WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 8.084
Volume 12, Issue 11, 1278-1330. Research Article ISSN 2277– 7105

TOXICITY STUDY OF FUNGAL CONTAMINATED BREWER'S SPENT GRAIN IN RATS

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Article Received on
18 May 2023,
Revised on 08 June 2023,
Accepted on 28 June 2023
DOI: 10.20959/wjpr202311-28873

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ABSTRACT

The present study was undertaken to evaluate the toxicity of fungal contaminated brewer's spent grain in rats. Repeated dose 28-day oral toxicity study of mycotoxins present in mixed fungal culture filtrates of *Rhizopus oryzae* 25%, *Aspergillus flavus* 50% and *Aspergillus nomius* 25%, was conducted by gavaging it to rats. 40 Wister albino rats were divided in to four groups, 10 rats in each group of either sex. Keeping one group as control, other three groups received the mixed fungal culture filtrates at low dose (0.5 ml/100 g), medium dose (1 ml/100 g) and high dose (2 ml/100 g) rates depending on their body weights respectively. Body weight parameter and blood samples were taken on day 0, 14 and 28 days of the study. Biochemical parameters like ALT, AST, BUN, creatinine and haematological parameters like TLC, TEC, Hb, PCV and Blood clotting time were analysed. Significant ($P<0.05$) decrease in body weight, significant ($P<0.05$) increase in ALT, AST, BUN and Creatinine serum concentration, a significant ($P<0.05$) fall in TLC, TEC, Hb and PCV with significant ($P<0.05$) increase in blood clotting time in

treatment groups compared to control group rats were observed. The gross and histopathological changes revealed, hepatotoxicity, nephrotoxicity, immunotoxicity, and cardiotoxicity in all the treatment groups. Thus, it was concluded from the observations in the present study that, the fungal culture filtrate had shown toxicity in rats, attributed to the presence of toxic principle in mixed fungal culture filtrates.

KEYWORDS: Brewer's spent grain, Toxicity, Rat, *Rhizopus oryzae, Aspergillus flavus, Aspergillus nomius.*

1. INTRODUCTION

Fungi are a kingdom of heterotrophic eukaryotic organisms that cannot produce their own food. Mycotoxicity in plants and animals was caused by fungal metabolites called mycotoxins. Rhizomorphs are fungi having hyphae, root-like threaded network that aid fungus to gather the substrate. Saprobes were fungi those feed on dead decomposing organic matter. However, fungi could also have symbiotic (mutually beneficial) relationships with photosynthetic algae or bacteria and with plant roots. A symbiotic association of a fungus and an animal that photosynthesizes is a Lichen, whereas a Mycorrhiza is a symbiotic relationship between a plant root and fungus.

The majority of fungi may reproduce both sexually and asexually. Asexual reproduction occurs through the release of spores or through mycelial fragmentation, which happens when the mycelium separates into several parts that develop independently. Separate individuals fuse their hyphae together in sexual reproduction. The duration of the life cycle varies depending on the species. Spores are microscopic cells or clusters of cells which are used by all fungus to reproduce and they are dispersed from their parent fungus by wind or water. Spores can remain dormant for a long period, until favourable conditions supports their growth.

Spores might stay latent until they can colonize a new food source. Fungi produce spores through both sexual and asexual reproduction.[1] Mold/Mould is a conspicuous mass of mycelium (masses of vegetative filaments, or hyphae) and fruiting structures produced by various fungi.

Fungi of the genera Aspergillus, Penicillium, Fusarium, and Rhizopus etc, form mould and are associated with food spoilage and plant diseases.

Moulds in numerous feedstuffs, including roughages, silages, and concentrates, infect dairy cattle causing mycosis, especially during stressful seasons when their immune systems are reduced. Harmful compounds produced by moulds referred as mycotoxins, which have an adverse effect on animals who consume mycotoxin-contaminated feed and resulting in disease called mycotoxicosis.[2]

Mycotoxins are produced by a variety of fungi in feed and feedstuffs. Mycotoxins are introduced into food systems by pre-harvest or post-harvest contamination of food,[3] i.e, during storage, transport, processing or feeding. Plant stress induced by climatic extremes, insect damage, and poor storage techniques, and incorrect feeding conditions are linked to mould development and mycotoxin generation. Temperature, water, and insect activity are the key elements that influence mycotoxin development in feed stuffs post- harvest.[4]

Environmental or extrinsic elements, such as relative humidity, temperature, and oxygen/CO2, play a vital role in mould development and subsequent mycotoxin formation. Intrinsic parameters such as ingredient composition, pH, grain moisture, and water activity, on the other hand, have a significant effect in mould development and mycotoxin formation,[5&6] maintaining proper preservative conditions are critical in ensuring quality forages.[7&8]

Mycotoxins are toxic chemical substances with low molecular weight (~700 Da) formed as secondary metabolites by a many fungal species produced mainly by species of Aspergillus, Penicillium or Fusarium genus.[9&10] These fungi readily colonize in crops and contaminate them with toxins in the field or post-harvest. The Food and Agriculture Organization (FAO) has estimated that world-wide 25% of crops are affected annually with mycotoxins.[11]

Because of the toxicological impact of mycotoxins on human and animal health, the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) have declared mycotoxins a high priority, leading to the establishment of legislative limits for mycotoxins in about 100 countries, with regional harmonisation for the European Union (EU).[12]

Surveys state that, aflatoxins (AFB1, AFB2, AFG1, AFG2, AFM1 and AFM2), ochratoxin A (OTA), fumonisin A (FB1), zearalenone (F-2), vomitoxin/deoxynivalenone (DON), diacetoxyscirpenol (DAS), nivalenone (NIV), trichothecenes, HT-2 and apicidin etc are the

principal contaminating mycotoxins in feed.[13&14] Mycotoxin surveys results highlighted two important issues of great concern for feed safety are mycotoxin co-occurrence, and modified emerging mycotoxin,[15][16] defined the diseases (mycotoxicoses) caused by mycotoxins are quite varied and involve a wide range of susceptible animal species including humans. Most of these diseases occur after consumption of mycotoxin contaminated grain or products made from such grains but other routes of exposure also exist. The diagnosis of mycotoxicosis might prove to be difficult because of the similarity in signs of disease to those caused by other agents. Therefore, diagnosis of a mycotoxicoses is dependent upon adequate testing for mycotoxins involving sampling, sample preparation and analysis.

