Molecular Identification, Mycotoxin Detection and Antifungal Activity of *Cassia fistula* Against Fungal Strains Isolated from Livestock Feed

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DECLARATION:

I certify that this research work titled "*Molecular Identification, Mycotoxin Detection and Antifungal Activity of Cassia fistula Against Fungal Strains Isolated from Livestock Feed"* is my own work. The work has not been presented elsewhere for assessment. The material that has been used from other sources it has been properly acknowledged / referred.

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DEDICATION

I want to dedicate this work to ALLAH Almighty, Holy Prophet (PBUH), my beloved parents and my family whose tremendous support and cooperation led me to this wonderful accomplishment.

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ABBREVIATIONS

AFB1	Aflatoxin B1
AFB2	Aflatoxin B2
AFG1	Aflatoxin G1
AFG2	Aflatoxin G2
BLAST	Basic Local Alignment Search Tool
Bp	Base pair
°C	Centigrade
DNA	Deoxyribonucleic acid
DON	Deoxynivalenol
DGGE	Denaturing gradient gel electrophoresis
ELISA	Enzyme-linked immune sorbent assay
FDA	Food and Drug Administration
GDP	Gross domestic product
ITS	Internal transcribed spacer
IARC	International Agricultural Research Center
ZE55A	Zearalenone
Ppb	Parts per billions
PCR	Polymerase chain reaction
Kg	Kilogram
SDA	Sabouraud dextrose agar
PDA	Potato dextrose agar
PDB	Potato dextrose broth

g	Gram
L	Liter
Ml	Milliliter
µl	Micro-liter
EDTA	Ethylene Diamine Triacetic Acid
HCL	Hydrochloric acid
SDS	Sodium Dodecyl Sulfate
NaCl	Sodium chloride
rpm	Revolution per minutes
DNTPs	Deoxy nucleotide Triphosphate
U	Unit
UV	Ultraviolet
TAE	Tris base, acetic acid and EDTA
Kb	Kilo base
PH	Potentia hydrogenii
NCBI	National Center for Biotechnology Information
HRP	Horseradish Peroxidase
TMB	Tetramethyl benzidine
s	Seconds
OD	Optical density
DMSO	Dimethyl sulphoxide
mm	Millimeter
µg	Microgram

OTA	Ochratoxin
rRNA	Ribosomal RNA

ABSTRACT

Fungi have fundamental significance on the earth ecosystem. Many fungal species play significant role e.g. as decomposers, pathogenic for many plants and animals and are vital for nutrient recycling in terrestrial habitat. Livestock feed can be infected with broad varieties of microbes. Feed ingredients may be infected with microbes through different ways, either transmitted through soil inoculation, rain fall; mechanical blending or insect's infection to the standing crops. For proper functioning and defense mechanism of animals, Quality of feed is compulsory. Thus it is very important to understand fungi in both morphological and molecular level. This study focused on molecular identification of livestock feed infecting fungal strains using PCR amplification approach. Five primers pairs; ITS1 & ITS4, ITS5 & ITS4, ITS3 &ITS4, ITS86F & ITS4, ITS1 & ITS86R were used in that research. The universal primer pair ITS1 & ITS4 were primarily utilized to amplify most of the samples, however, some isolates were successfully amplified through ITS86F and ITS4. Furthermore, Aflatoxins analysis of feed samples done through ELISA kit. Antifungal and antibacterial activity of characterized fungal isolates was checked on *Cassia fistula* plant extracts through wells diffusion method. The maximum zone of inhibition was shown by *Cassia fistula* Pods while acetone, water and ethanol extracts shows maximum activity.

Key Words: Livestock feed, Fungal identification, ITS region, Mycotoxin, ELISA

1. INTRODUCTION

Molds are of essential body in the earth. Fungi play many significance roles in the ecosystem e.g. as decomposers, symbionts, pathogenic and also involved in fundamental cycles. Molds are often abundant, and on the soil and environment can contain largest pool. After plant, fungi biomass is debatably counted as second on earth and in soil as they compete bacteria. From the statistic it is believed that about existing fungal species present on earth is about 1.5 million (Hawksworth, 2001) and various fungal species of them are significant regarding human, plant and animals pathogen. . Limited number of fungi has been found to date in comparison to the number of fungi that actually exist (Hawksworth, 1991). Out of roughly 1.5 million fungi species (as mentioned above), although the figure for fungi is still conventional, only 5 to 20 % species of fungi are known (Hawksworth, 1991).

Different fungi secrete the broad spectrum of enzymes which can break down the cell wall of plants, and can be used to convert the crops residue and different other biological wastes to useful biomaterials, bio fertilizer, bioenergy etc. While on other side fungal infection is spreading devastation in our earth ecosystem. Fungal contagious parasites get adjusted to the system used to fight them, e.g. resistance of wheat collapsed in Uganda against the stem rust when a new fungal strain UG99 was discovered in 1999.

Fungi are types of group which attack all kinds, parts and stages of plants, from their leaves and roots, from the seedling to fruits or grains and these diseases can be limited to a small leaf point or can damage the whole plant system. These pathogenic fungi can cause plant death very rapidly by inhibiting their seedling or can remain the part of plant till the time they have suitable energy resources from host, for example, high concentration of anther, seed or bulb in host

(Kendrick, 2001). Fungal characterization is vital for understanding all the characteristic of animals and plant pathogen (Lange, 2010).

Animal feed, the mixture of cereals, cotton seed, corn and other agricultural ingredients, is produced by feed mills has a great importance in livestock industry. The bulk of raw ingredients is purchased in a growing season and stored for the manufacturing of feed through the year.

Feed may be contaminated by different toxin producing fungi in different conditions; warm climate, in length post-harvest time, damp natural states favors fungal development.

For the proper physiological functioning and defense system of animals against different pathogens depends upon the quality of feed. Conventionally feed quality was defined by the nutritional assessment of feed ingredient(Fink-Gremmels, 2004). Quality of animal feed can be infected by various pathogens growing on it such as bacteria and fungi. In livestock feed industries every year major economic loss is due to the fungus contamination of agricultural feed ingredient and their byproducts in the American and Asian regions (Schmaile III and Munkvold, 2009).

Many fungal strains up to 100 different species are the natural contaminants of agricultural and food products. The larger part of the toxigenic fungal species belongs to *Aspergillus, Penicillium, Fusarium* and *Alternaria* (Bankole and Kpodo, 2005).

Contamination of different toxin producing fungi on the feed crude materials takes place for the period of pre harvest or the post-harvest stage. The principal way of fungal contamination to feed materials is through the transmission of soil by the wind or through the rain, mechanical fomentation and insects attack to standing crops. The favoring conditions for the growth of molds are approximately 12% moisture, 7°C or above temperature, oxygen and energy

requirement which affect feed production by direct loss of volume and quality of additives, production of mycotoxins and the breakdown of feed resulting in heating and dustiness.

Until the late 1980s, microbial ecologists and taxonomists were dependents on the physiological and morphological characteristics of culture to describe microbial communities and members. In the past decade through the advancement of DNA bar-coding techniques has brought great impact to assess microbial community structure and diversity in a range of feed samples(Anderson and Cairney, 2004)); ((Torsvik et al., 1990)); ((Dunbar et al., 1999)); ((Hedrick et al., 2000)); ((Hill et al., 2000)); ((Kowalchuk et al., 2003)); ((Liu et al., 1998)); ((Manter and Vivanco, 2007)); ((Marsh, 1999);(Smit et al., 1999). Molecular methods involving DNA study of compound communities are now well recognized and have become the successful alternative to culture based.

In Pakistan, most of the studies have been done on molds by focusing on agronomic sector. The most of the ingredients of animal feed are obtained from the similar resource as human food, so, any contamination of fungal strain in the environment put equally threat to the health of human being as well as animals and plants (Fink-Gremmels, 2005). This can be used as early caution for any epidemic of disease in the human population (Nyamongo and Okioma, 2005). Worldwide economic loss in the livestock industry due to the mycotoxin contamination in the feed has been reported to numerous hundred million dollars per year(Richard *et al*., 2003).

Mycotoxins are the secondary metabolites, produced by toxigenic fungi. A practical definition of mycotoxins is a fungal metabolite that produces an unwanted effect when animals or humans are exposed. Generally, exposure is all the way through uptake of polluted foods or feedstuffs. Mycotoxicosis is diseases produced by exposure to feeds or foods polluted with mycotoxins(Nelson *et al*., 1993). In animals, mycotoxins show a diverse range of biological

effects: toxicity of liver and kidney, estrogenic effects and effects of central nervous system etc. Worldwide mycotoxins which are present in the feed and feed ingredients are, ochratoxin, aflatoxin, and fumonisin, which are carcinogenic(Varga and Tóth, 2005).

Several species of these pathogenic fungi produces different types of mycotoxins that are diverse in nature and exhibit the wide range of chemical groups, furthermore their molecular weight varies from the 200 to 500 (Whitlow and Hagler, 2002). Numbers of mycotoxins are recognized, but just few of them have been precisely studied, briefly researched along with good analysis methods for only few are commercially economically accessible. Most familiar mycotoxins groups are aflatoxins and further groups include deoxynivalenol (DON), zearalenone (ZEA), trichothecenes and T2 toxin (T2), ochratoxin (OTA), fumonisin, and the ergot alkaloids. Main types of aflatoxins are AFB1, AFB2, AFG1 and AFG2, Total aflatoxin contamination is referred the amounts of these four aflatoxins. In the aflatoxin group aflatoxin B1 (AFB1) is mainly common. Aflatoxins are secondary metabolites of *A. flavus* and different parasitic *Aspergillus* species which contaminated different feed ingredients e.g. corn, nuts, maize.

Microbial resistance against the different antibiotics is worldwide promising hazard. These new manifestations of microbe's resistance are getting used to and developing with the time. At present only 10 total antifungal drugs are still approved by Food and Drug Administration (FDA) in USA. These approved drugs are chemically synthetic and these may cause the hormonal dysfunction, nephrotoxicity, neurotoxicity and some allergic reaction as these chemicals interact with the regular cellular functions (Taxvig *et al*., 2008).

Many pathogens acquired resistance against antibiotics because of the emergence of drug-resistance bacteria and fungi which pushed us to discover new drugs which are natural sources and best resentence against these bacteria and fungi. In developing countries, herbs are

commonly used as traditional medicine to cure diseases because of their high therapeutic potential. In the era between the 1981 to 2002, 61% new drugs were developed form herbs and very successful against infectious disease and cancer. The discovery of new active compounds is declining however; the products of higher plant with novel mechanism of action may use as antimicrobial agent. Although, the antibacterial activity of plant extracts has been studied but ethno medicinal plants required much attention in India.

The bulk of secondary metabolites are produced through wide variety of plants e.g. tannins, terpenoids, alkaloids, flavonoids, glycosides which have strong antimicrobial activity. *Cassia fistula* is well rooted in its folkloric uses and has currently well-known common receipt in the world for their use as broad-spectrum antimicrobial agents for the cure of several diseases. In recent studies, the antimicrobial assay of six extracts of *Cassia fistula* leaves, pods and seeds was performed against pathogenic fungi to identify new antimicrobial agents.

2. REVIEW OF LITERATURE

2.1. Fungi:

Fungi belong to the eukaryotic organism's groups which occur in the form of molds, yeasts as unicellular organisms or in the form of mushroom as multicellular. In 1949 fungi were first time classified as separate kingdom by Jahn and Jahn(Hibbett *et al*., 2007), and approximately 1.5 million species of fungi are known to present on earth ecosystem (Hawksworth and Mueller, 2005). Fungal growth plays significant part in our earth ecosystem. Fungi can also decompose the complex part of the plants e.g. cellulose and lignin, because of the production of many enzymes which play an important role in recycling process. Their dispersion from one place to other occurs through spore's movement and in the hard places they grown through hyphae. Fungi are very pathogenic in nature and can cause both mild and serious infection to animals, plants and human, instead can also developed the mutualistic relationship with many different organism e.g. mycorrhizae, lichen, hymenopteran, homopteran and isopteran insects. Pathogenic fungi have very well organized system for the penetration of their spores into the host cell and pass on from one organism to the others; many of these pathogenic species produce toxic compounds in the host body. Toxic fungi have lethal effects on animals and human health in many ways (Kendrick, 2001).

2.2. Classification of fungi:

The significant part of the mycologist in the taxonomy of the fungi is to predict fungal revolutionary relations among different species. Initially bacteria were to be placed in the fungi kingdom e.g. Gram-positive bacteria (actinomycetes) (Alexopoulos, 1952). After 10 years of this classification bacteria removed from this group and the protists groups; *Labyrinthulales*,

Trichomycetes and *Hyhochytrodiomycetes* were incorporated. Now only the *Trichomycetes* are included in kingdom Fungi while the rest two *Labyrinthulales* and *Hyhochytrodiomycetes has* removed from this kingdom, although these classifications are still not finalized (Alexopoulos, 1963).

With the invention of electron microscope, the third edition of the fungal classification was added by the phylogenetic analysis e.g. *Basidiomycetes, Zygomycetes, Deuteromycetes* and *Ascomycetes.* Slime molds were placed in the Fungi kingdom which included *Chytrids* and *Oomycetes* but in another isolated subdivision (Alexopoulos).

Fungus was separated into four different phyla by established monophyly excluding many groups which were incorporated earlier in classification apart from Chytrids which were definitely located in kingdom fungi (Alexopoulos *et al*., 1996). During that same period *Saccharomyces* and *Schizosaccharomyces* were shown as different organisms, *Mixia* was familiar as a member of the *basidiomycetes* and *Pneumocystis that* were identified as fungus (Taylor et al., 1999).

