proximate composition, AOAC standard methods were adopted (AOAC 1990). For mineral analysis, calcium (Ca) and iron (Fe) contents were analyzed by AAS (Hitachi Polarized Zeeman AAS, Z-8200, Japan) (AOAC 1990). Potassium (K) was determined using flame photometer (PFP7, Jenway, Japan), whereas phosphorus (P) was determined by UV-Vis spectrophotometer (Lambda-25, Perkin Elmer, USA) (Khalil and Mannan 1990).

Statistical analysis

All experiments were performed in triplicate and the data thus obtained were reported as means ± SD. Analysis of variance (ANOVA) was performed at $P < 0.05$ confidence level using Minitab statistical software (Minitab Inc. Pennsylvania, U.S.A).

Results

Antifungal activity of medicinal plant leaves

The antifungal activities of medicinal plant leaves are given in Table 1. All plants showed a considerable antifungal activity against *A. flavus* and variation among plants was significantly ($P < 0.05$). *Punica granatum* leaf extract showed highest activity (19 mm) followed by *Ziziphus jujuba* Mill., *Ficus benghalensis, Psidium guajava*, and *Gardenia radicans*. The MIC values against *A. flavus* are given in Table 1. The MIC values indicate that the medicinal plant has potent antifungal activities, inhibiting the growth of *A. flavus* at relatively small concentrations (Mahoney and Molyneux 2004).

Table 1. Antifungal activity and minimum inhibitory concentration (MIC) of medicinal plant leaf extracts against *Aspergillus flavus*.

Medicinal plant	Zone diameter (mm)	MIC (µg/ml)
Psidium guajava	$11.0 \pm 1.52^{d*}$	121.11 ± 0.76^{b}
Ficus benghalensis	15.0 ± 2.51^{c}	103.20 ± 1.09^{c}
Gardenia radicans	10.0 ± 2.51^{d}	132.50 ± 0.54^{a}
Punica granatum	19.0 ± 1.00^{b}	73.60 ± 0.73^{d}
Ziziphus jujuba Mill.	16.0 ± 1.52^{c}	77.50 ± 1.07^{cd}
Flucanozole (control)	26.0 ± 1.00^{a}	41.20 ± 1.00^{e}

MIC = Minimum inhibitory concentration, *Values are mean ± SD of triplicate runs. Superscripts within column are showing significant difference ($P < 0.05$) among plants

Aflatoxin inhibition

Feed samples treated with different concentrations of medicinal plant leaf powder were stored for a period of six months and the AFT (AFB1 and AFB2) concentrations were determined at the end of each month. The obtained results are shown