Many by-products have a high potential use as animal feed, because conventional feedstuffs are generally expensive, using agro-industrial by-products may be cost-effective. However, well known and widely used traditional by-products such as oil meals, bran, middlings, brewer's grains, distiller's grains, beet pulp and molasses, have been used historically as animal feed. Brewer's Spent Grain (BSG), a key leftover generated by the brewing industries, has good qualities for both animal feeding and thermal usage.[17]

Majority of spent brewer's grains are kept in a bunker silo or in plastic bags. The feed out time of spent brewer's grains is limited. Spoilage of leftover brewer's grains can occur within five to seven days of the bag or silo being opened, leading in increased mould development, reduced moisture content and poor palatability.[18]

The most commonly used fungal species in malting and brewing by-products are different Fusarium species, other fungal species invading cereals and making problems in malting and brewing industries include Alternaria spp., Epicoccum spp., Penicillium spp., Aspergillus spp.[19] The toxigenic organisms from the genera Fusarium, Aspergillus, Penicillium, Cladosporium, Geotrichum, and Alternaria were found in malted barley and brewer's spent grains.

Adult ruminants show a high ability to biodegrade mycotoxins, because of bacteria and protozoa settling in the rumen neutralize mycotoxins to fewer toxic compounds in the so called pre systemic biodegradation. Calves lack these capacities due to a non-functioning rumen; they are more susceptible to mycotoxins. However, it is incorrect to assume that rumen flora has limitless detoxifying capacities.

According to studies, a high dosage and/or a large number of different mycotoxins limits rumen flora population, resulting in more non-degradable and still- more toxic compounds being produced, which are then absorbed in the duodenum and cause internal organ damage by passing through further intestinal tract segments.

Feed may be contaminated by several fungal species and mycotoxins at the same time, and the toxicological effects can be different according to the type of mycotoxin interaction: less than additive, additive, synergistic, enhanced, or antagonistic.[20&21] For these reasons, mycotoxin concentrations in feed should be continuously monitored to support risk assessment and constant research, especially in agricultural and veterinary fields. The primary objective of the present research is to call attention to problems of mycotoxicosis, which appear in cattle, and very often are undiagnosed because of the lack of characteristic clinical signs.

During the disease investigation process and discussions with veterinarians working near brewing industries, informed that farmers were feeding brewery waste as concentrate feed to cattle. Veterinarians told that, long term feeding of this brewery waste to cattle resulted in sufferings from sub-acute rumen acidosis (SARA), ruminal atony, ruminal impaction, anorexia, weakness, diarrhoea, often uncontrolled coughing, dyspnea, poor milk quality, decreased milk production, gradual weight loss and infertility. On observations, feeding material was brewer's spent grain, a beer brewing industrial by-product and had mould growth on it. Thus, the conditions of ailing cattle were tentatively diagnosed to be resulting from ingestion of the mould infested brewer's spent grain (BSG). Based on these observations, the present study was taken to study the potential toxicity of fungal isolates from brewer's spent grain.

Keeping with this back ground the toxic potential of fungal contaminated Brewer's spent grain was evaluated in rats with the following objectives in the present study:

i. Culturing, isolation and identification of various fungi present in the fungal infected brewer's spent grain.
ii. Experimental induction of mycotoxicosis in rats with fungal isolates from brewer's spent grain.
iii. Screening for the presence of various mycotoxins.
iv. Assess the changes with serum biochemical parameters and hematological parameters and correlate the findings with gross and histopathological observations.

2. MATERIALS AND METHOD

The present study was undertaken to evaluate the toxicity of fungal contaminated brewer's spent grain in rats.

2.1. Collection of fungal contaminated Brewer's spent grain

Random samples of fungal contaminated brewer's spent grain were collected from the farmers at Kechhenahalli and B Durga villages of Davanagere district, Kabbenur of Dharwad district and Kumbharahalla of Bagalkot district, who were feeding the brewer's spent grain, as concentrate feed to cattle. They informed that on long-term use, the animals had developed sub-acute rumen acidosis (SARA), ruminal atony, ruminal impaction, anorexia, weakness, diarrhoea, often uncontrolled coughing, dyspnea, poor milk quality, decreased milk production, gradual weight loss and infertility. Fungal growths were discovered on brewer's waste grain during observations. Random samples of fungal infected brewer's spent grain were further processed for fungi isolation because it was suspected that fungal contamination of brewer's spent grain was the cause of animal diseases.

2.1.1.General laboratory procedures

The present investigation of isolation of the fungi from the fungal contaminated brewer's spent grain was done in the Laboratory of the Department of Veterinary Pharmacology and Toxicology, Veterinary College, Shivamogga.

a. Glassware cleaning

For all laboratory experimental studies, Corning and Borosil glass wares were used. The glass wares were boiled for half an hour and then washed with detergent powder followed by cleaning in tap water and then dried in an oven at 60^0 C overnight.

b. Sterilization

All the glassware used in the study were sterilized in an autoclave at 121^0 C,15 lb pressure for 15 min and then dried in hot air oven at 120 ^{0}C.

2.1.2. Toxicity study on laboratory animal (Rats)

2.1.2.1. Subacute toxicity in rats

Apparently healthy young Wistar albino rats procured from CPCSEA approved vender i.e, Adita Biosys Private Limited, Tumakur (Reg No: 1868/PO/RcBt/S/16/CPCSEA. Date of registration: 23.02.2016 Validity up to 08.04.2026, were used in the present study. They were

of the age group of 6-8 weeks having body weight about 160 ± 20 g. The animals were acclimatized to the experimental laboratory conditions for a week. They were maintained under hygienic laboratory conditions, providing standard laboratory animal feed (Nutrimix Std -1020, Manufactured by Nutrivet Life Sciences, Pune) and water ad libitum. The rats were grouped (n=5) and housed in polypropylene rat cages during the experiment. The approval of the Institutional Animal Ethics Committee was obtained prior to the start of the experiment. (**No.VCS/IAEC/SA-64/2020-21: Dated - 31/08/2021**).

