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Original article

Exploitation of selected plant extracts as bio-control against fungal contaminants in animal feed

Raza Ullah [a], Isfahan Touseef [a,*], Rameesha Abid [b,c], Arshad Farid [d,e], Sohail Ahmad [d], Hesham Ali El Enshasy [f,g,h], Adil Aksoy [i], Nada H. Aljarba [j], Tahani Mohamed Al–Hazani [k], Muhammad Muzammal [d], Shakira Ghazanfar [c,*]

[a] Department of Microbiology, Hazara University Mansehra, 21300, Pakistan
[b] Department of Microbiology, Quaid-i-Azam University, Islamabad 44100, Pakistan
[c] National Agricultural Research Center, National Institute for Genomics and Advanced Biotechnology (NIGAB) Islamabad, 44100, Pakistan
[d] Gomal Centre of Biochemistry and Biotechnology, Gomal University, D.I.Khan 29050, Pakistan
[e] Marine Biodiscovery Centre, Department of Chemistry, University of Aberdeen, Aberdeen AB24 3UE, UK
[f] Institute of Bioproduct Development (IBD), Universiti Teknologi Malaysia (UTM), Skudai, Johor Bahru, Malaysia
[g] School of Chemical and Energy Engineering, Faculty of Engineering, Universiti Teknologi Malaysia (UTM), Skudai, Johor Bahru, Malaysia
[h] City of Scientific Research and Technology Applications (SRTA), New Burg Al Arab, Alexandria, Egypt
[i] Eskil Vocation of High School, Laboratory Veterinary Science, Aksaray University, Aksaray, Turkey
[j] Department of Biology, College of Science, Princess Nourah bint Abdulrahman University, P. O. Box 84428, Riyadh 11671, Saudi Arabia
[k] Biology Department, College of Science and Humanities, Prince Sattam bin Abdulaziz University, P.O. Box: 83, Al-Kharj, 11940, Saudi Arabia

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ABSTRACT

Objectives: Pakistan is among the top agricultural countries around the globe, but mycotoxin contamination causes a major commercial loss every year. The present study aimed to isolate the fungi and their mycotoxins present in contaminated feed of animals in the local market of Peshawar.
Methodology: The antifungal activity of certain plant extracts was to be tested against pathogenic fungi. TLC method was used for mycotoxin analysis and it was found that Aflatoxin G1, G2, B1 and B2 were present in different amounts both qualitatively and quantitatively based on samples.
Result: This study found the presence of contamination in all the tested feed samples. All ten samples were found positive for *Aspergillus flavus*. B1 toxin was found in high quantity in all ten samples, while G2 was found in a lower quantity as compared to other toxins such as G1, B1 and B2. The quantity of aflatoxin was from 48.6 to 284.7 ppb in 25 g of samples. In the case of antifungal potency, all plant extracts showed important antifungal potency against tested fungi. The MIC and MFC values noted ranged from 110 to 300 mg/ml and 100–300 mg/ml respectively. *Citrus aurantium* and *Myrtus communis* were absorbed to have antifungal potency against all test fungi. *Citrus aurantium* extracts were also found to inhibit the growth of *Aspergillus flavus*.
Conclusion: According to the results of the present research, a variety of fungal strains and aflatoxins were present in animal feed in numerous parts of Peshawar, Pakistan and different plant extracts can be used in animal feed to reduce this type of contamination.

1. Introduction

Pakistan is mainly dependent on its agriculture sector. About half of Pakistan's population is dependent upon agriculture. Different researchers recommended that agricultural development is directly related to the national economy of the country (Ali and Iqbal, 2004). In addition to other agriculture products, livestock is also thought to be an important contributor to Pakistan's GDP because it adds almost 37% value-addition to the national GDP. According to the Food and Agriculture Organization (FAO) reports,

Abbreviations: FAO, Food and Agriculture Organization; PCSIR, Pakistan Council of Scientific and Industrial Research; PDA, Potato Dextrose Agar; PDB, Potato Dextrose Broth; MIC, Minimum Inhibitory Concentration; MFC, Minimum Fungicidal Concentration; TLC, Thin Layer Chromatography; UV, Ultra violet.

* Corresponding authors.
E-mail addresses: isfahan@hu.edu.pk (I. Touseef), shakira_akmal@parc.gov.pk, shakira_akmal@yahoo.com (S. Ghazanfar).

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25% of the cereals in the world are destroyed by mycotoxins (Klopfenstein,2000).

Mycotoxins are diverse types of molecules that are injurious to living things. They are generally present in cereals and grains during the pre or post-harvesting stage and due to their varied toxicity, they are considered injurious for the users (Yiannikouris and Jouany,2002). Many species of fungi account for the production of mycotoxins in animal feedstuff (Yiannikouris and Jouany,2002; Salari et al., 2012). The threat of mycotoxin contamination especially aflatoxin in cereals, grains and other field crops is a serious safety concern for the world (Reddy et al.,2009).

In olden times, many techniques were used to inhibit the progression of mycotoxins i.e. microorganisms, absorbents, chemicals and ionizing radiations (Sinha, 1998). Nevertheless, natural plants and plant extracts have expanded the attention of researchers due to their less toxic effects. Natural compounds from higher plants are cheap and ecofriendly and are considered as antimicrobial agents (Reddy et al., 2009; Gonçalez et al., 2003).