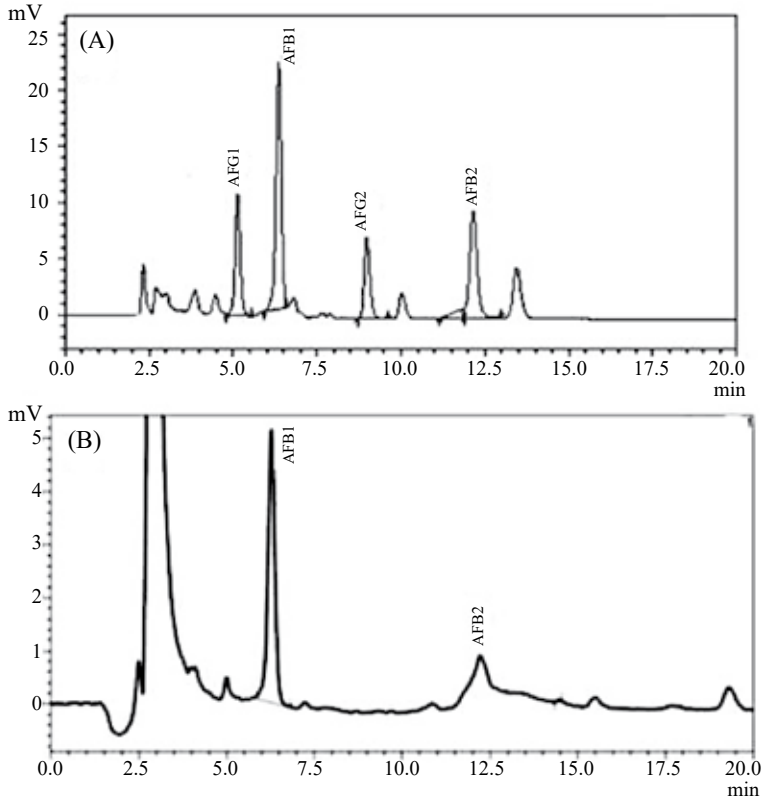

Fig. 1. HPLC chromatograms (A) standard aflatoxins, (B) aflatoxins (AFB1 and AFB2) in *A. flavus* contaminated feed samples

in Table 2 and typical HPLC chromatograms are shown in Fig. 1. All the treatments inhibited the AFTs in a concentration-dependent manner. *Punica granatum* at a 5% concentration inhibited the AFTs up to 100% throughout the storage period. The effect of *Z. jujuba* Mill. on AFTs inhibition was also promising and the values of *Psidium guajava* treated feed samples were significantly lower than the control. *Ficus benghalensis* was also found to be efficient for AFT control; the values of *Gardenia radicans* leaf treated feed samples were significantly lower than control. The detected AFTs in stored feed samples without leaf powder treatments were considerably higher than the recommended limits for feed.

Proximate composition and mineral contents of treated feed

The results of proximate analysis of the treated feed inoculated with *A. flavus* are given in Table 3. The proximate composition was recorded to be stable in the feed at the end of the six months of storage. Interestingly, it was observed that at higher concentration, the decrease in proximate composition was low, i.e. that the proximate composition changed in concentration dependent manner. Overall, it was observed that feed samples treated with plant leaf powder showed stability in proximate composition versus control.

Results revealed that feed treated with plant leaves did not change the mineral contents over the entire storage period of storage. Similarly to proximate composition, minerals also showed stability (Table 4).

Table 2. Contents (ppb) of aflatoxins (AFB1 and AFB2) in broiler feed treated with medicinal plant leaves for a period of six months.