2.1.2.2. Design of the experiment

Repeated dose 28-days oral toxicity study in rodents was undertaken as per the OECD-407 guidelines in 40 rats dividing into four groups of 10 animals each (5 males + 5 females). Grouping of rats was done as follows Control group, Group I (Low dose group), Group II (Medium dose group) and Group III (High dose group). Control group received sterile PD broth alone, whereas Group I, Group II and Group III received treatment with the mixed fungal culture filtrates at low dose, medium dose and high dose as mentioned in the below table respectively, over a period of 28 days.

Table no. 1: Experimantal groups.

Groups		**Dose (ml/100g oral)**	**No. of rats**
Control group	PD broth	2	10
Group I (Low dose group)	Mixed fungal culture filtratesof Rhizopus oryzae (25%) + Aspergillus flavus (50%) + Aspergillus nomius (25%)	0.5 (Low dose)	10
Group II (Medium dose group)	Mixed fungal culture filtratesof Rhizopus oryzae (25%) + Aspergillus flavus (50%) + Aspergillus nomius (25%)	1 (Medium dose)	10
Group III (High dose group)	Mixed fungal culture filtratesof Rhizopus oryzae (25%) + Aspergillus flavus (50%) + Aspergillus nomius (25%)	2 (High dose)	10

2.1.2.3. Procedure

The animals of each group were gavage with the mixed fungal culture filtrates at recommended doses designed to the respective group once daily, based on the hypothesis that, in field conditions, the animals might have consumed the brewer's spent grain contaminated with *Rhizopus oryzae*, *Aspergillus flavus* and *Aspergillus nomius* in equal or

different proportions in order to know the effects of mycotoxins in rats. The animals were weighed individually at the beginning of the study and at weekly interval till day 28. The feed leftover was measured daily.

2.1.2.4. Clinical observation

General clinical observations were made thrice a day. The health condition of the animals were recorded. Daily all the animals were observed for morbidity and mortality. Necropsy was done and organs were collected for histopathological studies at the end of the study.

2.1.2.5. Clinical biochemistry

Present study was done to investigate the toxic effect of the mixed fungal culture filtrates on different organs of the body especially on the liver, kidney, intestine, heart lung and spleen functions. The blood samples were drawn from rats by retro-orbital plexus puncture method using micro haematocrit capillary tubes. Serum biochemical parameters were estimated on day 0, 14, and 28 in sub-acute oral toxicitystudy. Using (HY-SAC Vet Version : A/6 Semi-auto Chemistry Analyzer, Hycel Handelsges Austria) and commercially available standard diagnostic reagent kits (Alpha Technologies, Chennai). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN) and creatinine (Cr) concentrations are determined.

2.1.2.6. Clinical haematology

The blood samples were drawn from rats by retro-orbital plexus puncture method using micro haematocrit capillary tubes. Haematological parameters were estimated on day 0, 14, and 28 in sub-acute oral toxicity study, using Vet exigo Auto Haematology Analyser, and Vet exigo diagnostic reagent kits. TLC, TEC, Hb%, PCV and blood clotting time were estimated.

2.1.2.7. Pathological study

At the end of the study period, all the surviving animals were sacrificed and gross changes in the organs were recorded. Representative tissue samples of liver, kidney, spleen, heart, lung and intestines were collected in 10 % neutral buffered formalin (NBF) for histopathological study.

2.1.2.8. Tissue processing

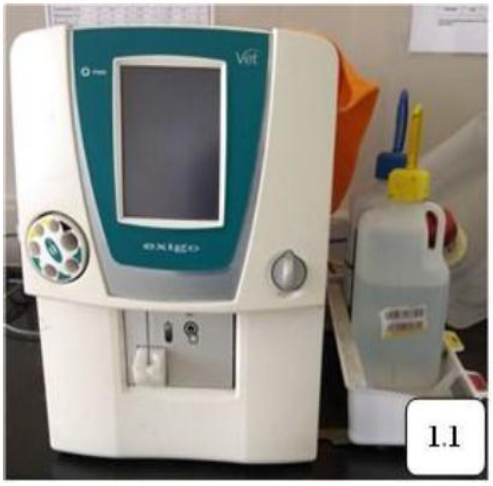
For histopathological examination, the tissues were processed by paraffin embedding technique. The sections (4µ-5µ) were cut using microtome and disposable blades. The

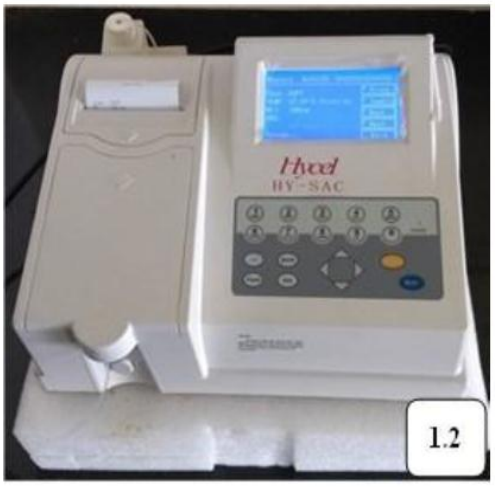
sections were stained using haematoxylin and eosin dye.

2.1.2.9. Statistical analysis

The values were expressed as mean SEM. The data was analyzed by one- way ANOVA with using GraphPad Prism Trial version 7 for Windows 10, GraphPad Software, San Diego California USA and P value, P< 0.05 was considered as level of significance.