Plants possessing powerful antimicrobial properties can be used to protect food from different types of toxins. Natural compounds from the plant can also be served as antifungal agents (Abid et al., 2022). Additionally, citrus plants are reported to have medicinal importance. Crude extracts from these plant's families can be used as natural remedies to cure many diseases i.e. diarrhea, constipation and vomiting, etc (Gonçalez et al., 2003). Citrus fruits have an ample amount of carotenoids, minerals, limonoids, essential oils, flavonoids, acridone alkaloids, and vitamin B and C complex. Limonoids, glycosides, flavones and polymethoxyflavones are the flavenoids naturally produced by citrus plants. (Gonçalez et al., 2003; Sinha, 1998). They have many health regulating activities i.e. anti-viral, anti-cancerous, anti-fungal, anti-bacterial, anti-oxidant, and anti-allergic potencies. Lemon juice is employed as a diuretic agent, antiscorbutic and also used against the common cold (Salari et al., 2012). Moreover, the antifungal and anti-aflatoxigenic efficacy of *Aloe vera*, *Zizyphusspina*, *Cassia italica*, *Lavandula vera*, *Olea europaea*, *Ricinus communis*, *Lawsonia inermis*, *Prosopisjuliflora*, *Datura stramonium* and *Eucalyptus globulus* is also documented to be used as inhibitors of aflatoxin production (Reddy et al.,2009). The current study aimed to isolate the fungal species and also analyze their mycotoxins in the samples of animal feeds available in the local markets of Peshawar. Also, it was aimed to test the antifungal activity of different plant extracts against those fungal species. The synergistic effects of toxin inhibition and mycoflora promotion were also aimed in the present study.

2. Methods and materials

2.1. Plant samples

Six different medicinal plant leaves i-e; *Aloe vera* (Aloe), *Myrtus communis* (Myrtle), *Citrus sinensis* (Sweet orange), *Emblica officinalis* (Amla), *Citrus limon* (Lemon) and *Citrus aurantium* (Sour orange) were collected from Pakistan Council of Scientific and Industrial Research (PCSIR) Peshawar, Pakistan (Table 1). The plant samples (N = 6) were shade dried and crushed to obtain a powder.

Table 1
List of plants used in the study.

Plant name	Family	Urdu/local name	English name
Aloe vera	Liliaceae	Aloe vera	Aloe
Citrus aurantium	Rutaceae	Narangi	Bitter orange
Citrus sinensis	Rutaceae	Malta	Sweet orange
Emblica officinalis	Phyllanthaceae	Amla	Indian gooseberry
Citrus limon	Rutaceae	Leemu	Lemon
Myrtus communis	Myrtaceae	Mehndi/hina	Myrtle

2.2. Preparation of aqueous extracts

Concisely, 50 g of dried powder of selected plants was soaked in about 500 ml sterile purified water. Then the mixture was filtered with a muslin cloth. After filtration, the mixture was centrifuged at 4000 rpm for 30 mins. The supernatant was further processed for filtration through Whatman filter paper No.1. The extract was then stored at 4 °C.

2.3. Sample collection areas

Different areas of Peshawar were visited and ten animal feed samples were purchased. The samples were processed within 48 h of being collected. Table 2 shows the animal feed samples.

2.4. Determination of total count of fungal colonies

The pour plate technique was used to determine fungal colonies. In 450 ml butterfield solution, 50 g of animal feed was added and mixed for 2 min after that, serial dilutions were made up to 10^{-6} folds. Potato Dextrose Agar (PDA) was used for the culturing of fungal isolates. A total of 20 ml media was transferred to each petri plate. After solidification of the media, the petri plates were incubated for 25 °C. Then 1 ml sample from each serially diluted tube was inoculated in sterilized petri plates. The plates were again placed in an incubator and after 3 to 5 days fungal colonies were appeared and calculated per 1 g of sample (Bhatti et al.,1990).

2.5. Isolated fungal colonies characterization

The isolated colonies were identified through microscopic analysis. The glass was prepared by adding 2 drops of lactophenol cotton blue dye on the glass. A small amount of the fungal sample to be tested was taken from the media and placed on the glass slide portion where the dye was added. A cover slide was placed over the sample and pressed a little until a small bubble was created in order to release the in-between air. To improve the observation, the hyphal growth was broken. The slides were observed under various magnifications. Characteristics like conidial head, conidial organization and hyphae were observed and noted (Habib et al., 2015).

2.6. Maintenance of fungal culture

The cultures were placed in PDA media and were served for further analysis (Satish et al., 2007).

2.7. Inoculum preparation

The strains were grown on PDA media at 27 °C and 7 days after their, the spores of these fungal species were grown on agar plates. The cells from the stored cultures were transferred and diluted

Table 2
List of locations and codes of samples collected during study.

S No	Location
1	Board bazzar
2	Bacha khan chouk
3	Bashir-abad
4	Firdos-stop
5	Fakkir-kely
6	Hashtnaagri
7	Palosi-bazar
8	Terahi-bala
9	Tehkaal
10	Warsaak-road

with fresh potato Dextrose Broth (PDB). The spores suspension was kept at the level of $2x10^5$cfu/ml spores (de Lima et al., 2013; Abril et al., 2008).

2.8. Antifungal activity assay

The leaf extract of six selected plants was analyzed for antifungal activities through the disc diffusion method (Yaseen et al.,2022). The process was performed in tight septic conditions and the tests were performed in triplicate to avoid errors. The zone of inhibition was measured in millimeters. The antifungal activity test was done under extreme aseptic conditions.