Treatments	Conc. (g/100g)	1st month[a]	2nd month[b]	3rd month[c]	4th month[d]	5th month[e]	6th month[f]
Control	00	9.40.04*	16.42 ± 0.03	27.39 ± 0.04	41.22 ± 0.04	64.61 ± 0.06	106.53 ± 0.04
P. guajava[c]	05	3.67 ± 0.06 (60.95)	5.83 ± 0.04 (64.94)	8.80 ± 0.03 (67.87)	13.24 ± 0.06 (67.88)	24.61 ± 0.05 (61.91)	50.80 ± 0.03 (52.31)
	10	1.86 ± 0.05 (80.21)	2.65 ± 0.02 (83.86)	5.06 ± 0.04 (81.52)	9.01 ± 0.05 (78.14)	15.86 ± 0.02 (75.45)	29.37 ± 0.02 (72.43)
	15	0.00 ± 0 (100)	0.00 ± 0 (100)	0.92 ± 0.03 (96.64)	1.82 ± 0.07 (95.58)	3.67 ± 0.04 (94.31)	5.93 ± 0.03 (94.43)
F. benghalensis[ab]	05	0.00 ± 0 (100)	0.00 ± 0 (100)	0.00 ± 0 (100)	0.71 ± 0.03 (98.27)	2.16 ± 0.03 (96.65)	4.11 ± 0.05 (96.14)
	10	0.00 ± 0 (100)	0.00 ± 0 (100)	0.00 ± 0 (100)	0.00 ± 0 (100)	0.00 ± 0 (100)	0.87 ± 0.02 (99.10)
	15	0.00 ± 0 (100)	0.00 ± 0 (100)	0.00 ± 0 (100)	0.00 ± 0 (100)	0.00 ± 0 (100)	0.64 ± 0.01 (99.32)
G. radicans[d]	05	4.12 ± 0.04 (56.17)	7.41 ± 0.05 (54.87)	10.93 ± 0.02 (60.09)	17.8 ± 0.04 (56.81)	29.24 ± 00.02 (54.74)	48.35 ± 0.04 (54.61)
	10	3.31 ± 0.02 (64.78)	6.63 ± 0.02 (59.62)	9.48 ± 0.04 (65.38)	16.63 ± 0.02 (59.65)	24.74 ± 0.05 (61.71)	38.09 ± 0.02 (64.25)
	15	1.54 ± 0.03 (83.61)	3.14 ± 0.03 (80.87)	4.95 ± 0.02 (81.92)	6.78 ± 0.05 (83.55)	10.95 ± 0.03 (83.05)	23.09 ± 0.04 (78.32)
P. granatum[a]	05	0.00 ± 0 (100)	0.00 ± 0 (100)	0.00 ± 0 (100)	0.00 ± 0 (100)	0.00 ± 0 (100)	0.00 ± 0 (100)
	10	0.00 ± 0 (100)	0.00 ± 0 (100)	0.00 ± 0 (100)	0.00 ± 0 (100)	0.00 ± 0 (100)	0.00 ± 0 (100)
	15	0.00 ± 0 (100)	0.00 ± 0 (100)	0.00 ± 0 (100)	0.00 ± 0 (100)	0.00 ± 0 (100)	0.00 ± 0 (100)
Z. jujuba Mill.[b]	05	0.00 ± 0 (100)	0.00 ± 0 (100)	0.00 ± 0 (100)	1.53 ± 0.03 (96.28)	2.39 ± 0.02 (96.30)	5.17 ± 0.03 (95.15)
	10	0.00 ± 0 (100)	0.00 ± 0 (100)	0.00 ± 0 (100)	0.00 ± 0 (100)	0.00 ± 0 (100)	2.64 ± 0.02 (97.52)
	15	0.00 ± 0 (100)	0.00 ± 0 (100)	0.00 ± 0 (100)	0.00 ± 0 (100)	0.00 ± 0 (100)	1.26 ± 0.03 (98.81)

*Values are mean ± SD of three samples analysed individually in triplicate. Superscripted lowercase letters within the row indicate significant difference ($P < 0.05$) in aflatoxin level at various incubation periods while superscripted uppercase letters within the column depict the inhibition potential ($P < 0.05$) of different medicinal plant leaves. Values in parentheses represent inhibitory effect (%) of medicinal plant leaves as compared to control, ppb = parts-per-billion, *P. guajava* = *Psidium guajava*, *F. benghalensis* = *Ficus benghalensis*, *G. radicans* = *Gardenia radicans*, *P. granatum* = *Punica granatum* and *Z. jujuba* Mill.

Discussion

Contaminated feed can be harmful to poultry as well as to the consumers because of the toxins contained in poultry products (Iqbal et al. 2015b). *Ziziphus jujuba* Mill. contains flavonoids, vitamin C, triterpenic acids, phenolics, and polysaccharides (Gao et al. 2013), whereas ellagic acid, ellagitannins (including punicalagins), punicic acid and other fatty acids, flavonoids, anthocyanidins, anthocyanins, estrogenic flavonols, and flavones are the

Table 3. Proximate composition (%) of fresh and stored broiler feed treated with medicinal plant leaves.