Plate 1: (Instruments)





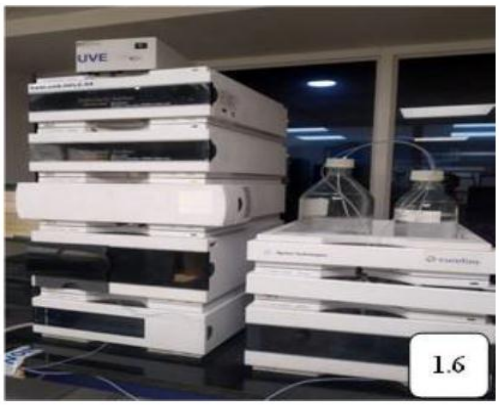
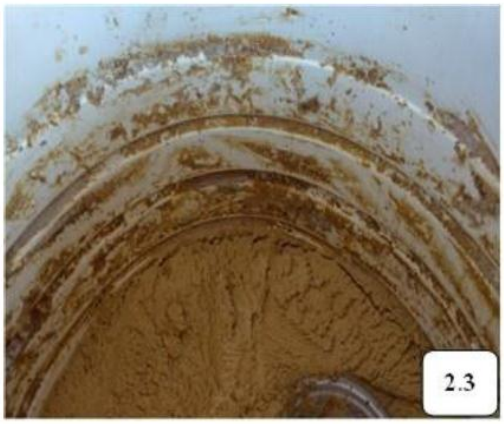


Plate 2: (Brewer's spent grain sample)



3. RESULT

3.1. Identification of the fungi

Molecular identification of the fungi species in the culture was done using 18srRNA method (BioEdge Solutions, Bengaluru). The identified species were: (Fig. 1, 2, and 3) *Rhizopus oryzae*, *Aspergillus flavus* and *Aspergillus nomius*.

>BF1_ITS

AGTCGAACCTGCGGAAGGATCATTAATTATGTTAAAGCGCCTTACCTTAGGGTTTCC
TCTGGGGTAAGTGATTGCTTCTACACTGTGAAAATTTGGCTGAGAGACTCAGACTGG
TCATGGGTAGACCTATCTGGGGTTTGATCGATGCCACTCCTGGTTTCAGGAGCACCC
TTCATAATAAACCTAGAAATTCAGTATTATAAAGTTTAATAAAAAACAACTTTTAAC
AATGGAAGTCTCTCTTGGTTCTCGCATCGATGAAGAACGTAGCAAAGTGCGATAACT
AGTGTGAATTGCATATTCAGTGAATCATCGAGTCTTTGAACGCAGCTTGCACTCTAT
GGTTTTTCTATAGAGTACGCCTGCTTCAGTATCATCACAAACCCACACATAACATTT
GTTTATGTGGTGATGGGTCGCATCGCTGTTTTATTACAGTGAGCACCTAAAATGTGT
GTGATTTTCTGTCTGGCTTGCTAGGCAGGAATATTACGCTGGTCTCAGGATCGGTTC
GCCCAGGAAGTAAAGTACAAGAGTATAATCCAGTAACTTTCAAACTATGATCTTCA
GGTGGGATTACCCGCTGAACTTAAGCATATCAATAGCGGAGGAAAGTC

Hit: *Rhizopus oryzae* strain 783018 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 28S ribosomal RNA gene, region
Percent identity: 97.27%
E value: 0.0
Query coverage: 98%
Accession no.: GU594768.1

Phylogenetic tree:

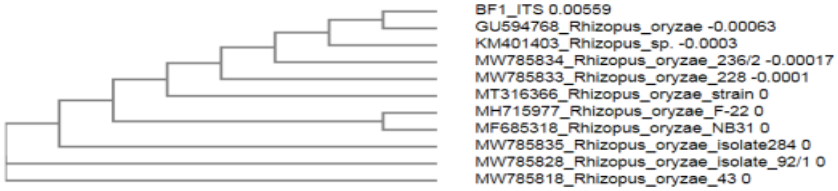


Figure 1: Molecular identification report, genomic Sequence and Phylogenic tree of fungus Rhizopus oryzae.

Samples: BF2,

Fungal sequence:

>BF2_query

AAAGGGGGCGGCGAGCGCGGCGGGCGACAACAAGAGCGACCCGTTGTTTAACTTGTACCTT
AGTTGCTTCGGCGGGCCCGCCATTCATGGCCGCCGGGGGCTCTCAGCCCCGGGCCCGCGCCC
GCCGGAGACACCACGAACCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGTAGGGTTC
CTAGCGAGCCCAACCTCCCACCCGTGTTTACTGTACCTTAGTTGCTTCGGCGGGCCCGCCAT
TCATGGCCGCCGGGGGCTCTCAGCCCCGGGCCCGCGCCCGCCGGAGACACCACGAACTCTG
TCTGATCTAGTGAAGTCTGAGTTGATTGTATCGCAATCAGTTAAAACTTTCAACAATGGATC
TCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAACTAGTGTGAATTGCAGAA
TTCCGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCC
TGTCCGAGCGTCATTGCTGCCCATCAAGCACGGCTTGTGTGTTGGGTCGTCGTCCCCTCTGC
GGGGGGGACGGGCCCCAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGC
TTTGTCACCCGCTCTGTAGGCCCGGCCGGCGCTTGCCGAACGCAAAT

Hit: *Aspergillus flavus* voucher AI_5_ITS small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1

Percent identity: 99.98%

E value: 0.0

Query coverage: 94%

Accession no.: MT093450.1

Phylogenetic tree:

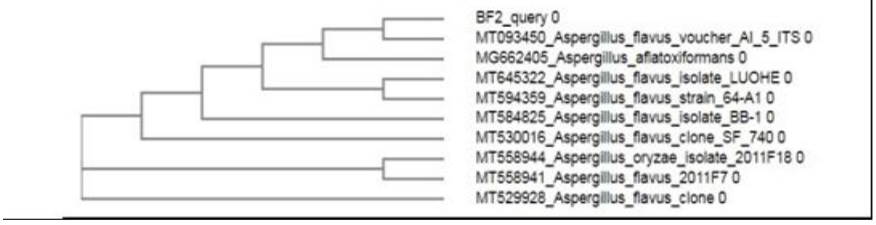


Figure 2: Molecular identification report, genomic Sequence and Phylogenic tree of fungus *Aspergillus flavus.*