2.9. Determination of minimum inhibitory concentration (MIC) of prepared extracts

The MIC value was determined using the standard methodology documented by (Awang et al., 2011).

2.10. Determination of minimum fungicidal concentration (MFC) of prepared extracts

MFC value was determined using the standard method documented by (Dahham et al., 2010). The minimum concentration required to inhibit the growth of fungi was recorded.

2.11. Percent mycelial inhibition of A. flavus using poison food technique

To check the antifungal activity of selected plants against *A. flavus, aqueous* extracts of these plants were made. The food poison technique previously used by (Mohana and Raveesha, 2007) was used for this process. Autoclaved distilled water was taken as a control sample. Four replicates were maintained for each concentration of aqueous extract. The efficacy of these extracts was observed in terms of percent inhibition (%) of the fungal mycelial growth using the following formula: I% = (dc-dt) × 100/dc (Thippeswamy et al., 2014).

dc = 'average diameter of mycelial growth in the control'.

dt = 'average diameter of mycelial growth in the treatment'.

2.12. Analysis and quantification of mycotoxins/aflatoxin by TLC technique

For the analysis of mycotoxins in the feed samples, the thin layer chromatography (TLC) technique was used (Bhatti et al., 1990). For visualization of aflatoxins on TLC plates, the plates were observed under Ultra-Violet light at 366 nm and 254 nm in a closed system and their spot intensities were compared.

2.12.1. Calculation

Effective Weight:

$$\frac{150ml\,of\,1^{st}filtrate \times 250ml\,of\,2^{nd}filtrare \times 8ml extracttaken}{Sampletaken(grams)todryness}$$

$$\frac{}{M1ofH2Oinslurry \times 150ml + 170ml + 30ml \times 20mlchloroform + 200ml\,of\,acetone\,1^{st}filtrate NaOH FeCl_3} = \text{Effective Weight}$$

Comparison of Standard:

$$\text{Aflatoxin Conc.in ppb or } \mu g/kg = \frac{Standardspot\,vol.in\mu l \times Conc.ofstandardin\%dilutionofextractin\mu l}{\text{Sample spot vol. in } \mu l \times \text{Effective Weight}}$$

2.13. Statistical analysis

Experiments were performed in triplicates and the values of these experiments were presented as mean values ± standard errors. The analysis was done with ANOVA using SPSS 20 software. The difference of 0.05 between values was considered statistically significant.

3. Results

3.1. Quantification of fungus in animal feed

All the samples were found to have a variable number of fungal colonies that were presented as cfu/g in Fig. 1. The highest TPC value of $4x10^{21}$ was shown by sample C while A was found to have the lowest TPC value of $5x10^{14.}$ All other samples showed moderate TPC values.

3.2. Fungal flora of animal feed samples

The plating technique was used for the investigation of feed samples. A representative figure of a couple of isolates is shown in Fig. 2 and Fig. 3. Table 3 shows the list of isolated fungi samples. Seven species of *Aspergillus* i-e; *A. parasiticus, A. flavus, A. niger, A. ochraceus, A. clavatus, A. fumigatus, A. carbonarius* and three species of *Penicillium* i-e; *P. citrinum, P. verrucosum* and *P. notatum* were obtained from feed samples. All ten samples were found positive for *Aspergillus flavus* while only one sample was positive for *Aspergillus notaum* i-e; sample G.

3.3. Antifungal activity of plant's leaves aqueous extracts

Table 4 shows the antifungal activity of plant extracts. *Citrus sinensis* showed the highest activity 13 ± 0 mm) against *Aspergillus niger* while *Aloe vera* was reported to have the least inhibition (07 ± 1 mm). *Aspergillus niger* and *Citrus lemon* showed no antifungal activity. In the case of *Aspergillus flavus*, the widest zone of inhibition (15 ± 1) was shown by *Citrus aurantium*., while the least activity (09 ± 1 mm) was shown by *Citrus limon*. *Emblica officinalis* showed no activity. The activity of *Citrus sinensis* was highest (15 ± 0 mm) against *Aspergillus parasiticus,* and lowest was that of *Citrus lemon* (07 ± 1 mm) against *Aspergillus parasiticus*. The antifungal activity of all the water extracts of selected plants against tested fungi is summarized in Table 4.

3.4. MIC of plant's leaves aqueous extracts

The Minimum Inhibitory Concentration (MIC) of the selected plant's leaves aqueous extracts was carried out by microdilution method which is listed as MIC value mg/ml in Table 5. The MIC value *Citrus sinensis* 160 mg/ml against *Aspergillus niger,* MIC value of *Citrus aurantium* was 240 mg/ml against *Aspergillus niger*. In the case of *Aspergillus flavus, Citrus aurantium* exhibited MIC at 130 mg/ml, whereas *Citrus limon* had the highest MIC (210 mg/ml). Against *Aspergillus parasiticus*, the lowest MIC was observed in *Citrus Sinensis* (140 mg/ml) whereas the highest MIC was observed in *Emblica officinalis* (210 mg/ml). Against *Aspergillus ochraceus* the lowest MIC value was found in *Citrus aurantium* (110 mg/ml) whereas the highest MIC value was found in *Emblica officinalis* (210 mg/ml) and *Aloe vera* (210 mg/ml). A summary of the MIC value of all the test plants against the isolated fungi is described in Table 5.