2.2.1. Division *Ascomycetes*:

It is expected that *Ascomycetes* are about 75% of total fungi of underwater substance(Das *et al*., 2007). All fungal species belongs to *Ascomycetes* replicate both ways sexually as well as asexually. These ascomycetes fungi group produce in small sacs which are called asci that’s why called Ascomycota. Mature sac fungal spores are known as ascospores; that are rupture at top of the ascus. Most common sac fungi are yeast which are produced by asexual process that is called budding. These buds are produced at side of parent cell, which pinch off and grows to the new cell new parent like cell. Fungal belongs to *Ascomycetes* group have the stable haploid phase (anamorphic);

Asexual spores called conidia are borne in the conidiophores(Wang *et al*., 2005). When the two haploid conidia fuse to each other then they generate dikaryotic teleomorph phase, after this teleomorph fungus, through meiosis, produce sexual spores (Pöggeler, 2001). These *Ascomycetes* have enormous influence on the human life which includes beneficial as well as pathogenic fungi of this kingdom, e.g. Ergot fungi has the poisonous properties and its contamination is always threat to human and animals (Horvath *et al*., 2004).

2.2.2. Division *Zygomycetes*:

Zygomycetes are zygote forming fungi that's why called as *zygomycota* and these fungi consist of the haploid fungi with Coenocytic hyphae and they have the capability to replicate both sexually as well as asexually (Schwarz *et al*., 2006). Inside a ball like structure, called zygosporangium, numerous spores called zygospores are formed. Mating of the hyphae of two anamorphic fungi causes the production of a diploid fungus; as a result, fungi develop and by the process of meiosis haploid spores are formed. These Zygospores are extremely resistant and can remain in resting phase for a long time period (Benjamin, 1979). These zygomycetes are commonly present on the decaying food, bread and cheese. Mycosis can be caused in the immune compromised patient by the *Absidia corymbifera* (Ribes *et al*., 2000). The most common Zygomycetes are *Mucor and Rhizopus.*

2.2.3. Division Basidiomycetes:

In this basidiomycetes division around 30,000 fungal species are present and further classified in three classes: *Pucciniomycetes* which includes rusts, smuts, anther and diverse yeast; *Ustilaginomycetes class* contains the true smuts and yeast ; while *Hymenomycetes* class consist of mushrooms and the other molds (Matheny *et al*., 2002). Newly, the *Wallemiomycetes,* the fourth class of basidiomycetes had been suggested to address the several remarkable xerophilic

(which are bearable to the dry situations) fungi (Ainsworth, 2008). This phylum Basidiomycota has much importance from the taxonomic view and also has huge significant importance in ecology terms. Basidiomycetes have mutualistic relation with the green algae, cyano bacteria and also with the *bryophytes*. *Basidiomycetes* fungi are mostly pathogenic and decomposers nature of the plants, animals and of other fungal species (Wasser, 2005). They are capable of reproducing both sexually and asexually. Basidiomycetes through sexual reproduction produce the four meiotic cells e.g. Basidiospores, on the tip of basidia (meiosporangia), within the fruiting body called basidiocarp. Polyphyletic yeasts are anamorphic *basidiomycetes* and reproduce asexually, including *Malassezia*, *Trichosporon*and and *Pseudozyma* species (Morrow and Fraser, 2009). The rusts fungi which are efficiently vital plant pathogen only constitute 7000 species and can infect flowering plants, ferns and conifers (Aime, 2006). The mushrooms, which consists of about 20,000 species, forms large collection of pathogenic along with symbiotic fungi (Henk and Vilgalys, 2007),(Matheny *et al*., 2007).

2.2.4. Division *Deuteromycetes*:

Deuteromycetes fungi are known as the imperfect fungi, due to these fungi have not sexual reproduction process. They produce through the asexual process and their spores are known as conidia. Most of the fungi in this division are pathogenic for humans and can cause several diseases as ringworm and athlete foot while many important fungal species are also present in this division as *Aspergillus* and *Penicillium* as decomposer and many important industrial uses. Others fungi included in this division are *Trichoderma*, *Alternaria* and *Colletotrichum*.

2.3. Animal feed:

Fungus and their mycotoxins contamination occur worldwide in the feed and feed ingredients because of the ubiquitous nature of fungi and they cannot be totally eliminated from these feed

ingredients (Trenholm and Charmley). In the survey of 1985 world food and agriculture organization estimated that 25 % total agriculture grain supply annually contaminated with the fungi and their mycotoxins(Whitlow and Hagler, 1995).

Conditions to control the moisture cannot stop the growth of fungi in the feed because the energy required to control moisture less than 14 % is very expensive. If once the temperature the storage change, evaporation will be occurred inside the silo. Once the fungus starts to grow in the storage feed its will start to generate moisture and as a result metabolism will start which convert grain sugar to the carbon dioxide. Energy and water released in this process favored the growth of fungus inside the storage feed(Osho *et al*., 2007).

Fungus contamination remains always be the problem on the livestock feed ingredients, as the storage time of feed increase the chances for the fungal growth are also increase. Fungus contamination causes different impacts on the livestock industry as this cause the economic loss of industry by reduction of uptake of these ingredients, immunity decreased and cause the damage of different organs(Doerr and Hamilton, 1981). The most harmful effects of the moldy feed on the livestock as mentioned by (Oyejide *et al*., 1987) is that it cause the loss of energy in feed and cause depletion of the many essential nutrients (e.g. essential amino acids and vitamins) and mycotoxicosis.

Crude ingredients used in the livestock feed are derived from the similar source as from human food products are made, animals and their products are ultimately source of human food thus any infectious or the non-infectious contamination in the feed may equally manifest in the health of animals and cause ultimately threat to human health and can serve as the early warning symptom of an approaching epidemic in the human populations.

Livestock feeds can be the carrier of wide types of microorganisms. Fungus may be incorporated in feed material at any instant during growing or harvesting the crops, either during processing or storage of the feed ingredients. The prime way of fungal spread in feed includes the transmission of top soil through the air, rainfall, environmental effects or either through different microorganisms attack to the standing crops.

Several of these microbes are inhabited to dried and comparatively nutrient poor environment in the soil and lives in the similar niche on the emerging crops. Fungal occurrence and development on the livestock feed products is one of the main threats to the livestock economics and health issue.

Instead of the harmful impact on dietetic and the organoleptic properties, Fungi can produce many types of toxigenic mycotoxin. Many species of fungi are considered as the natural pollutants of the food and food products and maybe harmful for different crops and their productivity. It is estimated that approximately 30-40 % of the presented fungi are able to produce their toxic substrate when conditions are favorable. The majority of toxin producing fungal species are belongs to some species of *Alternaria, Aspergillus, Penicillium* and *Fusarium*.

The purpose of work was to commence a study upon toxigenic mycoflora and Molecular identification of animal feed in Pakistan which comprise of enumeration and identification of fungi that naturally contaminate various types of livestock feed and exposure and quantification of the aflatoxin in the feed samples.

Fungus and mycotoxins infectivity of the agriculture crude ingredients occur at the stage of pre harvest or the post-harvest stage. The prime approach of inoculation of feed materials is the transference of soil by wind, rain, mechanical agitation, or insects to standing crops.

Regular monitoring of toxigenic mycoflora of the agricultural based feeds and foods is an essential pre-requisite for development of strategies to control or prevent mycotoxins exposure of feed animal and human population.

Fungi play a major role in the recycling of organic material. Fungal contamination can be responsible for major loss of animals feed; particularly feed which contains any moisture. Moldy feeds are less palatable and may reduce dry matter intake. This in turn leads to a reduction of nutrient intake, reducing weight gains or milk production. Performance losses of 5 - 10% are typical with moldy feeds even in the absence of mycotoxins. Dry feed can usually be stored successfully, but the minute they become damp, molds are likely to render them inedible. This is obviously a problem where large amount of feed is being produced seasonally and then require storage until they are needed(Adams *et al*., 1993).

Various animal and poultry feed raw materials are however derived from the same sources as human food, thus any fungal problem in an environment would equally manifest in the health of animals and may serve as early warning sign of an impending outbreak in human populations

As mentioned above that the fungal growth is the main cause of considerable economic loss in the feed industry as it easily grows on feed having tiny bit of moisture in it. World compound feed production is approaching 1 billion tons per year according to the International Feed Industry Federation (IFIF). By identifying the fungi present in livestock feed we can know the type of fungi and come up with better techniques to control this. Through this we will try to minimize the effect of fungus in livestock feed.

2.4. Molecular identification of fungi:

Various fungal species cause many diseases to plants, animals and human. For different diagnosis and inspection of these fungi, efficient recognition and quantification methods are very significant. These valuable methods help for the detection and understanding on those complex and pathogenic fungi which are not easy to grow and are threat for ecosystem. In the recent few years many techniques has been developed for fungal amplification e.g. qPCR and advancement of various probes development(Tsui *et al*., 2011).

Commonly laboratory techniques used for the identification of fungi was mainly focused on fungal culturing, isolation and disease diagnosis was through morphologically or biochemically techniques. Although these techniques are primary used for fungal identification but through the invention of accurate and authentic molecular techniques, scientist are focusing on these methods.

These molecular techniques are focused on RNA and DNA probe hybridization techniques and polymerase chain reaction (PCR). With the advancement of PCR techniques for fungal isolates identification, qPCR is very accurate and rapid tool to give quantified data for fungal species. These advance molecular techniques e.g. PCR, probe hybridization techniques give us much information for the identification of fungal isolates but still it is necessary to advancement in these methods for understanding their interaction with different organism in environment.

Conventional cultures based techniques were small potential to recognize the microorganism. Due to the lack of appropriate information, it was difficult to preserve and restore the cultural written heritage. Now advanced molecular techniques were used to identify the communities of fungus grow on paper samples of various age and composition. DNA based techniques focused

on inter transcribe spacer (ITS) regions that were present in nuclear rDNA. This technique was used to analyze the diversity of fungus grow on paper. These ITS regions were extremely specific in taxonomically different species of fungi or even within the species as well. It was focused on the optimization of many molecular techniques which include DNA extraction protocol and PCR techniques to amplify targeted DNA. The targeted DNA sequences were amplified with the help of specific primer set of ITS1 and ITS2 regions. These amplified regions were then examined by gel electrophoresis. Gel electrophoresis conditions such as voltage, current and time were measured to differentiate the fungi species in compound communities. Pure fungi samples were used as positive control for relative analysis of past papers (Michaelsen *et al*., 2006).

Modern molecular methods are effective way for fungal detection and identification for commercial, scientific and medical purposes. Many sequences of fungal genome were analyzed by using advanced techniques such as ribosomal DNA (rDNA) gene sequences. These were relatively preserved regions in fungi inter or intra species, consist of 18S, 5.8S and 28S and code for rRNA. There were many variable sequences of inter transcribe regions (ITS) such as ITS1 and ITS2. Though these were not translated regions but played a major role in production of functional rRNA, with specific sequence variations in species representative potential as fungi biological markers for molecular assays. They demonstrated the assessment of clinical methods for using the ITS sequences as biological markers. Many approaches for using fungal ITS were evaluated including culture identification methods, phylogenetic analysis, direct identification of clinical sample or environmental and molecular typing methods. Analysis of these approaches indicates that ITS regions were effective to fungi molecular based assays for their identification and characterization. Advances in ITS based amplification methods were used as an effective

way to diagnose infections caused by fungi which ultimately improve the output for affected patients(Iwen *et al*., 2002).

The Two ITS primer pairs ITS1, ITS86R and ITS86F, ITS4 were investigated for DNA amplification, extracted from fungal isolates. The amplified DNA region of 300bp – 500bp were then sequenced, for the construction of libraries and further these libraries were compared with the different data already present in GenBank.

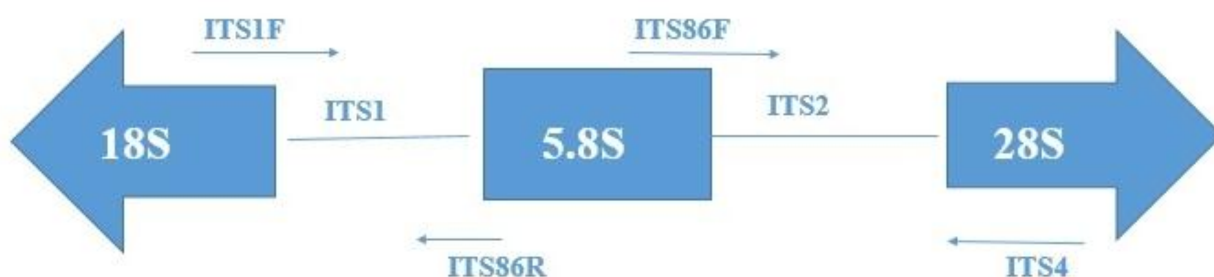


Figure 1: Representation of fungi ribosomal genes bridged by ITS regions targeted by the two primer pairs that were assessed in this study(Vancov and Keen, 2009).

2.5. Mycotoxins:

Quality of products is the main focused on those ingredients which have high nutritive values for the livestock industry. In national economy of Pakistan livestock industry play an important role in providing dairy and meat products. In Pakistan dairy products contribute up to 46% in the total agriculture sector and 11% of our total GDP comes from these dairy products(Sultana and Hanif, 2009). Agricultural productivity also enhanced due to the livestock sector and also plays an important role to reduce the poverty in the rural areas of Pakistan. Various factors that affect the health of animals are contaminated feed ingredients, poor storage condition, and any disease or climate changes. Livestock feed is always on the risk of contamination by different microbes e.g. Fungi, which cause to produce different toxins compounds which are harmful to animals. In past

mostly research was done on the viruses and bacterial diseases of animals. Whereas fungal and mycotoxins research was done only on monogastric animals species. Toxigenic fungi are present in anywhere on the ecosystem and when these get suitable condition grow and produce mycotoxins. Various animal and poultry feed raw materials are however derived from the same sources as human food, thus any fungal problem in an environment would equally manifest in the health of animals and may serve as early warning sign of an impending outbreak in human populations. Due to high cost of these feed ingredients mixing of bakery and kitchen wastes are common in the feed. In 2007 a tragedy happened in cattle colony of Karachi in Landhi, in which 493 animals died and about 1200 animals fall in sickness due to high level of aflatoxin and T2 toxin in the cattle feed (Sultana and Hanif, 2009). Pakistan has all types of weather conditions hot, cool and humid environment and the chances of occurrence of mold contamination and presence of different mycotoxins like aflatoxin, fumonisin, trichothecenes and zearalenone are mostly present in the livestock feed. These mycotoxins cause adverse effects to the health of animals because some are carcinogenic and some effect different organs e.g. liver and reproductive organ(Sultana and Hanif, 2009) .