>BF3_query

ACCGGGGGATGGCGGGGGGGGGCGACCGATACACCCGTGATAGTGTACCTTAGTTGCTTCG
GCGGGCCCGCCATTCATGGCCGCCGGGGGCTCTCAGCCCCGGGCCCGCGCCCGCCGGAGAC
ACCACGAACTCTGTCTGATCTAGTGAAGTCTGAGTTGATTGTATCGCAATCAGTTAAAACTT
TCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAACTAAT
GTGAATTGCAGAATTCCGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTC
CGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCATCAAGCACGGCTTGTGTGTTGGGTCG
TCGTCCCCTCTCCGGGGGGGACGGGCCCCAAAGGCAGCGGCGGCACCGCGTCCGATCCTCG
AGCGTATGGGGGCTTTGTCACCCGCTCTGTAGGCCCGGCCGGCGCTTGCCGAACGCAAATC
AATCTTTTCCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATA
AGCGGGAGAGAACTGCGCGCTGTGTGATAGGAGGTGGGCGGTGGTTGGACGCAGAGAGGG
AGGGTAGAGAGGAGGGGAAGGAGGGGTGGGGGGGGGTGTGTGTGGGGGGGGGGGGGGGG
GGGGGGAGGAGGGGGAGGGGGGGGTGGGAGAGGGGAGTGGGGAGGGGGGGAGGGTGGGG
AGGGAAGGGAGGAGCTGAGGGGAGGGAGGGGAGAGGAGGAGGGGGGGAGGGGAGGAGA
GGTGAGGGGAGGTGAGGGGAGGGGGATGGAGGATGAGTGGGAGGAGGAGGTGAGTGGGG
GGAGGAGGAGGTGGGGGGGTGGTGGGTAGGGGCG

Hit: *Aspergillus nomius* isolate BAB-6552 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence
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E value: 0.0
Query coverage: 60%
Accession no.: MF319897.1
Phylogenetic tree:

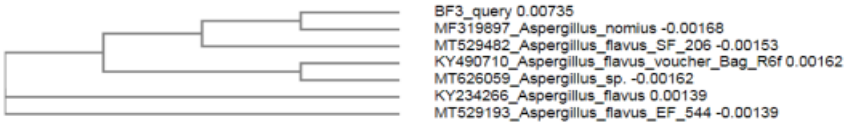
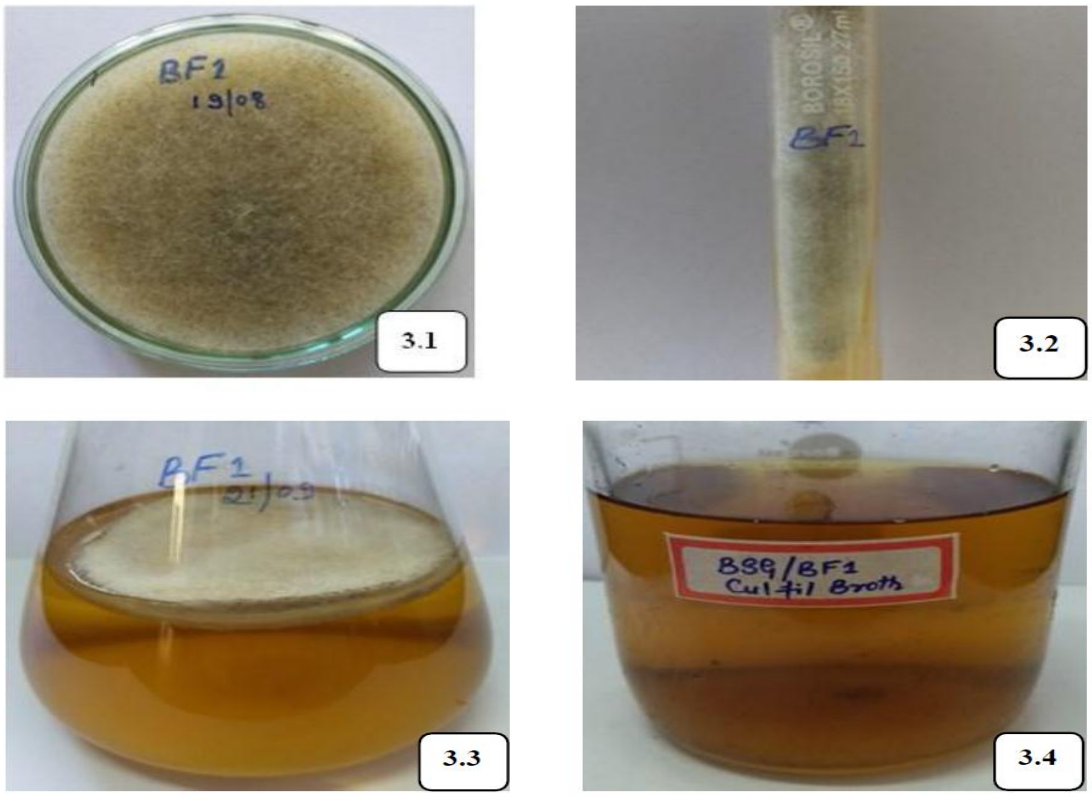
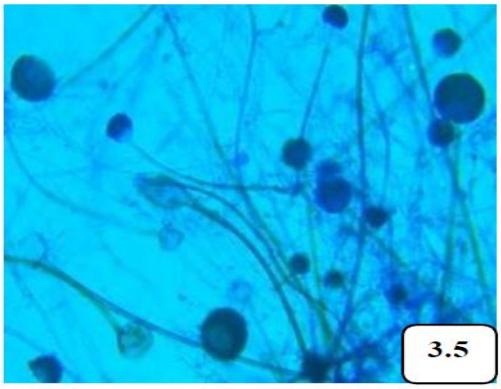


Figure 3: Molecular identification report, genomic sequence and phylogenic tree of fungus *Aspergillus nomius.*

Plate 3: (*Rhizopus oryzae*)