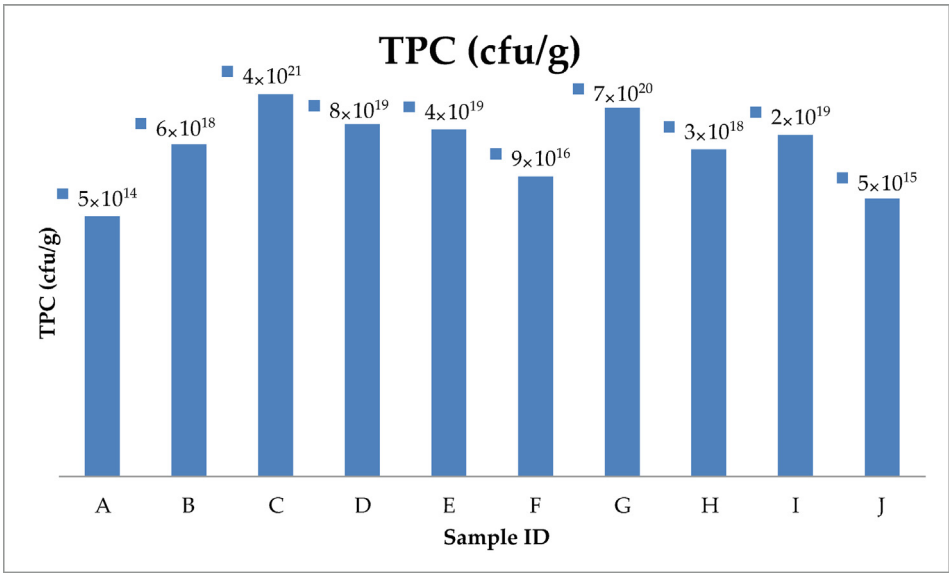

Fig. 1. All samples showing different TPC values.

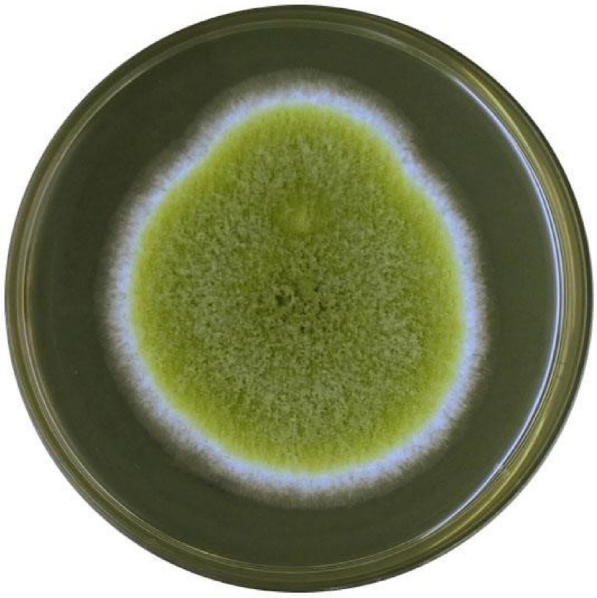

Fig. 2. Growth of *Aspergillus flavus* on PDA medium.

Fig. 3. Growth of *Aspergillus ochracheus* on PDA medium.

3.5. MFC of plant's leaves aqueous extracts

The MFC value of the aqueous extracts of selected plants was performed by the microdilution method. In the case of *Aspergillus niger* MFC value was 180 mg/ml in *Citrus sinensis*, while the highest MFC value was noted in *Aloe vera* i-e; 240 mg/ml. In the case of *Aspergillus flavus, Citrus aurantium* showed the lowest MFC value i.e 150 mg/ml, however, *Citrus limon* showed the highest MFC value i.e. 230 mg/ml. In the case of *Aspergillus parasiticus* the least MFC value was shown by *Citrus sinensis* (160 mg/ml), while the highest MFC was observed in *Emblica officinalis* (290 mg/ml). A summary of the MIC value of all the test plants against the isolated fungi is described in Table 6.

3.6. Toxicological analysis of animal feed

In the current study, the amount of aflatoxin was calculated by the TLC method in 25 g of each feed sample. Feed samples were screened for the presence of aflatoxins B1, B2, G1 and G2. The number of aflatoxins found in ppb in each 25 g is summarized in Table 7. Results showed that aflatoxin B1 was found in the highest quantity in sample C and also present in all the samples in variable amounts. Whereas aflatoxin G2 was found to rarely occur with the lowest quantity and also present a small quantity in sample I (12.7 ppb). Furthermore, the highest quantity of total aflatoxin was detected in sample C i.e. 248.7 ppb, whereas the lowest quantity of aflatoxin was detected in sample E i-e; 48.6 ppb.

Table 3
Identification of fungal flora.