Treatments	Conc. (g/100g)	Ash	Decrease (%)	Protein	Decrease (%)	Fat	Decrease (%)	Fibre	Decrease (%)
Control	00	6.54 ± 0.02		23.56 ± 0.03		3.53 ± 0.05		3.67 ± 0.04	
		(5.05 ± 0.01)	22.78	(19.40 ± 0.01)	17.65	(1.43 ± 0.04)	59.49	(1.69 ± 0.01)	53.95
P. guajava[c]	05	6.39 ± 0.01		22.93 ± 0.02		3.18 ± 0.07		3.20 ± 0.03	
		(5.54 ± 0.09)	13.32	(21.28 ± 0.02)	7.19	(1.86 ± 0.02)	41.51	(1.96 ± 0.04)	38.75
	10	6.31 ± 0.08		22.79 ± 0.08		3.29 ± 0.02		3.36 ± 0.09	
		(5.64 ± 0.08)	10.62	(21.78 ± 0.03)	4.43	(2.02 ± 0.03)	38.60	(2.23 ± 0.03)	33.63
	15	6.23 ± 0.03		22.67 ± 0.08		3.41 ± 0.03		3.52 ± 0.06	
		(5.53 ± 0.01)	11.23	(21.95 ± 0.03)	3.17	(2.51 ± 0.02)	26.39	(2.89 ± 0.06)	17.89
F. benghalensis[ab]	05	6.34 ± 0.03		22.61 ± 0.06		3.86 ± 0.08		4.09 ± 0.08	
		(5.92 ± 0.09)	6.62	(22.12 ± 0.07)	2.16	(3.11 ± 0.05)	19.43	(3.38 ± 0.03)	17.36
	10	6.23 ± 0.01		22.48 ± 0.06		4.20 ± 0.04		4.51 ± 0.01	
		(6.12 ± 0.03)	1.79	(22.29 ± 0.04)	0.84	(3.95 ± 0.03)	5.95	(4.21 ± 0.06)	6.65
	15	6.14 ± 0.06		22.27 ± 0.08		4.55 ± 0.02		4.93 ± 0.07	
		(6.06 ± 0.02)	1.30	(22.14 ± 0.04)	0.58	(4.33 ± 0.09)	4.83	(4.67 ± 0.04)	5.27
G. radicans[d]	05	6.17 ± 0.08		22.86 ± 0.04		3.18 ± 0.08		3.23 ± 0.03	
		(5.23 ± 0.08)	15.37	(20.89 ± 0.09)	8.61	(1.68 ± 0.04)	47.17	(1.87 ± 0.02)	42.11
	10	6.04 ± 0.07		22.71 ± 0.02		3.24 ± 0.03		3.39 ± 0.09	
		(5.30 ± 0.01)	12.25	(21.32 ± 0.02)	6.21	(1.93 ± 0.05)	40.43	(2.11 ± 0.04)	37.15
	15	5.93 ± 0.09		22.54 ± 0.03		3.29 ± 0.03		3.76 ± 0.05	
		(5.19 ± 0.08)	12.47	(21.47 ± 0.02)	4.74	(2.01 ± 0.02)	38.90	(2.85 ± 0.02)	24.20
P. granatum[a]	05	6.19 ± 0.02		22.84 ± 0.05		3.82 ± 0.06		4.53 ± 0.06	
		(6.18 ± 0.07)	0.0	(22.83 ± 0.06)	0.0	(3.82 ± 0.07)	0.0	(4.55 ± 0.06)	0.0
	10	6.02 ± 0.02		22.07 ± 0.06		4.11 ± 0.03		5.40 ± 0.09	
		(6.03 ± 0.04)	0.0	(22.07 ± 0.01)	0.0	(4.10 ± 0.07)	0.0	(5.42 ± 0.03)	0.0
	15	5.85 ± 0.03		21.43 ± 0.01		4.41 ± 0.04		6.26 ± 0.08	
		(5.84 ± 0.03)	0.0	(21.42 ± 0.07)	0.0	(4.42 ± 0.04)	0.0	(6.24 ± 0.19)	0.0
Z. jujuba Mill.[b]	05	6.21 ± 0.02		22.84 ± 0.08		3.54 ± 0.02		4.12 ± 0.09	
		(5.86 ± 0.09)	5.63	(22.31 ± 0.08)	2.32	(2.87 ± 0.09)	18.93	(3.67 ± 0.03)	10.92
	10	6.06 ± 0.04		22.63 ± 0.03		3.87 ± 0.05		4.31 ± 0.06	
		(6.02 ± 0.03)	0.66	(22.51 ± 0.01)	0.53	(3.59 ± 0.06)	7.23	(4.11 ± 0.07)	4.64
	15	5.89 ± 0.02		22.48 ± 0.08		4.23 ± 0.08		4.78 ± 0.03	
		(5.81 ± 0.08)	1.36	(22.46 ± 0.09)	0.08	(3.97 ± 0.02)	6.15	(4.61 ± 0.04)	3.56