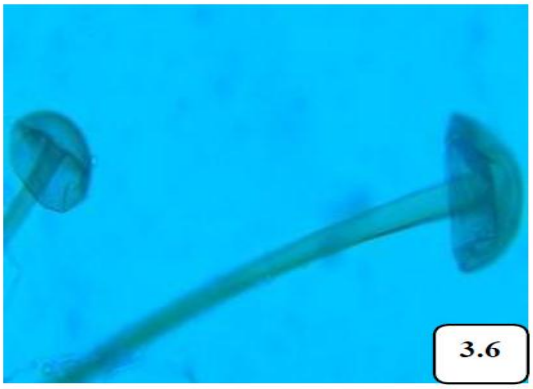
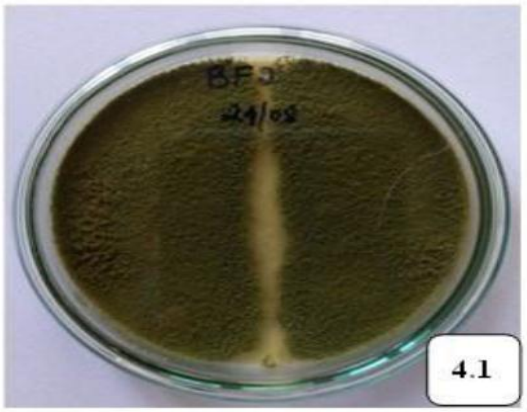
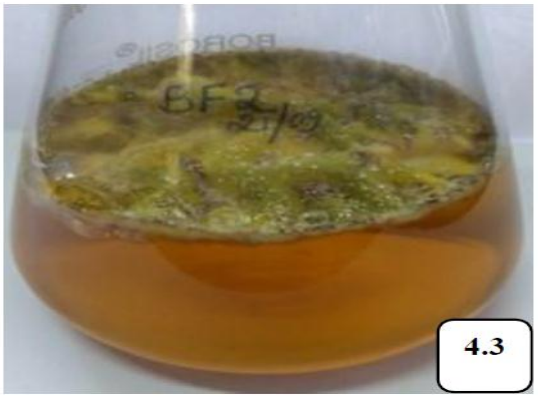
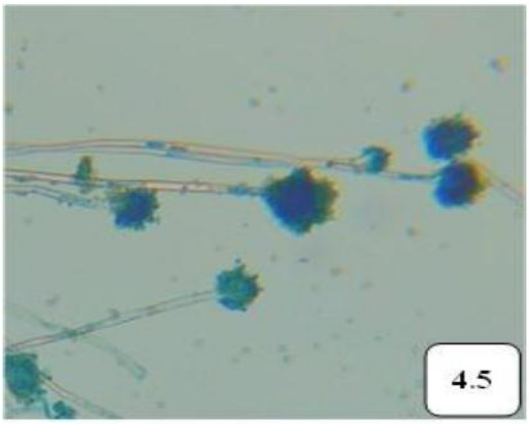


Plate 4: (*Aspergillus flavus*)







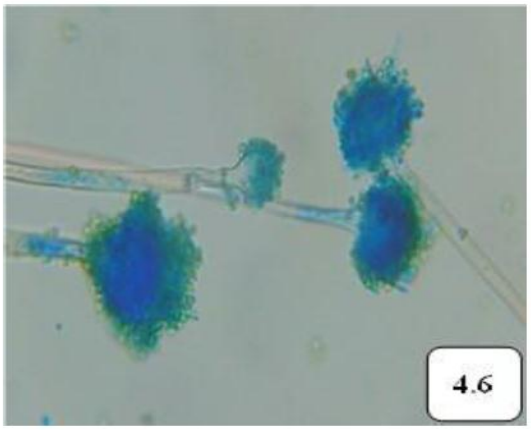
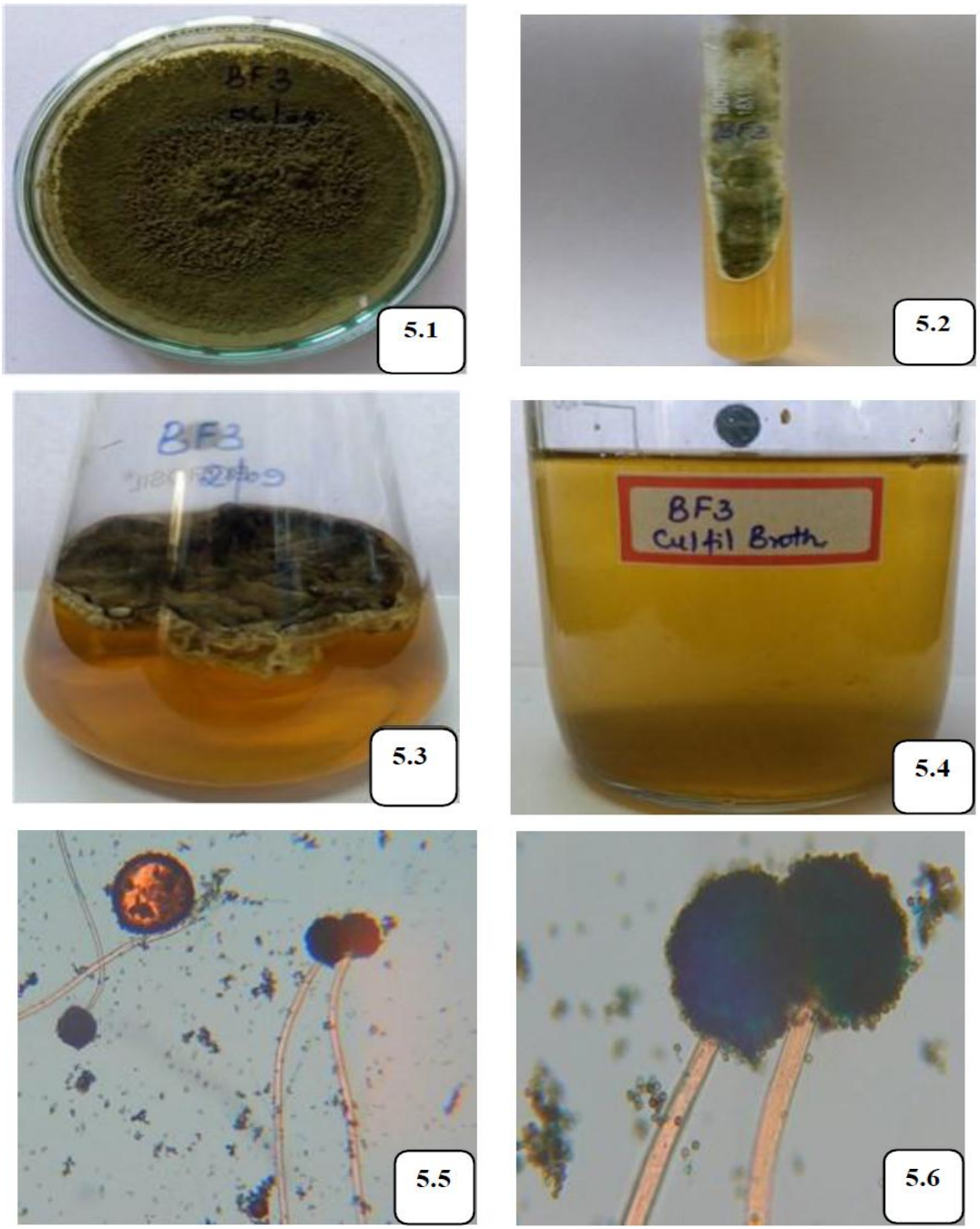