Table 1: Mycotoxins and their effects on buffaloes and cows

Effects	Mycotoxins
Immune suppression	Aflatoxin, trichothecenes
Hepatotoxicity	Aflatoxin, fumonisin
Carcinogenicity	Aflatoxin, fumonisin
Nephrotoxicity	Ochratoxin
Neurotoxicity	Trichothecenes
Decreased performance	Aflatoxin, trichothecenes, Zearalenone
Hematopoiticity	Trichothecenes
Dermal effects	Trichothecenes
Teratogenic effects	Aflatoxin, Zearalenone
Gastrointestinal effects	Aflatoxin, trichothecenes

2.5.1. Aflatoxin:

Aflatoxins are the fungus secondary metabolites produced by mostly *A. flavus* and *A. parasiticus* and another *Aspergillus* species.

They are mostly produced at temperature up to 12-40 °C and moisture at 3-18 %. The most commonly found aflatoxins includes aflatoxin B1, B2, G1 and G2and in these aflatoxin B1 is potent liver toxin and classified as the class1carcinogen for human beings by IARC (Rahmani *et al*., 2009). In livestock mycotoxins effect vary with their concentration, duration of exposure, species or the nutritional status. Calves are more susceptible to the mycotoxins than older animals. Aflatoxin cause different effects e.g. carcinogenic, mutagenic effects and also reduced

the immune system of animal's. These aflatoxin causes chronic and acute effects in livestock industry they cause infection in liver, reduce the milk production and cause anemia.

Feed consumption is also reduced if feed is contaminated with toxigenic fungi and also suppress the growth of animals. Aflatoxin contaminated feed may reduce the milk production up to 25%. Aflatoxin residue released from milk in the form of aflatoxin M1 within 12 hours and it's equal to 1.7 % of the dietary aflatoxin. FDA reported that Aflatoxin M1 limit in the milk should not be more than 0.5 ppb and aflatoxin B1 limit up to 20ppb (Rashid *et al*., 2012).

2.5.2. Ochratoxin:

Ochratoxin A mycotoxins released by *Aspergillus* and *Penicillium* species and the mostly cause kidney damages. They cause the production of extensively dilute urine, depression reduce the weight gain and water loss. These toxins easily degraded in the rumen that why little effect on the ruminants.

2.5.3. Zearalenone:

Zearalenone mycotoxins is secondary metabolites of many *Fusarium* species and mostly known as astrogenic metabolite. Zearalenone is present in many feed ingredients like corn, wheat, soya bean, sorghum and nuts. This mycotoxins occurrence depends upon the weather conditions, when condition is moist they widely grow. Zearalenone cause various effects to the animals e.g. estrogen response and also cause abortion in animals, vaginal secretion, affect reproductive system and also enhancement of mammary glands. Minute amount up to 660 ppb Zearalenone can reduce the consumption of feed, reduced the production of milk, damage the reproductive tract and also cause diarrhea.

2.5.4. Fumonisin:

In mycotoxins family Fumonisin B1 is most widely produced toxin and produced by the *F. verticillioides and F. proliferratum species.* Fumonisin B1 is present in everywhere on ecosystem and widely present on the maize or maize prepared products. Dairy animals are more susceptible to Fumonisin B1than beef animals because of the more production stress factors. Fumonisin B1 is very toxic and cause different carcinogenic effects, liver damage, reduce the production of milk and feed consumption.

2.5.5. Trichothecenes:

Trichothecenes mycotoxins are produced by the *Fusarium* species and they are approximately group of 180 sesquiterpenoid mycotoxins which are structurally related and mostly present in the livestock feed and ingredients. Trichothecenes mostly affect dairy cattle's rather than beef which include following toxins, T-2 toxin, nivalenol, HT-2 toxin, diacetoxyscirpenol and deoxynivalenol. These toxins also cause the reduction of feed consumption, milk production, estrous cycle, gastroenteritis, necrosis and intestinal hemorrhages. If Animals used feed that contain 640 ppb T-2 toxin for twenty days will be threat to bloody feces, abomasal, ruminal ulcers, enteritis which ultimately goes to death of animals. Trichothecenes also effects many different systems e.g. immune system, kidney failure, bone marrow and lymphoid system. Trichothecenes can reduce the consumption of feed, enhance the production of somatic cells of milk, and damage the reproductive tract even in 300 ppb of DON mycotoxins.(Sultana and Hanif, 2009)

Mycotoxins are secondary metabolites of fungi in feed and feed ingredients and are estimated that about 25 % of world crop is infected each year due to the contamination of fungi. Most mycotoxins producing fungi belong to *Aspergillus, Penicillium* and *Fusarium*. 300 mycotoxins

are known which cause toxicity of animals and humans. Mycotoxins are produced in cereals, corn and different agricultural products during growing, harvesting and storage at different environmental conditions. They are ubiquitous in nature and in the world no any region is escaped from the problems of mycotoxins. Whether the corn grows in the hot or cold areas and weather rainfall or humidity is present or not during the harvesting condition, fungal contamination to the corn crops is common.

In the farm animals feed contaminated with the toxigenic fungi have many negative impacts in the performance of animals, their growth rate, immune system, teratogenic effect, carcinogenic effect, mutagenic effect or damage to the nervous system and also fatal for liver and kidney infection. Occurrence of the mycotoxins everywhere in the environment is global issue, however in different areas of the world occurrence of different mycotoxins vary some are present more and some less. These are mostly present in the different ingredients of the feed stuff which is used in the livestock feed. In the most Europeans countries aflatoxin is not present commonly while presence of aflatoxin is most common in humid conditions e.g. in Asia and Africa(Akande *et al*., 2006).

Table 2**:** Global geographic occurrence of mycotoxins

Location	**Mycotoxins**
Western Europe	Ochratoxin, vomitoxin, zearalenone
Eastern Europe	Zearalenone, vomitoxin
. North America	Ochratoxin, vomitoxin, zearalenone, aflatoxins.
South America	Aflatoxins, fumonisin, ochratoxin, vomitoxin, T-2 toxin.
Africa	Aflatoxins, fumonisin, zearalenone
Asia	Aflatoxins
Australia	Aflatoxins, fumonisin

The problem is not just that the mycotoxins infect animals feed and cause different problems in animals systems and instead they can further transfer to the meat, milk, eggs ,different products of animals and cause the different diseases and threats to human beings(Vancov and Keen, 2009).

Table 3**:** Some food of animal origin which may be naturally contaminated with mycotoxins(Akande *et al*., 2006).

Mycotoxins	**Potential Effects on Humans**	**Occurrence**	**Maximum Level Reported (ppb)**
Aflatoxin B Pig liver Pig muscle Pig kidney	Hepatic cancer	Eggs	0.4 0.5 1.04 1.02
Aflatoxin M1	Carcinogenic	Cow milk	0.33
Ochratoxin A	Renal damage	Pig liver Kidney Sausages	98 89 3
Zearalenone	Oestrogenic	Pig liver Pig muscle	10 10

2.6. Antimicrobial activity:

Medicinal plants were traditionally commonly used as medicine and are the very rich source of Bio-medicine, food supplement, pharmaceutical intermediates, folk medicines, nutraceuticals, modern medicines and chemical entities for synthetic drugs. The use of medicinal plants is very extensive by all fields of people either in the form of modern medicines or by folk remedies(Srinivasan *et al*., 2001).

The widespread use of antibiotics and enhanced microbial resistance demands investigation for new antimicrobial agents with less resistance. Medicinal plants are provided with many active

components which include peptides, aldehydes, alkaloids, phenols etc. that can be used in the treatment of human diseases. *Cassia fistula* has been documented for the treatment of various diseases which include liver troubles and skin infections among various others. It has significant effects against various microbial agents and in accordance with literature it can be used as an antimicrobial agent for the treatment of various infectious diseases. *Escherichia coli* and *Klebsiella* spp has shown high resistance towards broad spectrum cephalosporin's.

Cassia fistula; a beautiful ornamental tree having yellow flowers, is found around the world. It is being widely used by the tribal people for the treatment of various fungal infections. The plant has various pharmacological uses and is recommended for the control of pests and diseases. Diarrhea would be treated using the whole plant. However, seeds are used for the treatment of skin ailments and fruits as well as flowers were used for the treatment of abdominal pain, leprosy, fever in addition with skin diseases. These treatments were being practiced by traditional people. Phenolic compounds are important extracts of *Cassia fistula* plant. These are basically the secondary metabolites. The heartwood of the plant contains fistucacidin. Whereas, the acetone extract of the flower contains proanthocyanidin and kaempferol. In addition to phenols, alkaloids have also been observed in the flowers.

A variety of microorganisms are the cause of diseases and despite the advancements in science, these diseases served as the leading cause of various health problems in developing countries. *Cassia fistula* and *Messua ferrea* plants are used in the treatment of a variety of diseases such as dysentery, rheumatism, fever, gout, sore throat etc. However, very few findings have been reported in literature about the medicinal value of these plants. There is an intense demand for the investigation of medicinal value of these plant species.

Antibiotics are the major weapon used for the treatment of different bacterial and fungal infections since their introduction. However, due to intense utilization of these weapons the intensity of benefits provided by them reduced to a great extent. The demand for the investigation of alternative sources involves the utilization of natural products. Plants are a rich source of a variety of secondary metabolites. These include alkaloids, terpenoids, flavonoids, tannins, glycosides etc. *Cassia fistula* has the potential to serve as a strong antimicrobial agent and treat a variety of bacterial infections. The plant is useful in the treatment of liver troubles, skin diseases and diabetes among various others.

3. METHODOLOGY

3.1. Study region:

Samples were collected from the Tehsil Kot Addu, District Muzaffargarh which is located in the southern Punjab region and in the center of Pakistan. Alluvial plain and is ideal for agriculture. From the total 8, 77,989 Acres land, 4, 24,521 acres' land is under the cultivation of different crops and major crop production is corn, cotton and rice, key ingredients of animals feed.

3.2. Sample collection:

26 different companies' livestock feed samples were collected which consist of commercially packed feed and farm mixed open feed. Samples of livestock feed were collected two at regular duration spread over the study period. For each sample, 0.5 kg feed was collected from each sample. After the mixing of whole feed ingredients 0.5kg feed sample was packed in the zipper bag and stored at 4°C before culturing on the media.

3.3. Fungal identification:

This research focused on molecular identification of livestock contaminated fungal strains using PCR amplification approach. Five primers pairs; ITS1 & ITS4, ITS5 & ITS4, ITS3 &ITS4, ITS86F & ITS4, ITS1 & ITS86R were studied in this research. The primer pair of ITS86F and ITS4 were primarily used to amplify mostly samples, however, some isolates were successfully amplified through ITS1F and ITS4 and ITS1 & ITS86R.

3.3.1. Fungal growth media:

Two media usually used for the growth of fungal strains SDA and PDA. SDA was prepared by 65 g SDA in 1 L distilled water while PDA was prepared by 39 g PDA powder in 1L distilled water. After preparing media, this will be autoclaved at temperature 121 °C and pressure at 15

psi for 15 minutes. Petri plates are prepared in the safety cabinet using these media and pour approximately 25 ml media in each plate and incubated these plates at temperature 37°C for overnight to confirm that there is no contamination in the plates. Fungal contaminated samples inoculated in these plates for the growth of fungi and incubate these plates at temperature 25 °C for about 3-5 days for proper growth of fungi.

Potato dextrose broth (PDB) is used for the growth of mycelia and this broth is made either through directly powdered, 24 g PDB powder is mixed in 1L distilled water or prepared through mixing 20 g anhydrous dextrose and 200 g potato infusion in 1 L distilled water at pH 5.1 and autoclave at same conditions (temperature 121 °C and pressure at 15 psi for 15 minutes). Fungal spores are mixed in the PDB media and place on the shaking incubator temperature 25 °C for 4-5 days. After 4-5 days when mycelia grown in PDB then these mycelia are filtered through mira cloth and stored these mycelia at -80 for nucleic acid extraction.

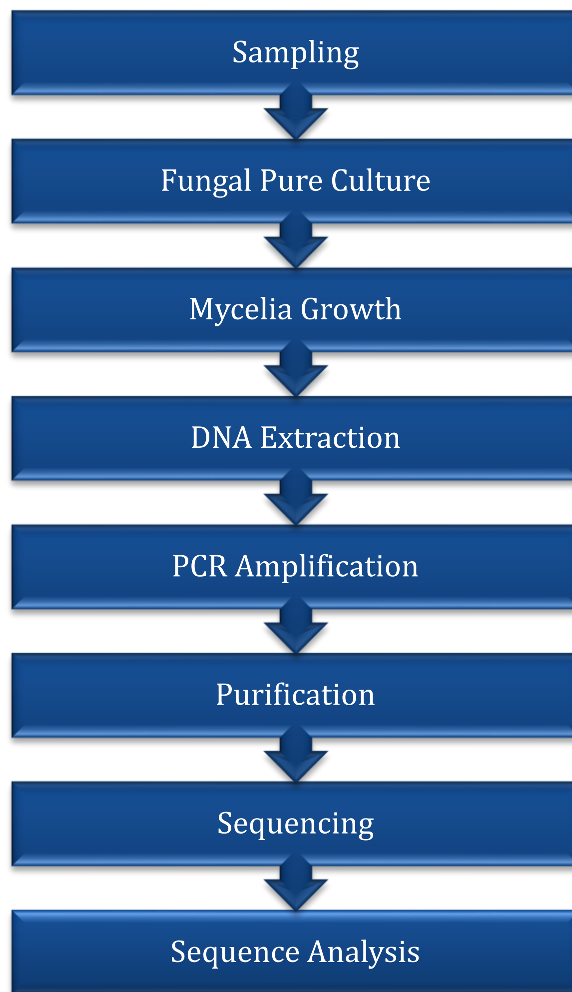


Figure 2**:** Step wise scheme for current study.