Values are mean ± SD of three samples analysed individually in triplicate at $P < 0.05$. Superscript uppercase letters within the column depicted significant difference ($P < 0.05$) among different medicinal plants. Values given in parentheses represent treated feed samples at end of storage. *P. guajava* = *Psidium guajava*, *F. benghalensis* = *Ficus benghalensis*, *G. radicans* = *Gardenia radicans*, *P. granatum* = *Punica granatum* and *Z. jujuba* Mill. = *Ziziphus jujuba* Mill.

Table 4. Mineral composition (ppm) of fresh and stored broiler feed treated with medicinal plant leaves.

Treatments	Conc. (g/100g)	Iron	Decrease (%)	Calcium	Decrease (%)	Phosphorus	Decrease (%)	Potassium	Decrease (%)
Control	00	164.4 ± 1.23*		1806.3 ± 1.34		1664.2 ± 1.64		4721.5 ± 4.35	
		(49.20 ± 1.23)	70.07	(1395.6 ± 1.75)	22.73	(1113.6 ± 8.64)	33.08	(4302.5 ± 3.13)	8.87
P. guajava[c]	05	168.6 ± 3.32		1778.4 ± 2.43		1593.4 ± 1.47		5121.3 ± 4.42	
		(120.7 ± 3.24)	28.41	(1556.7 ± 1.25)	12.46	(1380.5 ± 2.38)	13.36	(4805.3 ± 2.24)	6.17
	10	173.4 ± 2.65		1749.5 ± 1.52		1543.5 ± 1.62		5234.1 ± 3.17	
		(118.6 ± 2.47)	31.60	(1536.4 ± 2.23)	12.15	(1397.5 ± 1.85)	9.45	(4947.8 ± 3.53)	5.46
	15	178.7 ± 1.84		1718.2 ± 1.74		1491.7 ± 1.15		5323.7 ± 3.64	
		(142.9 ± 4.26)	20.03	(1572.5 ± 3.43)	8.47	(1409.5 ± 4.65)	5.51	(5156.6 ± 4.26)	3.13
F. benghalensis[ab]	05	177.2 ± 4.37		1783.2 ± 1.82		1614.5 ± 1.38		5131.2 ± 3.58	
		(152.4 ± 4.12)	14.02	(1659.4 ± 1.65)	6.94	(1496.3 ± 3.36)	7.32	(5014.8 ± 4.54)	2.26
	10	181.5 ± 1.42		1751.4 ± 1.36		1568.3 ± 1.76		5207.8 ± 3.73	
		(174.6 ± 1.38)	3.86	(1721.5 ± 1.76)	1.71	(1541.6 ± 3.83)	1.70	(5157.1 ± 3.37)	0.97
	15	188.3 ± 3.57		1708.7 ± 1.14		1512.4 ± 1.59		5318.3 ± 2.49	
		(186.2 ± 2.76)	1.12	(1684.2 ± 2.65)	1.43	(1496.2 ± 1.59)	1.07	(5286.3 ± 3.42)	0.60
G. radicans[d]	05	165.9 ± 3.56		1796.2 ± 2.37		1587.2 ± 1.47		4931.5 ± 1.15	
		(108.9 ± 2.65)	34.5	(1591.4 ± 2.98)	11.42	(1313.5 ± 3.79)	17.24	(4653.4 ± 3.74)	5.63
	10	168.4 ± 4.36		1753.6 ± 1.64		1532.4 ± 1.68		5014.7 ± 3.47	
		(110.4 ± 1.47)	34.4	(1542.4 ± 2.76)	10.91	(1362.9 ± 4.14)	11.06	(4732.3 ± 4.86)	5.63
	15	170.6 ± 1.14		1728.4 ± 2.13		1478.3 ± 1.46		5130.7 ± 3.63	
		(114.7 ± 1.33)	32.76	(1494.2 ± 1.45)	13.55	(1307.4 ± 1.35)	11.56	(4858.6 ± 3.46)	5.30
P. granatum[a]	05	177.5 ± 4.26		1788.3 ± 1.27		1640.3 ± 1.93		5014.6 ± 3.62	
		(177.5 ± 2.32)	0.0	(1788.4 ± 2.45)	0.0	(1640.4 ± 4.54)	0.0	(5014.3 ± 4.65)	0.0