Plate 5: (*Aspergillus nomius*)



3.2. Screening for the presence of mycotoxins

The fungal contaminated brewer's spent grain sample and the fungal culture filtrate PD broths were analyzed for the presence of mycotoxins like aflatoxins (B1, B2, G1 and G2) ochratoxins, and other mycotoxins, by HPLC-FLD method at Eurofins Analytical Services India Private Limited, Whitefield, Bengaluru. The results are given in Table no.2 chromatogram reports in (Fig. 4, 5, 6, and 7)

The Rhizopus oryzae inoculated PD broth culture filtrate material was screened for the presence of various mycotoxins and did not show any mycotoxins. (Fig. 4).

The Aspergillus flavus inoculated PD broth culture filtrate material showed the presence of AFB1, AFB2 AFG1 and AFG2. (Fig. 5).

The Aspergillus nomius inoculated PD broth culture filtrate material showed the presence of AFB1, AFB2, AFG1 and AFG2. (Fig. 6).

Fungal contaminted brewer's spent grain sample showed the presence of AFB1, AFB2, AFG1 and AFG2. (Fig. 7).

HPLC Report

```
Data file       : C:\Chem32\1\Data\2021\December\07Aflatoxins.S\        ->
Injection Date  : Wed, 8. Dec. 2021             Seq Line   :          34
Sample Name     : 258-2021-1200001776           Location   :          28
Acq Operator    : SYSTEM                        Inj. No.   :           1
                                                Inj. Vol.  :      100 µl

Acq. Method     : C:\Chem32\1\Data\2021\December\07Aflatoxins.S\      ->
Analysis Method : C:\Chem32\1\Data\2021\December\07Aflatoxins.S\      ->
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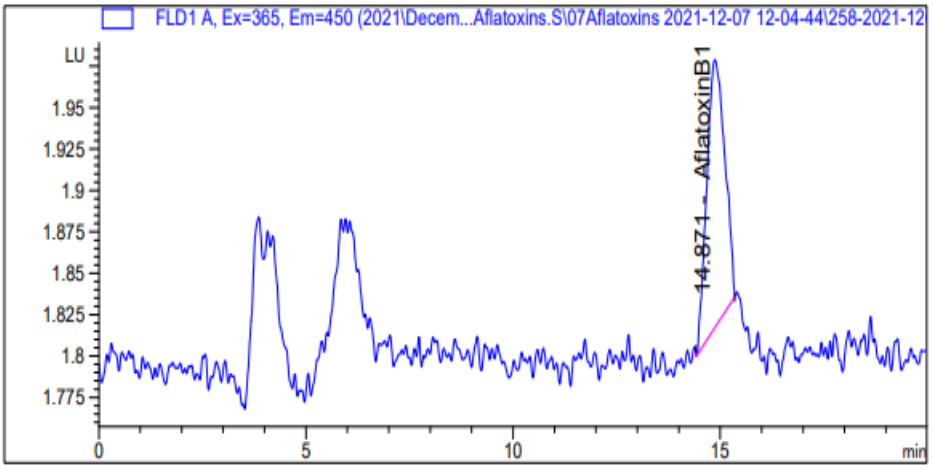


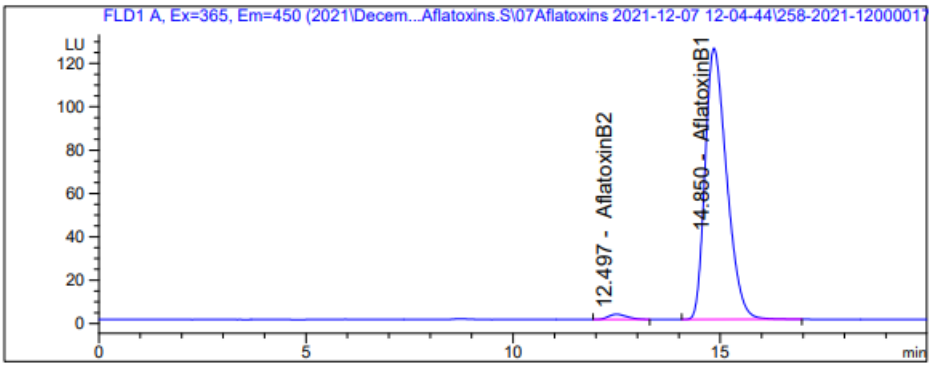
Signal 1: FLD1 A, Ex=365, Em=450

Figure 4: Chromatogram of aflatoxins quantification in *Rhizopus oryzae* inoculated PD broth culture filtrate.

HPLC Report

Data file : C:\Chem32\1\Data\2021\December\07Aflatoxins.S\ ->
Injection Date : Wed, 8. Dec. 2021 Seq Line : 35
Sample Name : 258-2021-1200001777 Location : 29
Acq Operator : SYSTEM Inj. No. : 1
Inj. Vol. : 100 µl

Acq. Method : C:\Chem32\1\Data\2021\December\07Aflatoxins.S\ ->