3.4. Nucleic acid extraction:

Nucleic acid extraction was done through (Coenen *et al*., 1997) protocol which is rapid, reliable, authentic and inexpensive procedure for the total nucleic acid extraction. Grown mycelium is grinded into powdered form using the liquid nitrogen in mortar and pestle. Mycelia powder about 0.3-0.4 g is transfer into the micro centrifuge tube of 2ml with addition of 350 µl extraction buffers which contain (10 ml; about 400 µl of the 0.5 mM EDTA contain [pH 8], and 200 µl of 1 MTris-HCl [pH 7.5], 1 ml of 10 % Sodium dodecyl Sulphate (SDS), 5 ml 1 % Sodium Chloride (NaCl) and 3.4ml autoclave distill water). This

2ml centrifuge tube is then vortex to homogenized sample and then placed at temperature 70 °C for 1-hour incubation. After this incubation, we add 350 µl of the phenol and equal amount of sevag (chloroform ratio to isoamyl alcohol is 24:1) in same tube for nucleic acid purification and centrifuge this tube at 11000 rpm for the time 15 min. Supernatant (upper layer) about 500 µl transferred to the other centrifuge tube with addition of 500 µl sevag and again centrifuge this tube at same 11000 rpm for the time 10 min. Supernatant (upper layer) of about 400 µl was transferred to other new 1.5 ml tube with addition of the 800 µl of chilled ethanol for the nucleic acid precipitation. This tube is kept for incubation at temperature -20 ° C for 4 hours. After 4-hour incubation thread like structure of nucleic acid precipitate will be appeared in this tube and after centrifugation at 11000 rpm for time 15 min nucleic acid will be pellet down and this pellet was air dried at the room temperature and re-suspended in 40µl of autoclave water. The extracted DNA pellet was then stored at -20 °C for further use. For the nucleic acid presence, we further checked it on 1 % agarose gel.

3.5. Primer design:

The amplification of fungal ITS region was done by using different ITS primers (Table 1). The primers target the region ITS region consist of 28S rRNA, 18S rRNA and 5.8S rRNA gene. All primers were synthesized by Eurofin USA

.

Table 4: Reported oligonucleotide primer used for amplification of ITS regions in current study.

Primers names	**Primer sequence (5'-3')**	**References**
TS1 (F)	TCCGTAGGTGAACCTGCGG	(White *et al.*, 1990)
ITS2 (R)	GCTGCGTTCTTCATCGATGC	(White *et al.*, 1990)
ITS3 (F)	GCATCGATGAAGAACGCAGC	(White *et al.*, 1990)
ITS4 (R)	TCCTCCGCTTATTGATATGC	(White *et al.*, 1990)
ITS 5 (F)	GGAAGTAAAAGTCGTAACAAGG	(White *et al.*, 1990)
ITS86-F (F)	GTGAATCATCGAATCTTTGAA	(Turenne *et al.*, 1999)
ITS86-R (R)	TTCAAAGATTCGATGATTCAG	(Vancov and Keen,

3.5.1. PCR Assay

A volume of 50 µl of reaction mixture was prepared for amplification of PCR which contain 2µl nucleic acid. Each reaction mixture consists of nucleic acid, PCR water, 10X Taq DNA polymerase buffer (Thrmoscientific), 25 mM of $mgcl_2$, 0.2 mM dNTPs (Invitrogen), 0.2 µM of the forward and reverse primers and 5U/µl Taq polymerase enzyme.

Each PCR reaction mixture was firstly denatured at temperature 95°C for 7 minutes. A total of 35 cycles were programmed on the thermocycler (Maxpro) to denature DNA at temperature 95 °C for time 50 seconds. The annealing temperature is at temperature 60 °C for time 60 seconds. The primers extension was at the temperature 72 °C for 60 second time and final extension occurred at temperature 72 °C for time 10 minutes.

Table 5**:** PCR Mixture.

Reagents	**Quantity Used (µL)**	**Final Concentration**
De-ionized d.d.H2O	33 µL	
Taq polymerase buffer	5 µL	10X
Mgcl$_2$	3 µL	25 mM
dNTPs	2 µL	0.2 mM
Primer F	2 µL	74.8 nmole
Primer R	2 µL	74.4 nmole
Template	2 µL	
Taq polymerase	1 µL	5 U/µl
Total volume	**50 µL**	

3.5.2. Agarose gel electrophoresis:

1 % agarose gel is used for separation of the amplified PCR amplicon of 18S ribosomal RNA region in 1 X TAE buffer and ETBR in 20 mg /ml. To check the size of PCR amplicon we used 1 kb DNA ladder. DNA bands was visualized through UV Tran illuminator or through gel documentation system.

3.5.3. Purification of PCR products and DNA sequencing:

For the purification of PCR product PCR purification kit (Thrmoscientific) was used. PCR mixture and binding buffer was added with equal proportion 1:1 and gently mixed them, change in color in the mixed solution indicate the PH of DNA binding. Optimal color of DNA binding is yellow while in case of orange or violet PH of solution is maintained by adding 10 µl of 3M

sodium acetate. The mixed solution is centrifuged at 12000 rpm and flow through is discarded after centrifugation. Now add 700 µl washing buffer in the same column and again centrifuge at 12000 rpm for 1 min and after discarded flow through again centrifuge the empty column to confirm all the residue are discarded. After discarded flow through 40 µl autoclave distilled water was added to mix DNA in water and again centrifuges this at 12000 rpm for 60 seconds. This purified PCR product was then stored at temperature -20 ° for further used.

3.5.4. Sequencing of full length ITS region:

Purified PCR product was commercially sequenced by the Eurofins USA. The sequences were BLAST with the other sequence via NCBI nucleotide data base.

3.6. ELISA Assay:

This ELISA Assay was performed using the standard kit protocol (Elabscience). This kit was used for the detection of the total Aflatoxins by using competitive ELISA by using livestock feed. Elabscience ELISA kit was composed of 96 wells Micro ELISA Plate, antibody, HRP conjugate, standards and other supplementary reagents.

Micro titer ELISA Plate in that Elabscience ELISA kit has been pre coated with the aflatoxins. During the ELISA assay the total aflatoxin in the feed sample compete with the fixed amount of aflatoxin in Micro titer ELISA Plate on the solid phase supporter for sites of antibody to aflatoxin. After the addition of HRP conjugate enzyme in micro titer plate, TMB substrate is added for the development of color, there is a negative correlation between the optical density value of sample and the concentration of aflatoxin. Total aflatoxins concentration of the feed samples is measured by measuring the optical density of the samples to the standard curve. The ELISA reproducibility in the analysis largely depends upon the consistency of the washing plate, the correct washing plate is the point of determination in ELISA program.

All the reagents must be brought to room temperature before starting the assay. The concentrated wash buffer (40ml) needs to dilute into 800 ml with deionized distilled water. The assay includes six major steps which were Labeling, Washing, Color development, Adding of Stop Solution and Optical Density measurement.

3.6.1. **Numbering**: number the sample and standard in order (multiple well), and keep a record of standard wells and sample wells.

3.6.2. **Add sample**: add 50μL of Standard, Blank, or Sample per well, then add 50μL HRP conjugate to each well, then add 50μL antibody working solution, cover the plate with sealer we provided, shake for 5s softly to mix thoroughly, incubation for 30min at 25°C.

3.6.3. **Wash:** uncover the sealer carefully; remove the liquid of each well. Immediately add 250μL wash working buffer to each well and wash sufficiently, 30s interval/time, repeat the process five times. Invert the plate and pat it against thick clean absorbent paper (If bubbles exist in the wells, clean tips can be used to prick them).

3.6.4. **Color development**: add 50μL substrate solution A to each well, and then add 50μL substrate solution B, shake for 5s softly to mix thoroughly, light-resistant incubation for 15min at 25°C.

3.6.5. **Stop:** addition of 50μL stop solution to each well, shake softly to mix thoroughly, in order to stop reaction.

3.6.6. **OD measurement**: optical density (OD) value of each well was determined at once by using the micro plate reader which is adjusted at 450nm. This step must be finished in 10min after stop reaction. User must open the micro plate reader in advance to preheat instrument, and set all the testing parameters.

3.6.7. **Result analysis**: To make standard curve of Absorbance against concentration of standard in ppb we need Average absorbance percentage which is calculated by this formula.

Absorbance percentage (%) =Absorbance of standard/Absorbance of ppb Standard x100%

The standard curve is plotted on Microsoft Excel with absorbance rate percentage of each sample on y-axis against the concentration of standard on the x-axis to make their fit curve. To obtained OD values of samples can used to calculate unknown concentration using standard curve. The obtained value needs to be multiplied by dilution factor if the samples were diluted. Graphs were plotted on MS Excel for the analysis of results.

3.7. Antimicrobial activity:

3.7.1. Collection of sample:

Cassia fistula seeds, leaves, flowers and pods were collected from the sports ground of NUST and stored at room temperature until the next procedure.

Cassia fistula seeds, leaves, flowers and pods were collected and dried at the room temperature. After drying these organs were grinded into fine powder using mortar and pestle, six different solvents extracts were prepared to check antimicrobial activity.

3.7.2. Extracts formation:

3.7.2.1. Aqueous extracts:

20 g of seeds, leaves and pods powder of *Cassia fistula* were soaked in the 200 ml of distilled water separately in the flasks, furthermore these flasks were set on the shaking incubator toward room temperature for 2,3 days. The solutions were filtered through the filter paper and the extracted filtrate was further dried into the rotary evaporator at the temperature 80 °C awaiting the entire water evaporated. The collected dry extract was stored at 4°C until the further use.

3.7.2.2. Methanolic extracts:

30 g of seeds, leaves and pods powder of *Cassia fistula* were soaked in the 150 ml of 100% methanol separately in the flasks, furthermore these flasks were set 2,3 days on the shaking incubator toward room temperature. The solutions were filtered through the filter paper and the extracted filtrate was further dried into the rotary evaporator at the temperature 60 °C awaiting the entire methanol evaporated. The collected dry extract was stored at 4°C until the further use.

3.7.2.3. Chloroform and ethanolic extracts:

30 g of seeds, leaves and pods powder of *Cassia fistula* were soaked in the 300 ml of chloroform and 300 ml 100% ethanol separately in the flasks, furthermore these flasks were set 2,3 days on the shaking incubator toward room temperature. The solutions were filtered through the filter paper and the extracted filtrate was further dried into the rotary evaporator at the temperature 60 °C for chloroform solution and 70 for ethanolic solution and waiting till all the solvents get evaporated. The collected dry extract was stored at 4°C until the further use.

3.7.2.4. Acetonic extracts:

30 g of seeds, leaves and pods powder of *Cassia fistula* were soaked in the 100 ml of acetone separately in the flasks, furthermore these flasks were set 2,3 days on the shaking incubator toward room temperature. The solutions were filtered through the filter paper and the extracted filtrate was further dried into the rotary evaporator at the temperature 50 °C awaiting all the acetone evaporated. The collected dry extract was stored at 4°C until the further use.

3.7.2.5. Hexane extraction:

25 g of seeds, leaves and pods powder of *Cassia fistula* were soaked in the 150 ml of n-hexane separately in the flasks, furthermore these flasks were set 2,3 days on the shaking incubator toward room temperature. The solutions were filtered through the filter paper and the extracted filtrate was further dried into the rotary evaporator at the temperature 60 °C awaiting all the n-hexane evaporated. The collected dry extract was stored at 4°C until the further use.

3.7.3. Dilutions of the extracts:

The whole dried extracts formed by seeds, pods, flowers and leaves of *Cassia fistula* were weighted separately and their dilution was made by adding 100mg extract in 1ml Dimethyl sulphoxide (DMSO) solvent.

3.7.4. In-vitro antifungal activity:

3.7.4.1. Media preparation:

Two media usually used for the growth of fungal strains SDA and PDA. SDA was prepared by 65 g SDA in 1 L distilled water while PDA was prepared by 39 g PDA powder in 1L distilled water. After preparing media, this will be autoclaved at temperature 121 °C and pressure at 15 psi for 15 minutes. Petri plates are prepared in the safety cabinet using these media and pour

approximately 25 ml media in each plate and incubated these plates at temperature 37°C for overnight to confirm that there is no contamination in the plates.