	10	181.3 ± 1.85		1756.4 ± 1.42		1617.1 ± 1.25		5327.8 ± 3.38	
		(181.4 ± 3.47)	0.0	(1756.6 ± 1.65)	0.0	(1617.8 ± 3.98)	0.0	(5327.1 ± 3.48)	0.0
	15	186.9 ± 3.26		1727.5 ± 2.35		1594.7 ± 1.47		5682.7 ± 4.75	
		(186.8 ± 3.65)	0.0	(1727.2 ± 3.34)	0.0	(1594.1 ± 2.68)	0.0	(5682.3 ± 1.73)	0.0
Z. jujuba Mill.[b]	05	170.8 ± 1.68		1788.3 ± 3.39		1609.2 ± 1.96		5057.3 ± 3.68	
		(137.6 ± 2.65)	19.4	(1693.5 ± 1.43)	5.30	(1519.3 ± 1.67)	5.58	(4974.3 ± 4.75)	1.64
	10	174.5 ± 3.74		1761.4 ± 1.86		1561.4 ± 1.47		5131.4 ± 3.74	
		(167.9 ± 1.73)	3.78	(1721.5 ± 1.12)	2.26	(1540.2 ± 2.37)	1.35	(5087.4 ± 1.32)	0.85
	15	180.2 ± 3.34		1749.3 ± 3.75		1508.4 ± 1.46		5247.5 ± 2.59	
		(172.4 ± 2.45)	3.34	(1723.5 ± 2.23)	1.47	(1494.2 ± 3.35)	0.94	(5223.7 ± 4.75)	0.45

*Values are mean ± SD of three samples analysed individually in triplicate at $P < 0.05$. Superscripted lowercase letters within the row indicate significant difference ($P < 0.05$) of the incubation period while superscripted uppercase letters within the column depict significant difference ($P < 0.05$) among different medicinal plants. Values given in parentheses represent treated feed samples at end of storage, ppm = parts-per million, *P. guajava* = *Psidium guajava*, *F. benghalensis* = *Ficus benghalensis*, *G. radicans* = *Gardenia radicans*, *P. granatum* = *Punica granatum* and *Z. jujuba* Mill. = *Ziziphus jujuba* Mill.

major constituents of *P. granatum* (Viladomiu et al. 2013). *Ficus benghalensis* contains flavonoids, coumarins, leucocyanidins, terpenes as major components (Sankaranarayanan and Sampathkumar 2012). Similarly, allic acid, catechin, chlorogenic acid, caffeic acid, epicatechin, rutin, quercitrin, isoquercitrin, quercetin, kaempferol, glycosylated campeferol, tocopherol, β-carotene and lycopene are the major constituents of *P. guajava* (Araújo et al. 2015). So far, the FATs inhibition in *A. flavus* inoculated feed sample treated with leaf powder might be due to the bioactivity of these bioactive compounds. The components present in plants have beneficial effects but toxic effects of certain plant components have also been reported, i.e., the oestrogenic effect of isoflavonoids or the toxicity of coumarins. Tannins in the feed of monogastric animals could reduce bioavailability and utilization of proteins (Roy et al. 1988; Bankole 1997; Abou-Arab et al. 1999; Soliman and Badeaa 2002; Romagnoli et al. 2007).