S. No	Sample ID	Identified Fungus
01	A	*Aspergillus niger* *Aspergillus flavus* *Aspergillus parasiticus* *Aspergillus carbonarius*
02	B	*Aspergillus niger* *Aspergillus flavus* *Aspergillus ochraceus*
03	C	*Aspergillus niger* *Aspergillus flavus* *Aspergillus parasiticus* *Aspergillus carbonarius*
04	D	*Aspergillus flavus* *Aspergillus parasiticus*
05	E	*Penicillium citrinum* *Aspergillus parasiticus* *Aspergillus carbonarius* *Aspergillus flavus*
06	F	*Aspergillus niger* *Aspergillus flavus* *Aspergillus parasiticus* *Aspergillus carbonarius*
07	G	*Aspergillus notatum* *Aspergillus flavus* *Aspergillus parasiticus* *Aspergillus carbonarius*
08	H	*Aspergillus flavus* *Aspergillus parasiticus* *Penicillium citrinum*
09	I	*Aspergillus niger* *Aspergillus flavus* *Aspergillus parasiticus* *Aspergillus carbonarius* *Aspergillus ochraceus* *Penicillium citrinum*
10	J	*Aspergillus niger* *Aspergillus flavus* *Aspergillus parasiticus* *Aspergillus carbonarius*

3.7. Percent mycelial inhibition of A. flavus

The antifungal activity of the aqueous extract of each plant was determined against *A. flavus* by poison food technique for the determination of percent mycelial inhibition. The result is presented in Fig. 4. *Citrus aurantium* showed the highest (62.3 ± 0.33) mycelial inhibition of *A. flavus,* while *Citrus limon* showed the lowest mycelial inhibition (46.5 ± 0.46).

4. Discussion

The present study was carried out to check the antifungal effect of the plant leaves aqueous extract of specific plants to inhibit or lower the fungal growth and their mycotoxins in animal feed samples. In the current study, six medicinal plants (listed in Table 1) were used to investigate their antifungal and antimycotoxinigenic capacity. The highest activity among six plants was shown by *Myrtus communis* against *aspergillus clavatus*. It has been previously reported that *Myrtus communis* has significant antifungal ability against dermatophytic fungi. The research has confirmed that methanolic extract of *M.communis* leaves inhibits *Mycosporum canis* (Mehrabani et al., 2013). In another study, the growth of *R. solani* was inhabited by 60% through essential oils of *Myrtus communis* at a concentration of 1600 ppm (Curini et al., 2003). The difference between the results of our study and Curini et al., (2003) may be due to the techniques used to calculate antifungal activity and selection of test fungi.

Numerous studies exposed the noteworthy biological activity of *Aloe vera* against bacteria, viruses, and fungi. Ethanol and aqueous extract of *Aloe vera* had shown exciting results against a number of bacteria and fungi. (Nidiry et al., 2011). In the current study aqueous, extracts of aloe vera leaves were tested that inhibited the growth of different species of *Aspergillus* and *Penicillium*.

Shireen et al., (2015) documented that *Aloe vera* extract showed strong antifungal activity against *Candida albicans* (Shireen et al., 2015). In the present study, an aqueous extract of *Aloe vera* leaves was also subjected to the percentage (%) mycelial inhibition of *Aspergillus flavus* and results showed that about 51 ± 0.37% growth was inhibited. Coopoosamy and Magwa et al., (2007) documented the antifungal activity of *Aloe vera* and reported that *Aloe vera* restricts the growth of *Aspergillus niger* to 24.29%, 9.26% of *Aspergillus flavus* growth and 6.24% of *Penicillium digitatum* growth (Coopoosamy and Magwa et al., 2007). Moreover, Kawai and his group (1998) showed that crude extracts of *Aloe vera* controlled the production of aflatoxin (Kawai et al., 1998).

Sharma and Tripathi, 2009 reported that citrus essential oils comprise a complex mixture of volatile compounds having a potential antifungal activity that reduces the growth of fungi in a dose–response manner or inhibits fungal growth. In the present research, the *Citrus sinensis* leaves were found to be an effective inhibitor against *Penicillium* and *Aspergillus*. The highest antifungal activity of *Citrus sinensis* was recorded against *Aspergillus parasiticus* whereas MIC and MFC were recorded as 140 mg/ml and 160 mg/ml respectively and their results showed MIC 4.09.0 µl/ml against the *Trichoderma harzianum* and *Verticillium fungicola.*

In the present research, the *C. sinensis* leaves extract was also subjected to the percentage (%) mycelial inhibition of *Aspergillus flavus*. (Chutia et al., 2009) studied *C. sinensis* essential oil for the percentage (%) growth inhibition of diverse fungal species, they found that *C. sinensis* inhibited *Fusarium oxysporum Alternaria alternate* (84%) (42%), *Curvularia lunata* (93.25%) and *Helminthosporium oryzae* (54%). The variation of our results in comparison with

Table 4
Antifungal activity of plant's leaves aqueous extracts.

Name of Fungus	Zone of inhibition in milimeter (mm)					
	Aloe vera	***Citrus limon***	***Citrus aurantium***	***Citrus sinensis***	***Emblica officinalis***	***Myrtus communis***
A. parasiticus	13 ± 1.0	7 ± 1.0	11 ± 1.0	15 ± 00	6 ± 1.0	13 ± 00
A.. flavus	11 ± 0.6	9 ± 1.0	15 ± 1.0	11 ± 00	NZ	14 ± 1.0
A. ochracheus	09 ± 1.0	10 ± 00	17 ± 1.0	11 ± 1.0	9 ± 2.0	11 ± 1.0
A. fumigatus	14 ± 0.0	13 ± 1.0	10 ± 2.0	14 ± 2.0	11 ± 1.0	13 ± 00
A. niger	07 ± 1	NZ	9 ± 2.0	13 ± 00	10 ± 1	10 ± 1.0
A. carbonarius	10 ± 2.0	8 ± 2.0	12 ± 1.0	9 ± 2.0	6 ± 1.0	9 ± 00
A. clavatus	08 ± 0.0	11 ± 1.0	12 ± 00	8 ± 1.0	7 ± 2.0	17 ± 1.0
P. citrinum	NZ	9 ± 00	11 ± 1.0	11 ± 1.0	7 ± 00	8 ± 1.0
P. notatum	15 ± 00	7 ± 1.0	9 ± 1.0	10 ± 00	10 ± 1.0	14 ± 1.0
P. verrucosum	06 ± 00	8 ± 2.0	10 ± 0.5	7 ± 0.5	9 ± 1.0	11 ± 1.0

NZ: No zone of inhibition.