3.7.4.2. Inoculation of plates:

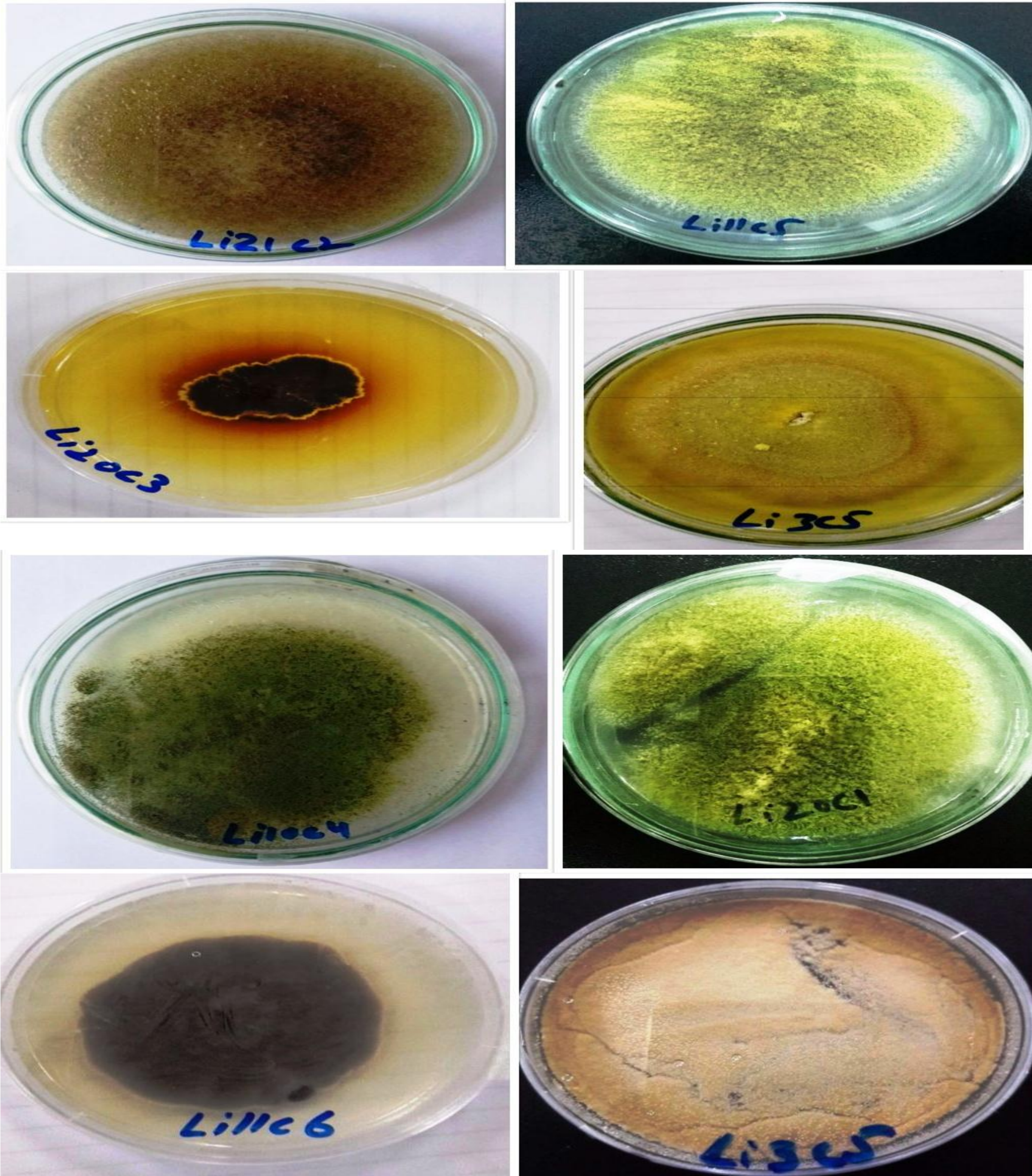
Contamination free plates of SDA/PDA were inoculated with the fungal strains using the streaking method. 50µl water, that containing spores of known fungi was poured on SDA/PDA plates and streak the plate with glass spreader. Surface of the solidified PDA/SDA plate was Inoculate by streaking the glass spreader three times over the whole agar plate surface and rotate the whole agar plate up to 45 degrees all time to make sure the smooth distribution of fungal spores. Sterilize the glass spreader with absolute ethanol after streaking the agar plate with every fungal strain.

3.7.4.3. Well diffusion method:

Well diffusion technique is used confirm the antifungal activity of fungal strain. 50µl dilution extracts of *cassis fistula* was poured on the 6mm wells made by autoclave yellow tips, by using micropipette. Eight wells with different solvent extracts (100 mg per ml) of same concentration of 50µl were poured in each Petri plate. This antifungal technique was complete in triplicate. One well was poured with standard antibiotic amphotericin B serve as a positive control for negative control 50% DMSO used. The plates were than incubated for 3-4 days at 25 °C after that zone of inhibition of extracts was measured.

4. RESULTS

Pure fungal cultures were obtained from the feed by streaking the respective fungal spores in the PDA plated.



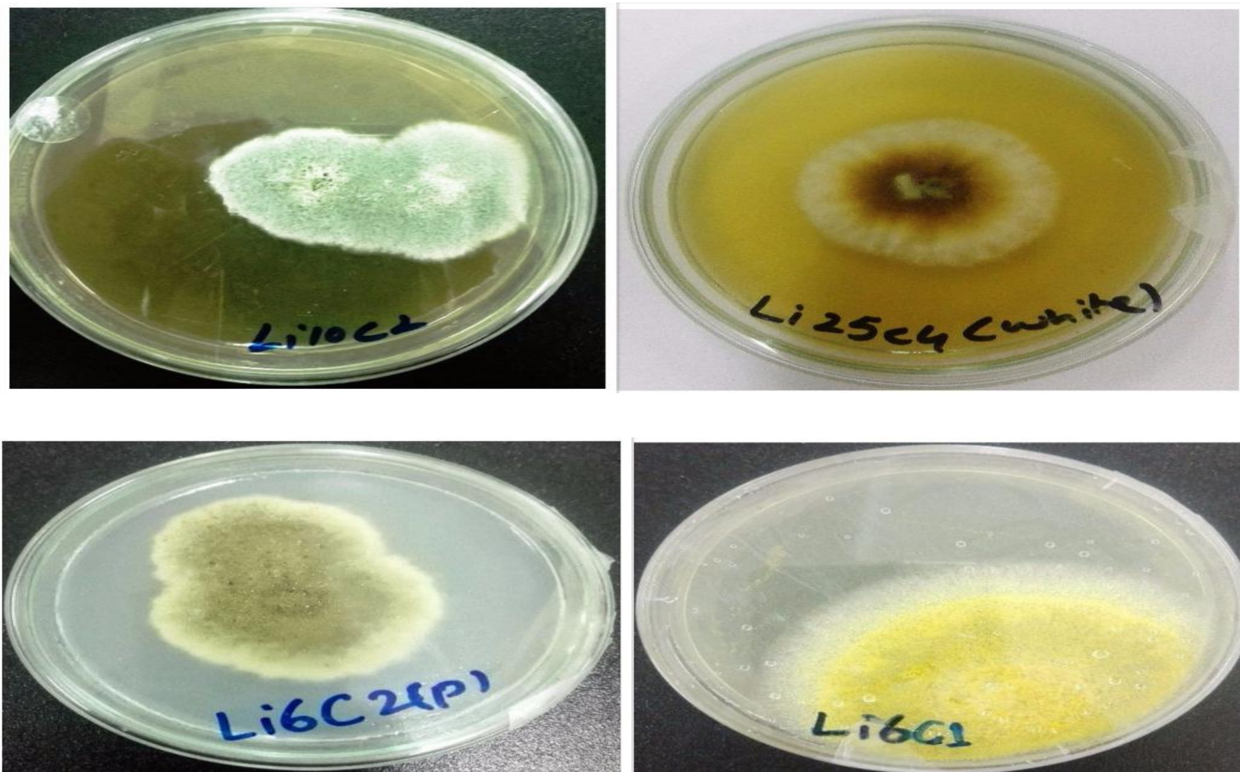


Figure 3: Representative pure cultures of livestock fungi.

Fungal isolates were analyzed morphologically to analyzed strains because strains color and pattern helped to get an idea about fungal genus. List of samples are mentioned in Table 6.

Table 6**:** List showing different colonies obtained from different feed samples.

Feed code	**Colonies retrieved**
Li1	No Fungus
Li2	No Fungus
Li3	Li3C1, Li3C2, Li3C3, Li3C4 , Li3C5
Li4	No Fungus
Li5	No Fungus
Li6	Li6C1, Li6C2
Li7	Li7C1, Li7C2
Li8	No fungus
Li9	Li9C1, Li9C2, Li9C3
Li10	Li10C1, Li10C2, Li10C3, Li10C4
Li11	Li11C1, Li11C2, Li11C3, Li11C4, Li11C5
Li12	Li12C1, Li12C2, Li12C3,
Li13	Li13C, Li13C
Li14	Li14C1, Li14C2, Li14C3, Li14C4
Li15	Li15C1, Li15C

Li16	Li16C1, Li16C1
Li17	Li17C1, Li17C, Li17C3
Li18	No fungus
Li19	LIi9C1, Li19C2, Li19C3
Li20	Li20C1, Li20C2, Li20C3
Li21	Li21C1, Li21C2

4.1. Molecular analysis:

For the identification of different fungal isolates from livestock feed DNA Profiling technique is used. Genomic DNA of subjective fungi strains was successfully extracted by using the above written method Coenen *et al.*,1997. After nucleic acid extraction the efficiency of DNA was checked through the specific ITS primers region via PCR amplification method. All fungal isolates give the amplifiable DNA, without the further purification stage.

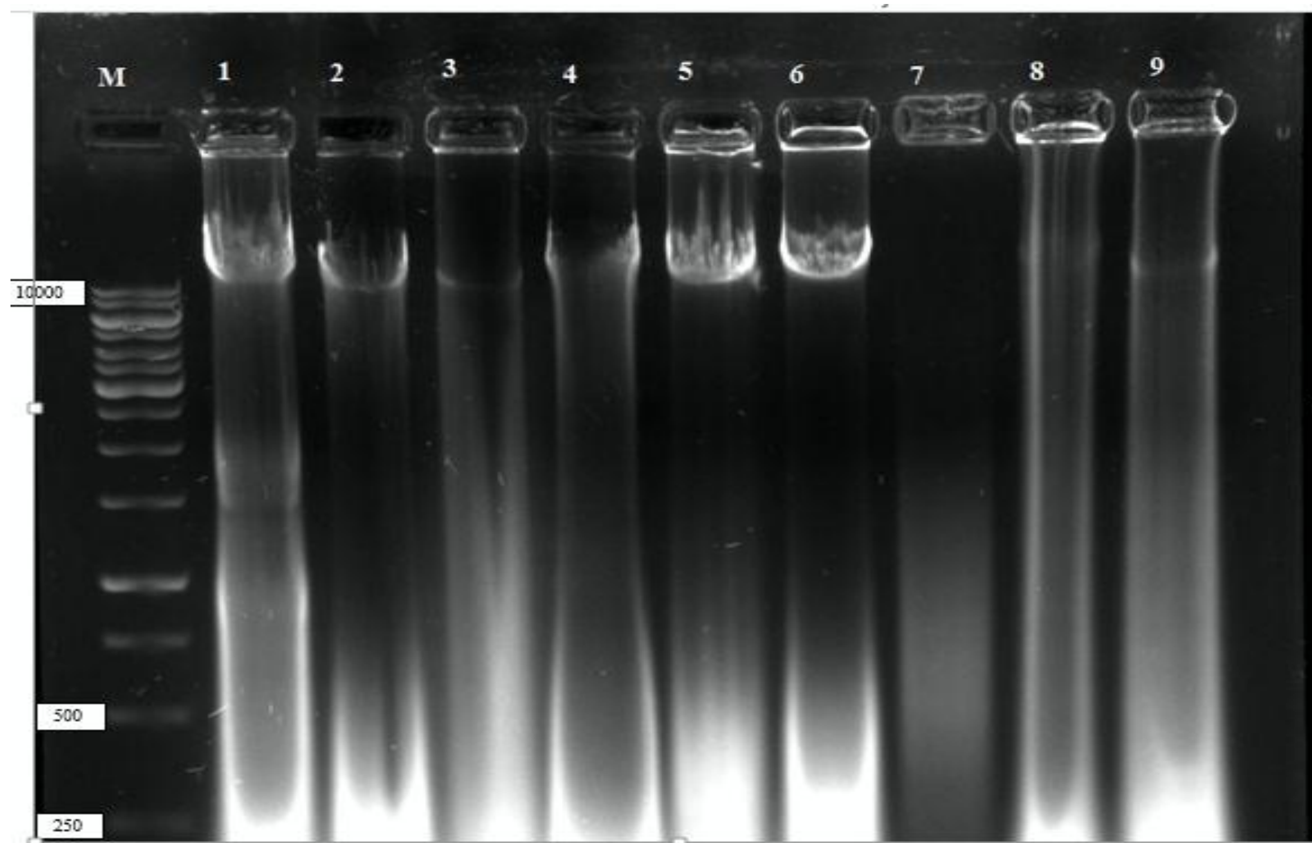


Figure 4: Genomics DNA on agarose gel. Lane M contain Ladder while 1-9 is number of samples.

4.1.1. PCR amplification:

Extracted DNA of collected fungi strains were successfully amplified using the six ITS primer sets. ITS1, ITS4 was commonly used and known as universal primer set were used for the amplification all 20 samples, however, only five isolates (15 %) were successfully amplified (Figure 8). Remaining samples were subjected following primers pairs, ITS86F &ITS4 and ITS1 &ITS86R.

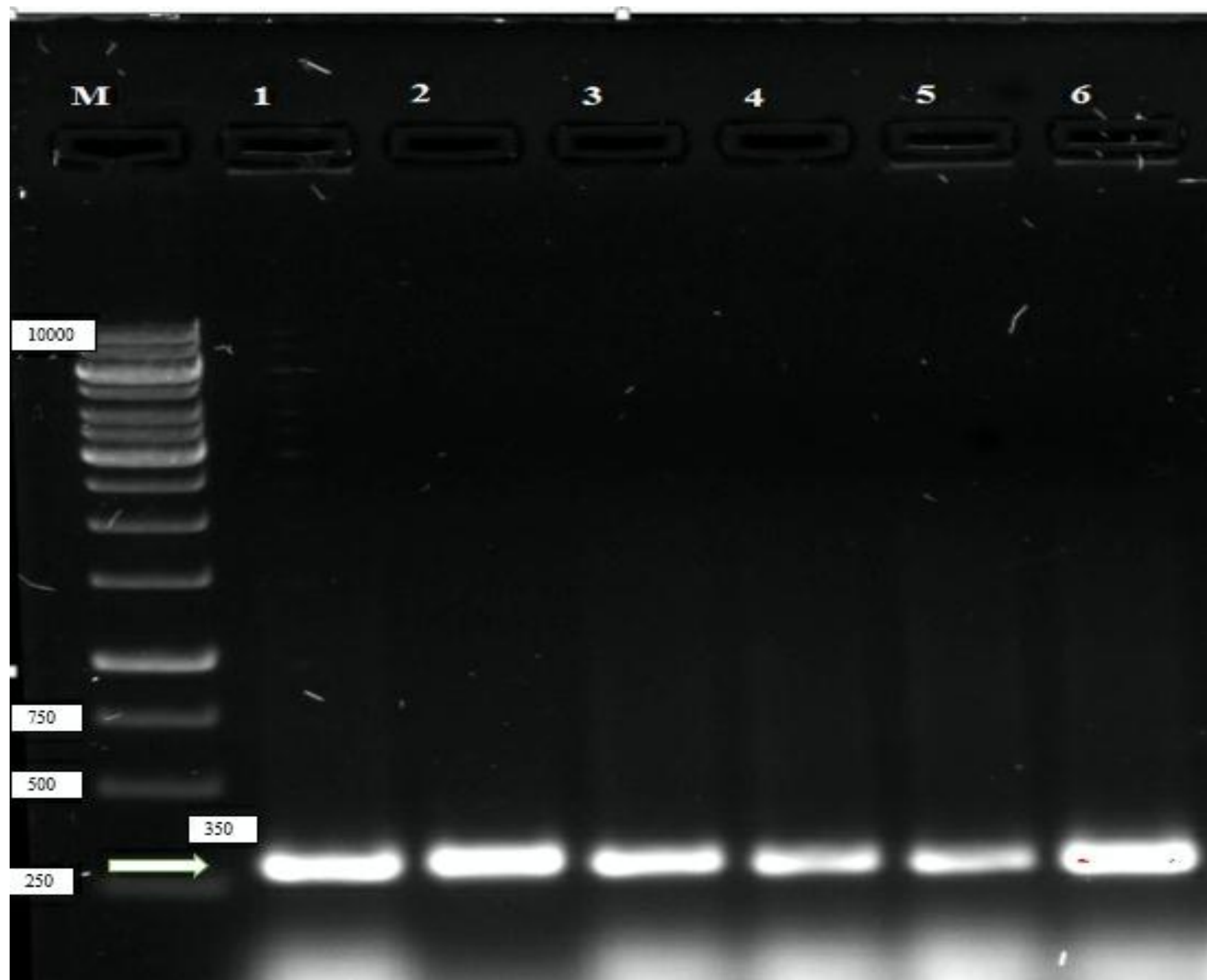


Figure 5: Fungal strains amplified using ITS primer pair (ITS1F & ITS 86R). Lane M contain Ladder while 1-6 contain amplified products.