The antimicrobial assay revealed that the plants under investigation have significant antifungal activities, revealing the presence of bioactive compounds in the extracts. *Psidium guajava* and *G. radicans* were found to have promising antifungal activities. The medicinal plants that inhibit the growth of *A. flavus* at very low concentration are considered potent antifungal agents. The antifungal activities of plant extracts can be correlated with the secondary metabolites having antifungal agents (Sharma and Kumar 2008; Sankaranarayanan and Sampathkumar 2012; Gao et al. 2013; Viladomiu et al. 2013; Araújo et al. 2015).

Poultry feed treated with plant powder revealed that the medicinal plants have promising efficiency for the inhibition of AFT production by *A. flavus.* Previous reports also revealed that spices, plant extracts, essential oils have proven to exert efficient antifungal activities against toxigenic microorganisms (Shi et al. 2006; Inan et al. 2007). The medicinal plants investigated in our study inhibited *A. flavus* growth efficiently and as a result, the contents of aflatoxins were also reduced.

Results of the present investigation are in line with Dahham et al. (2010), who reported that the methanolic extracts of *P. granatum* have a potent antifungal activity and correlated it with phytochemicals in extracts including phenols, flavonoids and tannins.

Treated feed samples showed a slight change in the proximate composition and this slight decrease in proximate contents could be due to AFTs production because AFTs affect proximate contents and ultimately, lead to bad nutritive quality. Results revealed that medicinal plant leaf supplementation reduced the risk of AFTs, production by *A. flavus* without compromising the proximate components. The decrease in protein contents was due to fungus growth because during proliferation the protein is assimilated in the synthesis of protoplasm (Aboloma and Moriike 2012). Previous findings also support these results i.e., change in proximate composition in almond, inoculated with *A. flavus* reduced crude fat from 11.5% to 14.7% (Ali et al. 2009). The decrease in fat contents of the feed might be due to the lipolytic activity of the fungus. Results revealed that feed treated with plant leaves did not change the mineral contents over the entire storage period. The decrease in mineral contents was significantly higher in control compared to treated samples. The detrimental effects of AFTs on the stored feed observed in the present investigation are in line with previous studies. Authors have reported a significant decrease in Fe, Ca, P and K contents in *A. flavus* contaminated *Dialium guineense* and the Fe, Ca, P and K contents were also decreased in *A. flavus* inoculated almond, and these findings are in line with previous studies that fungus may affects the mineral contents (Ali et al. 2009; Aboloma and Moriike 2012). A decrease in P contents in papaya and mango fruits by fungal contamination has also been reported because P is assimilated by microorganisms as inorganic phosphate and is incorporated into phospholipids and nucleic acids as well as ATP synthesis (Pawar 2012). In another report, a direct relationship between P contents and microbial growth was observed (Fagbohun and Faleye 2012). In the present investigation, *P. guajava*, *F. benghalensis*, *G. radicans*, *P. granatum* and *Z. jujuba* also showed a promising anti-aflatoxigenic activities and the growth

of *A. flavus* was inhibited efficiently; however, *P. granatum* at very low concentration activity was excellent for the inhibition of AFTs (AFB1 and AFB2) production by *A. flavus* over the period of six months of storage without compromising the proximate composition and mineral contents of the feed. So far, the plants under investigation could be potential candidates for the control of AFT production by *A. flavus* in stored poultry feed.

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