Table 5
Antifungal activity MIC of plant's leaves aqueous extracts.

Name of Fungus	MIC Value mg/ml					
	Myrtus communis	*Emblica officinalis*	*Aloe vera*	*Citrus sinensis*	*Citrus aurantium*	*Citrus limon*
A. niger	180	180	230	160	240	NA
A. flavus	160	NA	170	170	130	210
A. parasiticus	170	290	150	140	170	230
A. ochracheus	170	210	210	180	110	190
A. carbonarius	210	300	190	230	170	230
A. fumigatus	160	180	170	150	200	160
A. clavatus	100	240	230	240	160	170
P. notatum	150	200	150	190	210	160
P. citrinum	210	250	NA	180	170	150
P. verrucosum	170	240	280	250	180	180

NA: not applied.

Table 6
Antifungal activity MFC of plant's leaves aqueous extracts.

Name of Fungus	MFC Value mg/ml					
	Myrtus communis	*Emblica officinalis*	*Aloe vera*	*Citrus sinensis*	*Citrus aurantium*	*Citrus limon*
A. niger	200	200	240	180	240	NA
A. flavus	170	NA	200	190	150	230
A. parasiticus	180	290	180	160	190	240
A. ochracheus	190	230	230	190	130	210
A. carbonarius	230	300	210	230	200	230
A. fumigatus	170	200	180	170	210	180
A. clavatus	110	260	250	260	180	190
P. notatum	160	210	170	210	230	170
P. citrinum	230	260	NA	190	190	180
P. verrucosum	190	220	300	250	200	200

NA: not applied.

Table 7
Toxicological analysis of animal feed samples.

Sample ID	B1 (ppb)	B2 (ppb)	G1 (ppb)	G2 (ppb)	Total Aflatoxin (ppb)
A	48.6	26.7	ND	ND	75.3
B	53.8	30.6	ND	ND	84.4
C	136.5	63.9	67.7	16.6	284.7
D	57.6	23.1	17.6	ND	98.3
E	36.8	ND	11.8	ND	48.6
F	88.5	24.2	13.5	ND	126.2
G	67.3	19.6	9.7	ND	96.6
H	34.3	17.8	ND	ND	52.1
I	112.8	51.9	28.2	12.7	205.6
J	79.2	34.4	7.5	ND	121.1

ND = Not detected.

earlier studies is possible because of the selection of different fungal species and methods.

In our study, *Citrus aurantium* presented high antifungal efficiency. The highest activity was found in the case of *Aspergillus ochraceus*, the zone of inhibition was calculated as 17 ± 1 mm, where MIC and MFC were noted to be 110 mg/ml and 130 mg/ml respectively. (Hsouna et al., 2013) found that the antifungal potency of *Citrus aurantium* against the growth of, *Fusarium*, *Alternaria alternata* and *Aspergillus*. According to this research, *Citrus aurantium* essential oil inhibited the growth of *Fusarium graminearum* and *Aspergillus flavus* with a zone of inhibition of 22 mm and MIC value of 78 µg/ml respectively. Also, *Citrus aurantium* showed high antifungal potential against *Aspergillus niger*. *Fusarium oxyporum* and *Aspergillus flavus*. The zone of inhibition ranged 14–34 mm and MIC was 0.078–1.25 mg/ml.

In the current investigation, *Citrus limon* showed modest antifungal activity. The most important activity was recorded in the case of *Aspergillus clavatus* and its zone of inhibition was 11 mm. In the present research, the percentage (%) of mycelial inhibition of *A. flavus* was also done. *Citrus limon* inhibited the growth of *A. flavus* up to 46.5 ± 0.46%. Similarly, the percentage (%) of mycelial inhibition of *A. flavus* was also done by (Chutia et al., 2009). Furthermore, *Citrus limon* essential oil also restricted the growth of *A. flavus* up to 44.0 ± 0.07%. Fisher and Phillips, (2006) found that the spread of foodborne microbes can be controlled by using essential oils of different citrus plants such as *C. sinensis, C. limon, C. bergamia* and their constituents. Also, (Hosni et al., 2010) reported that three monoterpenes are effective antifungal components that are 1,8-cineole, thymol, and (S)-limonene. (Hosni et al., 2010) found that essential oils from *C. limon* showed antifungal activity against *Glomerella cingulata* and *Fusarium oxysporum*.

5. Conclusions

Despite the existence of plentiful agricultural-based food resources for livestock in Pakistan, some pathogenic fungal species are accountable for polluting livestock feed by making mycotoxins.