Eight fungal isolates (40 %) were amplified using primer pair ITS86F and ITS4R, while one isolate (5 %) gave positive results when primer set ITS1F and ITS86R were used and three samples are amplified by using full length region ITS1F and ITS4R. Eight isolates (40 %) were not amplified by any of +++primer set moreover; none of the isolate was amplified by following pairs of primer; ITS5-ITS4, ITS1-ITS2 and ITS3-ITS4.

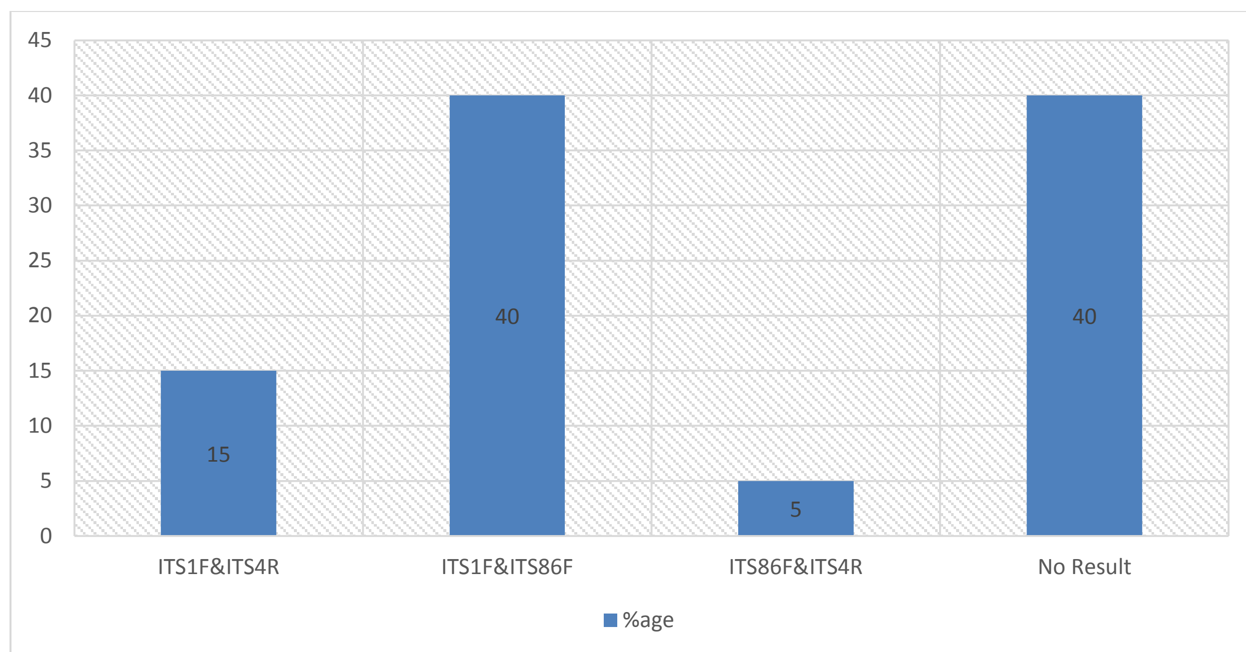


Figure 6**:** Graphic representation of ratio of fungal strains amplified by different set of ITS primers.

4.1.2. Agarose gel electrophoresis:

The amplified product of PCR when examined on agarose gel of 1% against the DNA ladder of 1kb, different band sizes were obtained. Samples that were amplified using primer set ITS1F & ITS4R gave product size of 500 bp to 700 bp; however, ITS86F & ITS4R and ITS1F & ITS86R gave amplicon size of 250 bp to 500 bp. Thus, the product obtained in this analysis was within range of reported data.

4.1.3. Purification of PCR products and DNA sequencing:

PCR products with primer dimmers were purified through PCR purification kit (Pure Link).

Purified PCR product after purification was analyzed on the agarose gel of 1% against ladder of 1 kb (Thrmoscientific) DNA ladder, clear band were obtained on gel which were further sent for sequencing.

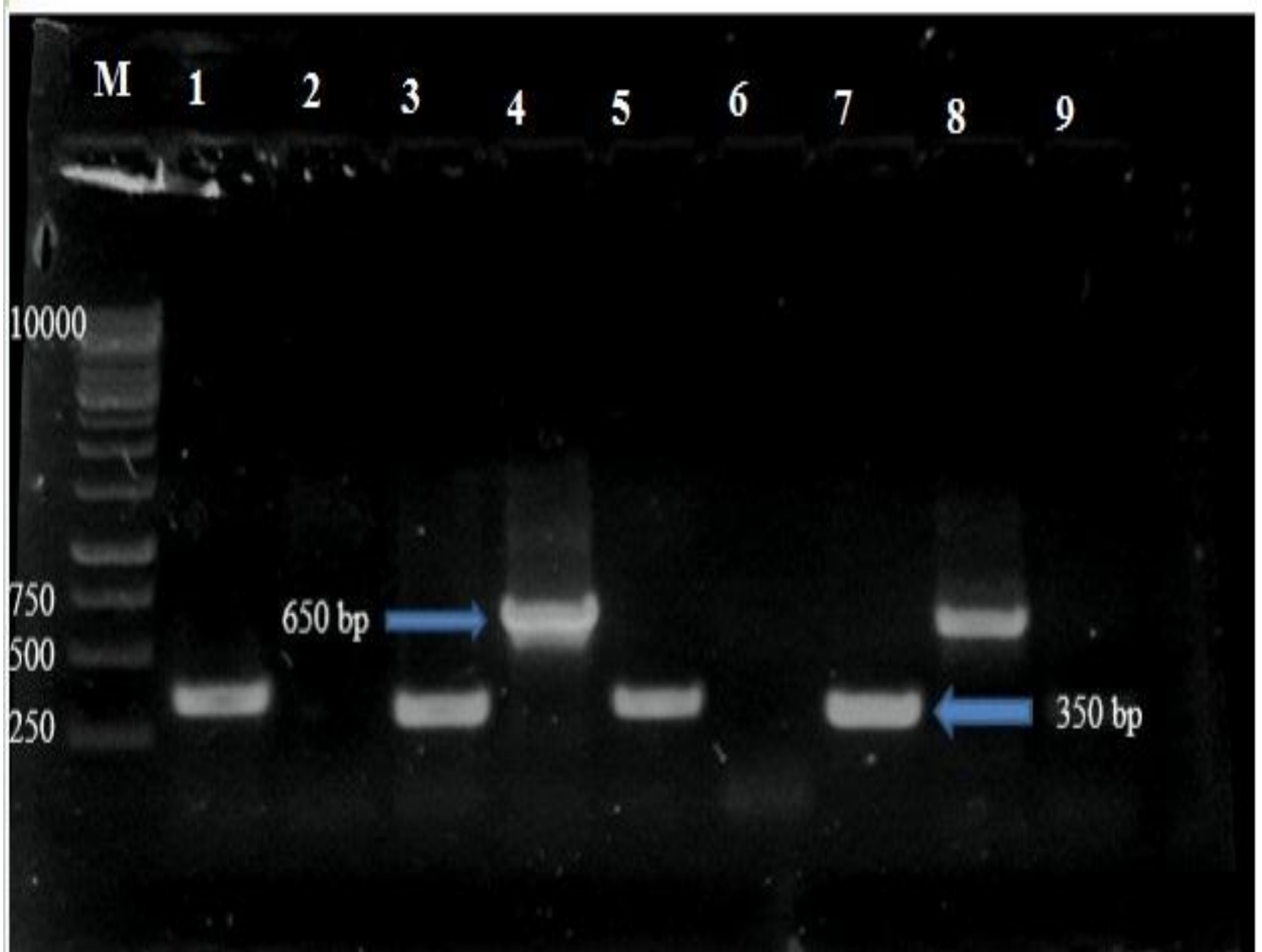


Figure 7: Agarose gel electrophoresis of PCR purified products. Lane M contain ladder and Lane 1-9 contain purified amplicon.

4.1.4. Sequencing of full length ITS region:

PCR purified products was commercially sequenced by the Eurofins USA. The sequences were BLAST with the other sequence *via* NCBI data base.

Table 7**:** List of mycofloral colonies identified.

Colony code	**Fungi name**	**Primer pair**	**Amplicon size**
Li11C6	*Aspergillus subolivaceus*	ITS1F &ITS4R	500-700 bp
Li21C2	*Mucor circinelloides*	ITS1F &ITS4R	500-700 bp
Li10C4	*Talaromyces funiculosus*	ITS1F &ITS86R	250-500 bp
Li11C5	*Aspergillus flavus*	ITS86F &ITS4R	250-500 bp
Li20C3	*Aspergillus tamari*	ITS1F &ITS4R	500-700 bp
Li10C2	*Penicillium funiculosum*	ITS86F & ITS4R	250-500 bp
Li6C1	*Aspergillus oryzae*	ITS86F & ITS4R	250-500 bp
Li20C1	*Aspergillus flavus*	ITS86F & ITS4R	250-500 bp
Li10C2	*Talaromyces calidicanius*	ITS86F & ITS4R	250-500 bp
Li10C4	*Aspergillus oryzae*	ITS86F & ITS4R	250-500 bp
Li20C3	*Aspergillus costiformics*	ITS86F & ITS4R	250-500 bp
Li3C5	*Aspergillus terreus*	ITS86F & ITS4R	2500-500 bp

4.2. Assessment of secondary metabolites

To assess the total aflatoxin concentration in feed samples Elabscience standard total aflatoxin kit was used. 26 livestock feed samples were pre-treated with 70% methanol and diluted with de-ionized water for processing. Optical density was measured at 450 nm in microplate reader followed by the protocol of Elabscience kit. Due to some handling error during protocol or ill

equipment use the microplate reader was able to detect to optical density of the standards and the samples but was not able to draw the standard curve which is needed to determine the concentration of aflatoxins in each sample. So a standard curve was drawn manually using Microsoft Excel 2007. A standard curve graph was plotted between concentration of standards in ppb and their average absorbance percentages. The values are shown in Figure 6.

The values of concentrations of unknown samples generated from this curve with optical density values obtained from micro plate reader are shown in tables below. They are also multiplied by 5 which is the dilution factor.

Table 8**:** Total aflatoxin concentrations of livestock samples

Feed code	**Company name**	**OD obtained**	**Aflatoxin Conc.**
Li1	Panjnad Wanda	0.132	4.5
Li2	Passo Milk Wanda	0.075	5
Li3	Zamindara Wanda	0.077	4.95
Li4	National Wanda	0.131	4.5
Li5	Mughal e Azam Wanda	0.197	3.95
Li6	National Wanda	0.055	3.45
Li7	Super Power Feed	0.168	3.15
Li8	Premium Wanda	0.067	3.4

Li9	Passo Milk Wanda	0.120	3.25
Li10	Chanon Wanda	0.072	3.4
Li11	Hassan Farmer Wanda	0.127	3.25
Li12	ICI Wanda	0.091	3.25
Li13	Super Power Feed	0.072	3.4
Li14	Chanon Wanda	0.057	3.45
Li15	Khal Wanda	0.126	3.25
Li16	National Wanda	0.077	3.35
Li17	Pak Cattle Wanda	0.065	3.4
Li18	ICI govt. Wanda	0.056	3.45
Li19	Matra Wanda	0.330	2.75
Li20	Corn Powder	0.081	3.35
Li21	Sultan Wanda	0.061	3.4
Li22	China feed	0.061	3.4
Li23	ICI	0.054	3.45
Li24	Metro	0.061	3.45
Li25	China feed	0.080	3.4
Li26		1.397	3.35

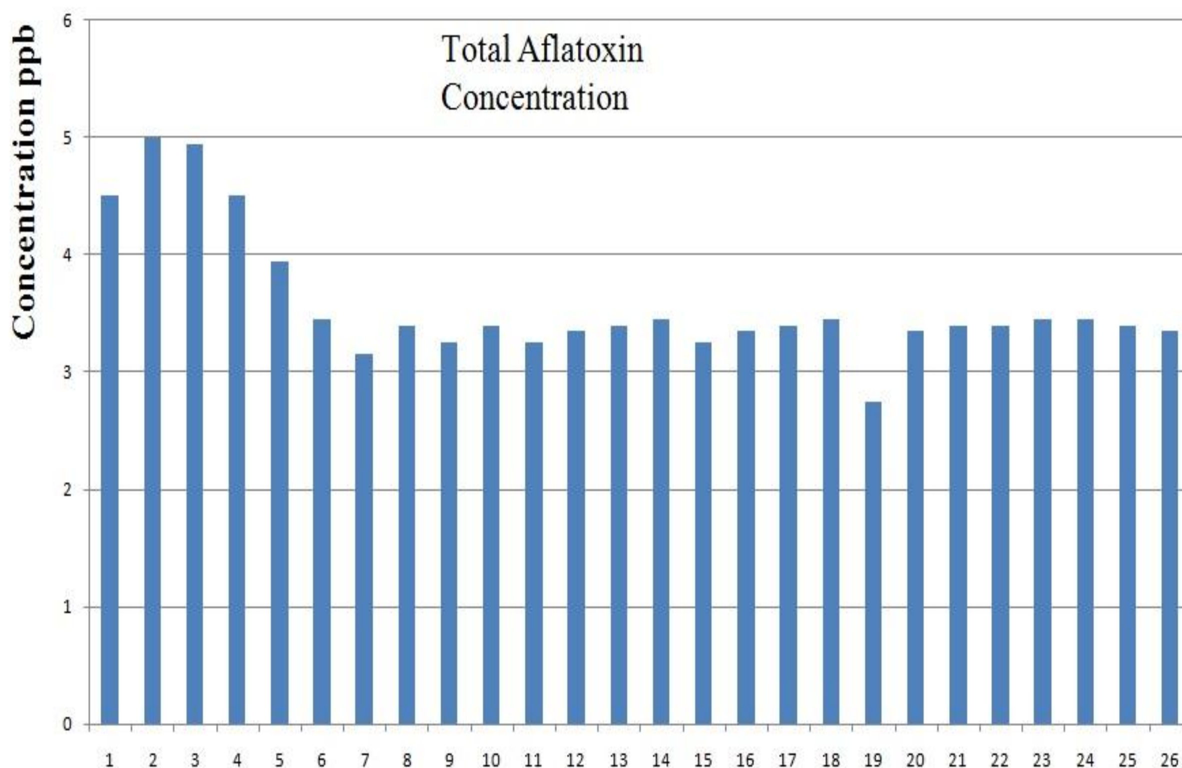


Figure 8: Graphical representation of total aflatoxin concentrations of processed livestock samples

In the 26 livestock samples that were processed the least concentration of aflatoxins was 2.75 ppb which is Li20 (raw corn) and the highest concentration is 4.95 ppb of Li04, LI06 National feeds).

According to the United States Food and Drug Administration the permissible level of the aflatoxins in food for human consumption is 2ppb and that of livestock is 100-300 ppb. Although the level of aflatoxins obtained from livestock samples falls under the permissible limits of livestock feed but it is above the permissible limit for human consumption. So the livestock animals consuming these feed samples later fed to humans may make them vulnerable to aflatoxicosis.

4.3. Antifungal activity:

Cassia fistula extracts activity was checked for antifungal assay against the pathogenic fungal strain *A. flavus*. *Cassia fistula* seeds, leaves, flowers and pods extracts was made against six different solvents: water, ethanol, methanol, n-hexane, chloroform and acetone. Drug for positive control amphotericin B is used while in negative control 50% DMSO solution is used.

4.3.1. *Cassia fistula* pods activity:

Antifungal activity of six extracts (water, ethanol, methanol, n-hexane, chloroform and acetone) was assessed in terms of zone of inhibition of fungal growth. Pods extract activity against the *A. flavus* increased with the concentration (µg/ml) of pods extracts of *cassia fistula*. Pods extracts of *Cassia fistula* from Hydrolic, ethanol and methanol solvent confirm significant zone of inhibition against *A. flavus,* while acetone extracts do shows inhibition but less than hydrolic ethanol and methanol extracts. Hexane and chloroform extracts does not show any positive response against *A. flavus.*

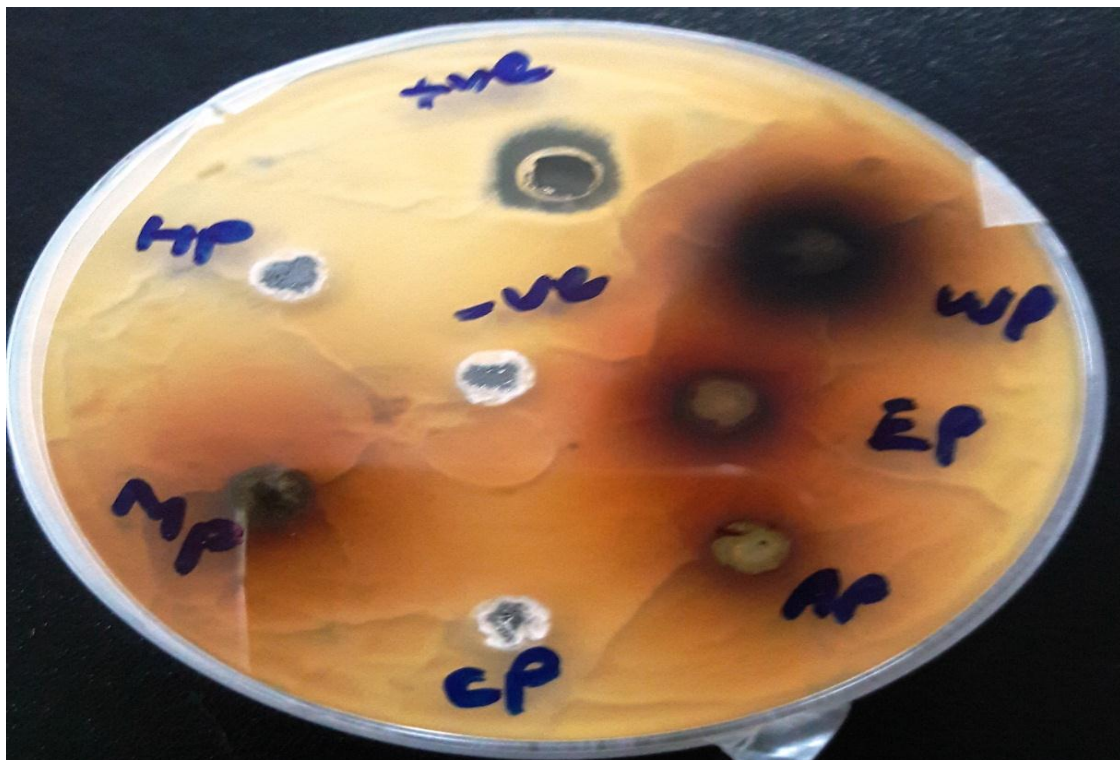


Figure 9: Antifungal activity of pods (dissolved in different solvents) Pods extracts activity against A. flavus grown on PDA medium. Whereas, MP is Methanolic extracts, CP is chloroform extract, WP is water extract, EP is ethanol extract, AP is acetone extract, and HP is hexane extract.

Table 9: Zone of inhibition of pods extracts:

Dilutions	**Zone of inhibition**
+ve control	6 mm
-ve control	0 mm
CP (Chloroform Pods)	0 mm
EP (Ethanol Pods)	4 mm
WP (Water Pods)	5 mm

AP (Acetone Pods)	2 mm
HP (Hexane Pods)	1 mm
MP (Methanol Pods)	2 mm

4.3.2. *Cassia fistula* Leaves Activity:

Leaves extract activity against the *A. flavus* increased with the concentration (µg/ml) of leaves extracts of cassia fistula. Leaves extracts of *Cassia fistula* from the hydrolic and ethanolic solvent confirm significant zone of inhibition against *A. flavus*, while N-hexane and ethanol extracts also shows inhibition but less than hydrolic and methanol extracts. Acetone and chloroform extracts does not show any positive response against *Aspergillus flavus*.

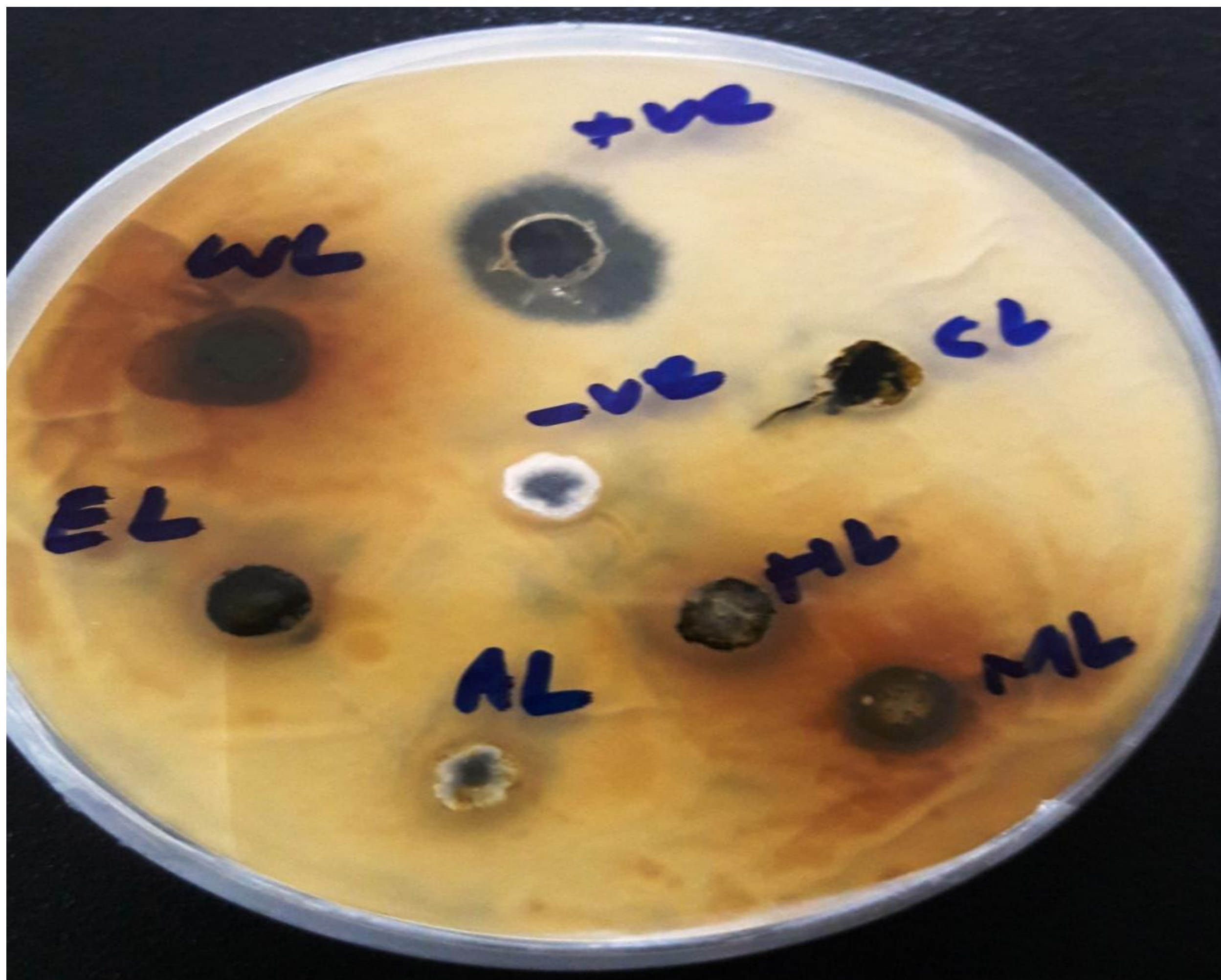


Figure 10: Antifungal activity of leaves (dissolved in different solvents) Leaves extracts activity against *A. flavus* grown on PDA medium. Whereas, ML is Methanolic extracts, CL is chloroform extract, WL is water extract, EL is ethanol extract, AL is acetone extract and HL contain N-hexane extracts.

Table 10**:** Zone of inhibition of leaves extracts

Dilutions	**Zone of inhibition**
+ve control	7 mm
-ve control	0 mm
CL (Chloroform Leaves)	0 mm
EL (Ethanol Leaves)	1 mm
WL (Water Leaves)	5 mm
AL (Acetone Leaves)	0 mm
HL (Hexane Leaves)	1 mm
ML (Methanol Leaves)	1 mm

4.3.3. *Cassia fistula* seeds activity:

Seeds extract activity against the *A. flavus* increased with the concentration (µg/ml) of seeds extracts of cassia fistula. Seeds extracts of *Cassia fistula* from Chloroform, ethanol and methanol solvents confirm significant zone of inhibition against *A. flavus*, while water and acetone extracts also shows inhibition but less than Chloroform, ethanol and methanol extracts. N. hexane extracts does not show any positive response against *Aspergillus flavus*.