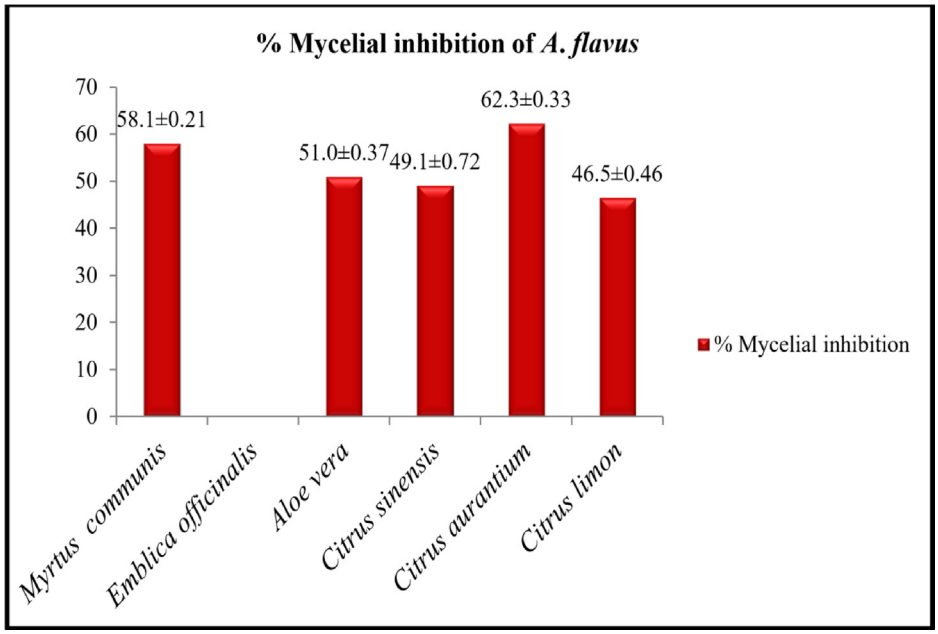

Fig. 4. % Mycelial inhibition of *A. flavus* by selected plant extract. Data given are the mean of four replicates ± standard error.

Moreover, mycotoxin contamination, mainly aflatoxins, is the main distress for the safety of grains and other field crops around the world. Natural plants and plant extracts, on the other hand, have captured the attention of the scientific community due to their less toxic effects. Natural products derived from higher plants are environmental friendly, biodegradable, substantial, and have antifungal and antimycotoxigenic properties. Results of the present study showed that all the aflatoxins i.e., G1, G2, B1 and B2 were present in varying amounts depending on the plants. The highest quantity was present of B1 in all ten samples while G2 was in a lower amount as compared to other samples. Relating to the antifungal properties of the samples *Citrus aurantium* and *Myrtus communis* were absorbed to have antifungal potency against all test fungi. *Citrus aurantium* extracts were found to inhibit the growth of *Aspergillus flavus*. Future research is needed to investigate the proper genomic understanding of these mycotoxins as an effective treatment therapy. Furthermore, good surveillance and proper hygienic measures should be implemented to reduce the likelihood of pathogenic contaminants in animal feed.

CRediT authorship contribution statement

Raza Ullah: Conceptualization, Methodology. **Isfahan Touseef:** Conceptualization, Methodology, Supervision. **Rameesha Abid:** Data curation, Writing – review & editing, Visualization, Validation. **Arshad Farid:** Writing – review & editing, Visualization, Formal analysis, Supervision. **Sohail Ahmad:** Writing – review & editing, Formal analysis. **Hesham Ali El Enshasy:** Writing – review & editing, Resources, Validation, Formal analysis. **Adil Aksoy:** Resources, Investigation, Supervision. **Nada H. Aljarba:** Funding acquisition. **Tahani Mohamed Al–Hazani:** Funding acquisition. **Muhammad Muzammal:** Resources, Investigation, Supervision. **Shakira Ghazanfar:** Validation, Formal analysis, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Abid, R., Ghazanfar, S., Farid, A., Sulaman, S.M., Idrees, M., Amen, R.A., Muzammal, M., Shahzad, M.K., Mohamed, M.O., Khaled, A.A., Safir, W., 2022. Pharmacological properties of 4′, 5, 7-Trihydroxyflavone (Apigenin) and Its Impact on cell signaling pathways. Molecules 27 (13), 4304.

Abril, M., Curry, K.J., Smith, B.J., Wedge, D.E., 2008. Improved microassays used to test natural product-based and conventional fungicides on plant pathogenic fungi. Plant Dis. 92, 106–112.

Ali, S., Iqbal, M., 2004. Total factor productivity growth in pakistan's agriculture: 1960–1996. Pak. Dev. Rev., 493–513

Awang, N., Baba, I., Yamin, B.M., Halim, A.A., 2011. Preparation, characterization and antimicrobial assay of 1, 10-Phenanthroline and 2, 2'-Bipyridyl adducts of Cadmium II N-Sec-Butyl-N-Propyldithiocarbamate: crystal structure of Cd [S CN i-CH CH] 2, 2'-Bipyridyl. World Appl. Sci. J. 12, 1568–1574.

Bhatti, N.N., Nasir, M.A., Randhawa, M.A., 1990. Association of aflatoxin producing strains of Aspergillus with some stored cereals. Pakistan J. Phytopathol. 2, 74–82.

Chutia, M., Bhuyan, P.D., Pathak, M.G., Sarma, T.C., Boruah, P., 2009. Antifungal activity and chemical composition of Citrus reticulata Blanco essential oil against phytopathogens from North East India. LWT-Food Sci. Technol. 42, 777–780.

Coopoosamy, R. M., & Magwa, M. L., 200. Traditional use, antibacterial activity and antifungal activity of crude extract of Aloe excelsa. African J. Biotechnol, 6.

Curini, M., Bianchi, A., Epifano, F., Bruni, R., Torta, L., Zambonelli, A., 2003. Composition and in vitro antifungal activity of essential oils of Erigeron canadensis and Myrtus communis from France. Chem. Nat. Compd. 39, 191–194.

Dahham, S.S., Ali, M.N., Tabassum, H., Khan, M., 2010. Studies on antibacterial and antifungal activity of pomegranate Punica granatum L. Am. Eurasian J. Agric. Environ. Sci. 9, 273–281.

de Lima, J.R., Gonçalves, L.R.B., Brandão, L.R., Rosa, C.A., Viana, F.M.P., 2013. Isolation, identification, and activity in vitro of killer yeasts against Colletotrichum gloeosporioides isolated from tropical fruits. J. Basic Microbiol. 53, 590–599.

Fisher, K., Phillips, C.A., 2006. The effect of lemon, orange and bergamot essential oils and their components on the survival of Campylobacter jejuni, Escherichia coli O157, Listeria monocytogenes, Bacillus cereus and Staphylococcus aureus in vitro and in food systems. J. Appl. Microbiol. 101, 1232–1240.

Gonçalez, E., Felicio, J.D., Pinto, M.M., Rossi, M.H., Medina, C., Fernandes, M.J.B., Simoni, I.C., 2003. Inhibition of aflatoxin production by Polymnia sonchifolia and its in vitro cytotoxicity. Arq. Inst. Biol. 70, 159–163.

Habib, M.A., Abdu, P., Kwanashie, C.N., Kabir, J., Negedu, A., 2015. Isolation and identification of Aspergillus species from poultry feeds in Kaduna State. Nigeria. Microbiol. Res. Int. 3, 27–32.

Hosni, K., Zahed, N., Chrif, R., Abid, I., Medfei, W., Kallel, M., Sebei, H., 2010. Composition of peel essential oils from four selected Tunisian Citrus species: Evidence for the genotypic influence. Food Chem. 123, 1098–1104.

Hsouna, A.B., Hamdi, N., Halima, N.B., Abdelkafi, S., 2013. Characterization of essential oil from Citrus aurantium L. flowers: antimicrobial and antioxidant activities. J. Oleo Sci. 62, 763–772.

Kawai, K., Beppu, H., Shimpo, K., Chihara, T., Yamamoto, N., Nagatsu, T., Yamada, Y., 1998. In vivo effects of Aloe arborescens Miller var. natalensis Berger Kidachi aloe on experimental tinea pedis in guinea-pig feet. Phytother. Res.: Int. J. Devoted Pharmacol. Toxicol. Eval. Nat. Prod. Deriv. 12, 178–182.

Klopfenstein, C.F., 2000. Nutritional quality of cereal-based foods. In: Handbook of cereal science and technology. CRC Press, pp. 705–723.

Mehrabani, M., Kazemi, A., Mousavi, S.A.A., Rezaifar, M., Alikhah, H., Nosky, A., 2013. Evaluation of antifungal activities of Myrtus communis L. by bioautography method. Jundishapur J. Microbiol. 6, 1T.

Mohana, D.C., Raveesha, K.A., 2007. Anti-fungal evaluation of some plant extracts against some plant pathogenic field and storage fungi. J. Agric. Technol. 4, 119–137.

Nidiry, E. S. J., Ganeshan, G., & Lokesha, A. N., 2011. Antifungal activity of some extractives and constituents of Aloe vera.

Reddy, K. R. N., Reddy, C. S., & Muralidharan, K., 2009. Detection of Aspergillus spp. and aflatoxin B1 in rice in India. Food Microbiol, 26 1 27-31..

Salari, R., Najafi, H., Boroushaki, M.T., Mortazavi, S.A., Fathi Najafi, M., 2012. Assessment of the microbiological quality and mycotoxin contamination of Iranian red pepper spice. J. Agric. Sci. Technol. 14, 1511–1521.

Satish, S., Mohana, D.C., Ranhavendra, M.P., Raveesha, K.A., 2007. Antifungal activity of some plant extracts against important seed borne pathogens of Aspergillus sp. J. Agric. Technol. 3, 109–119.

Shireen, F., Manipal, S., Prabu, D., 2015. Anti-fungal activity of Aloe vera: In vitro study. SRM J. Res. Dent. Sci. 6, 92.

Sinha, K.K., 1998. Detoxification of mycotoxins and food safety. Mycotoxins Agric. food Saf. 1, 381–406.

Thippeswamy, S., Mohana, D.C., Abhishek, R.U., Manjunath, K., 2014. Inhibitory activity of plant extracts on aflatoxin B1 biosynthesis by Aspergillus flavus. J. Agric. Sci. Technol. 16, 1123–1132.

Yaseen, M., Kamran, M., Farid, A., Ismail, S., Muzammal, M., Amir, K.A., Rashid, S.A., 2022. Antibacterial, Hemagglutination, and Insecticidal Activity Studies on the Solvent Extracts of the Roots of Olea ferruginea. Makara J. Sci. 26, 8.

Yiannikouris, A., Jouany, J.P., 2002. Mycotoxins in feeds and their fate in animals: a review. Anim. Res. 51, 81–99.