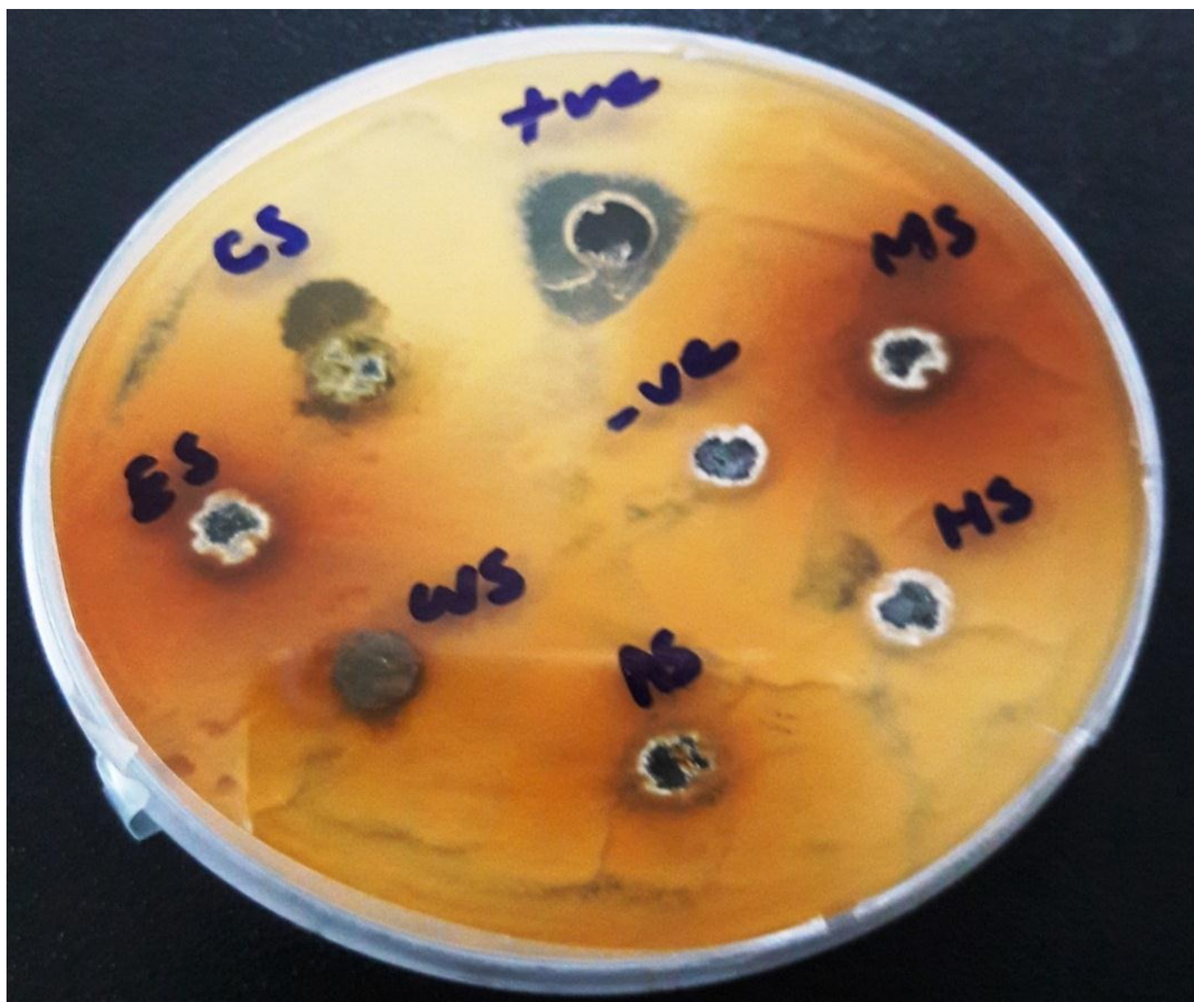


Figure 11: Antifungal activity of seeds (dissolved in different solvents) seeds extracts activity against A. flavus grown on PDA medium. Whereas, MS is Methanolic extracts, CS is chloroform extract, WS is water extract, ES is ethanol extract, AS is acetone extract, and HS is hexane extract.

Table 11**:** Zone of inhibition of seeds extracts.

Dilutions	**Zone of inhibition**
+ve control	7 mm
-ve control	0 mm
CS (Chloroform seeds)	3 mm
ES (Ethanol seeds)	2 mm
WS (Water seeds)	1 mm
AS (Acetone seeds)	1 mm
HS (Hexane seeds)	0 mm
MS (Methanol seeds)	2 mm

4.3.4. *Cassia fistula* flowers activity:

Flowers extract activity against the *A. flavus* increased with the concentration (µg/ml) of flowers extracts of cassia fistula. Flowers extracts of *Cassia fistula* from Chloroform, ethanol and methanol solvents confirm significant zone of inhibition against *A. flavus*, while water and acetone extracts also shows inhibition but less than Chloroform, ethanol and methanol extracts. N. hexane extracts does not show any positive response against *Aspergillus flavus*.

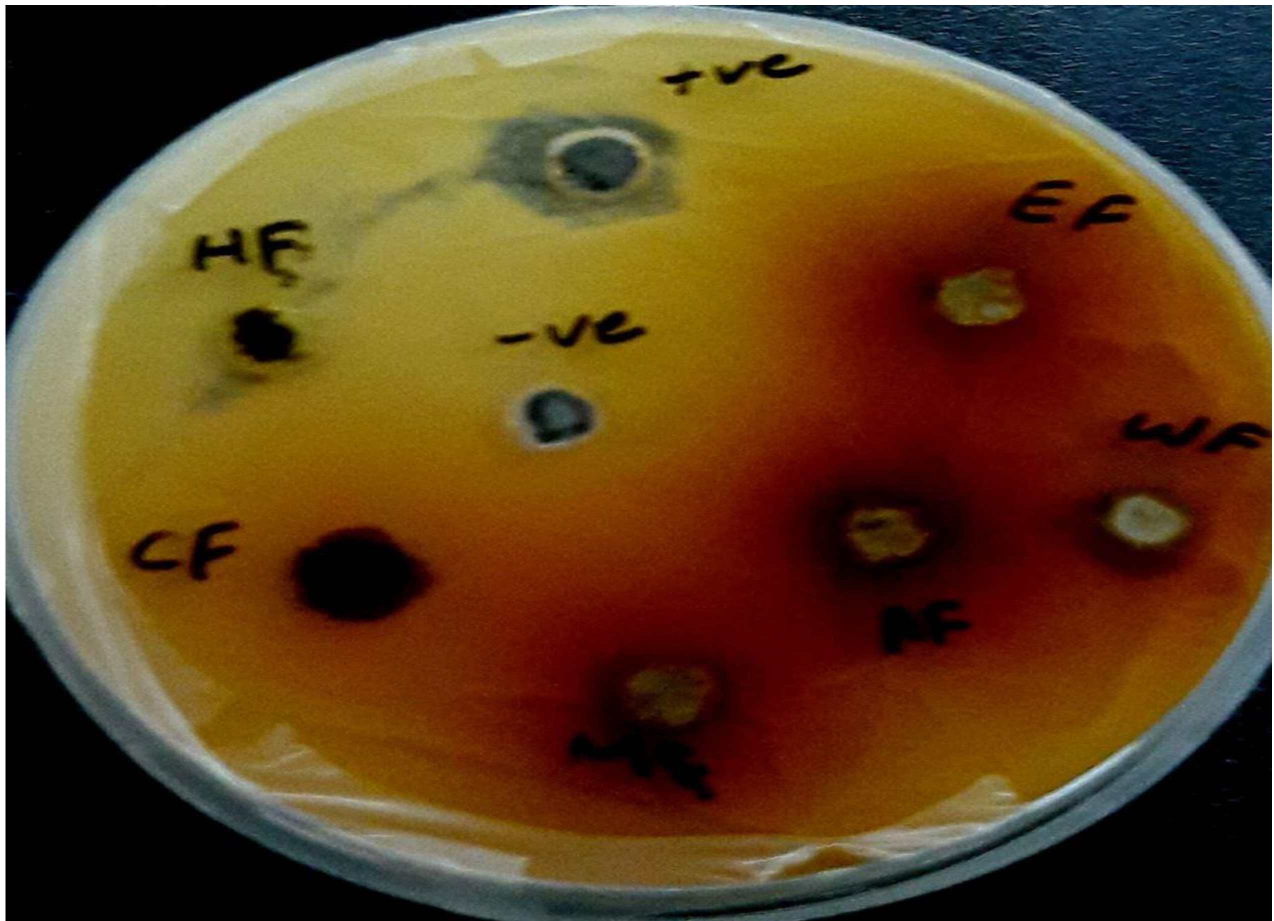


Figure 12**:** Antifungal activity of flowers (dissolved in different solvents) seeds extracts activity against *A. flavus* grown on PDA medium. Whereas, MF is Methanolic extracts, CF is chloroform extract, WF is water extract, EF is ethanol extract, AF is acetone extract, and HF is hexane extract.

Table 12**:** Zone of inhibition of seeds extracts.

Dilutions	**Zone of inhibition**
+ve control	4 mm
-ve control	0 mm
CF (Chloroform seeds)	2 mm
EF (Ethanol seeds)	3 mm
WF (Water seeds)	1 mm
AF (Acetone seeds)	0 mm
HF (Hexane seeds)	0 mm
MF (Methanol seeds)	2 mm

5. DISCUSSION

Animal infected feed which carry different toxigenic Fungi cause serious threat for humans and animals and pose a risk of infection (Sharma and Rajak, 2003). In the present research different isolates of fungi were collected from the animal feed and cultured these fungi by using media SDA and PDA. Extracted isolates from feed was identified by using molecular approaches. DNA was extracted from these fungal isolates by using (Coenen et al., 1997) method. For PCR amplification, the main important is to confirm that the targeted genome is purely extracted. Because mostly in DNA extraction, some impurities are also remains during extraction procedure along with our DNA which cause the non-specificity in the PCR amplification. This non specificity of DNA by inhibitors can be removed by increasing the quantity of DNA polymerase, which gives resistance to the inhibitor resistance (Bellemain et al., 2010).

For the identification and characterization of animal feed fungal isolates, DNA bar-coding is important tool (De Beeck et al., 2014). For fungal identification, authentic and rapid method is used in this present research. For DNA extraction easy and inexpensive method is used and at molecular lever for PCR amplification ITS priers are used. Most common ITS region primer that are used in molecular methods for identification of the fungi was studied in 90s (White et al., 1990) which was designed for the characterization of broad range of fungal isolates from feed.

In this study, six primer pairs; ITS1F and ITS4R, ITS86F and ITS4R, ITS1F and ITS86R were used to amplify 26 fungal strains. Every primer set showed specificity to certain strains. Five fungal species (26%) out of twenty were successfully amplified using ITS1F and ITS4R primer set and six fungal strains amplified by using ITS86F and ITS4R and four amplified with ITS1F

and ITS86R. Many studies have been reported for the characterization and amplification of wide range of fungal DNA using these primers set.

26 livestock samples were processed for aflatoxin concentration measurement, because Aflatoxins are secondary metabolites of *A. flavus* and different Parasitic *Aspergillus* species which contaminated different feed ingredients e.g. corn, nuts, maize. Main types of aflatoxins are Aflatoxin B1, Aflatoxin B2, Aflatoxin G1 and Aflatoxin G2, Total aflatoxin contamination is stated the amounts of these four aflatoxins. This aflatoxin is class 1 carcinogenic which may be harmful to liver, kidney, different tissues and organs of human as well as animals. The least concentration of aflatoxins was 2.75 ppb which is Li20 (Raw corn powder) and the highest concentration is 4.95 ppb of Li04, LI06 National feeds feed). According to the US-FDA (Food and Drug Administration) the acceptable level of the aflatoxins in food for human consumption is 2ppb and that of livestock is 100-300 ppb. Although the level of aflatoxins obtained from livestock samples falls under the permissible limits of livestock feed but it is above the permissible limit for human consumption. So the livestock animals consuming these feed samples later fed to humans may make them vulnerable to aflatoxicosis.

To discover new drugs which are natural sources and best resentence against pathogenic fungi; antifungal activity of the extracts of *Cassia fistula* was studied against pathogenic fungal strains *A. flavus*. *Cassia fistula* seeds, leaves and pods extracts was made against six different solvents: water, ethanol, methanol, n-hexane, chloroform and acetone. Drug for positive control amphotericin B is used while in negative control 50% DMSO solution is used.

Cassia fistula leaves and Pods show the maximum zone of inhibition against *A. flavus*, while seeds show less activity. It has been suggested that the leaves, pods and seeds of *Cassia fistula* would become effective against different pathogenic fungi in animal feeds.

In conclusion, present investigation and previous study showed that *Cassia fistula* plant extracts have effective antifungal activity against animal's and human pathogenic fungal strains.

6. CONCLUSION

The study suggested that the ITS regions of ribosomal DNA (rDNA) gene are significant and useful target for molecular approaches, thus providing an efficient tool for fungi identification and characterization. The major feature of this method is the ITS region location between two highly conserved genes which allow their amplification by fungi universal primers. Species to species probing, analysis of direct sequence and RFLP study using the ITS region in PCR based method have all facilities for the identification of pathogenic fungi. Thus, DNA- based approaches that use ITS region as molecular target for the fungal identification are effective for the diagnosis of invasive infection caused by fungal species. However, it is little doubtful that ITS as fungi molecular marker will increase our knowledge regarding the diversities of fungal communities in the next decade.

In Pakistan almost all the mixed feed is contaminated with toxins and these toxins can be entered into the food chain and cause negative impacts in the performance of animals as well as humans, their growth rate, immune system, teratogenic effect, carcinogenic effect, mutagenic effect or damage to the nervous system and also fatal for liver and kidney infection. Occurrence of the mycotoxins everywhere in the environment is global issue. Growth of harmful fungi can be prevented by using the medicinal plant *Cassia fistula* pods in the feed ingredient. *Cassia fistula* Pods have the maximum activity to inhibit the fungal growth. However, more studies are required for better understanding and advance applications.

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8. APPENDIX

Table 13**:** List of feed samples showing the place and date of collection.

Feed	Company	Place	Date
LI1	Panjnad Wanda	Kot Addu	28-06-2015
LI2	Passo Milk Wanda	Kot Addu	28-06-2015
LI3	Zamindara Wanda	Kot Addu	29-06-2015
LI4	National Wanda	Kot Addu	29-06-2015
LI5	Mughal e Azam Wanda	Kot Addu	29-06-2015
LI6	National Wanda	Kot Addu	29-06-2015
LI7	Super Power Feed	Kot Addu	29-06-2015
LI8	Premium Wanda	Multan	30-06-2015
LI9	Passo Milk Wanda	Kot Addu	30-06-2015
LI10	Chanon Wanda	Kot Addu	30-06-2015
LI11	Hassan Farmer Wanda	Kot Addu	30-06-2015
LI12	ICI Wanda	Tarnol	03-07-2015

LI13	Super Power Feed	Kot Addu	30-06-2015
LI14	Chanon Wanda	Kot Addu	03-07-2015
LI15	Khal Wanda	Tarnol	01-07-2015
LI16	National Wanda	Kot Addu	29-06-2015
LI17	Pak Cattle Wanda	Multan	29-06-2015
LI18	ICI govt. Wanda	Kot Addu	29-06-2015
LI19	Matra Wanda	Kot Addu	29-06-2015
LI20	Corn Powder	Kot Addu	29-06-2015
LI21	Sultan Wanda	Kot Addu	29-06-2015