Analysis Method : C:\Chem32\1\Data\2021\December\07Aflatoxins.S\ ->



Signal 1: FLD1 A, Ex=365, Em=450

Figure 5: Chromatogram of aflatoxins quantification in Aaspergillus flavus inoculated PD broth culture filtrate.

HPLC Report

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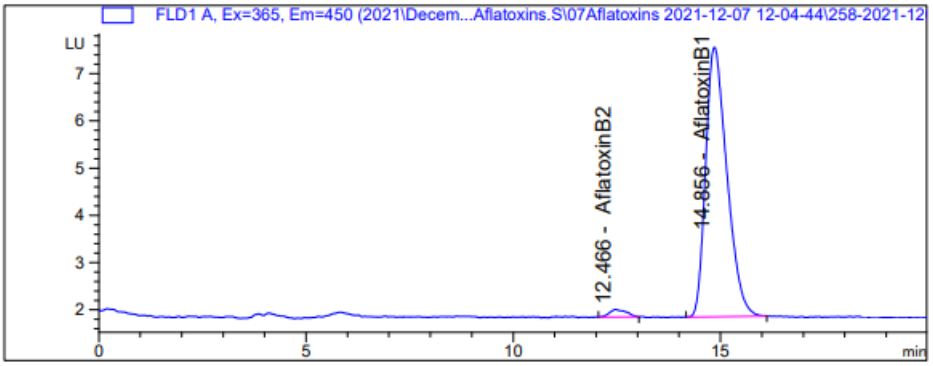
Injection Date : Wed, 8. Dec. 2021 Seq Line : 36
Sample Name : 258-2021-1200001778 Location : 30
Acq Operator : SYSTEM Inj. No. : 1
Inj. Vol. : 100 µl

Acq. Method : C:\Chem32\1\Data\2021\December\07Aflatoxins.S\ ->
Analysis Method : C:\Chem32\1\Data\2021\December\07Aflatoxins.S\ ->



Signal 1: FLD1 A, Ex=365, Em=450

Figure 6: Chromatogram of aflatoxins quantification in *Aspergillus nomius* inoculated PD broth cultue filtrate.

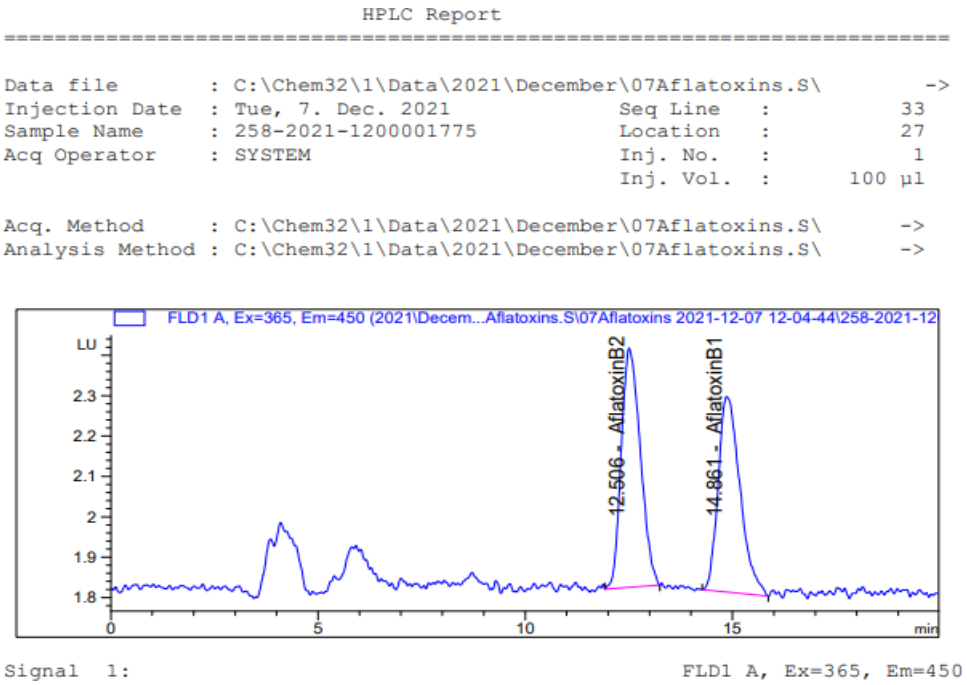


Figure 7: Chromatogram of aflatoxins quantification in *Brewer's spent grain* feed sample.

Table no. 2: Report of multimycotoxin analysis (µg/kg or ppb).

Mycotoxins	Sample Name			
	R.oryzae	***A.flavus***	***A.nomius***	**Brewer's spent grain**
Aflatoxin B1	No Peak	230.49	10.52	1.06
Aflatoxin B2	No Peak	1.66	< 0.5	< 0.5
Aflatoxin G1	No Peak	< 0.5	< 0.5	< 0.5
Aflatoxin G2	No Peak	< 0.5	< 0.5	< 0.5
Ochratoxin	No Peak	No Peak	No Peak	No Peak
T-2 Toxi	No Peak	No Peak	No Peak	No Peak
H-2 Toxin	No Peak	No Peak	No Peak	No Peak
DAS	No Peak	No Peak	No Peak	No Peak
DON	No Peak	No Peak	No Peak	No Peak
Fuminosins	No Peak	No Peak	No Peak	No Peak
Zearalenone	No Peak	No Peak	No Peak	No Peak
Citrinin	No Peak	No Peak	No Peak	No Peak
Beauvericin	No Peak	No Peak	No Peak	No Peak
Apicidin	No Peak	No Peak	No Peak	No Peak

3.3. Repeated dose 28-day oral toxicity study

3.3.1. Clinical Signs

General clinical observations were made thrice a day. The health condition of animals were recorded. All the animals were observed daily for morbidity and mortality. Necropsy of all the animals was done at the end of study and organs were collected for histopathological

studies. All the treated group rats were observed for 28 days. All animals showed similar type of clinical signs. They showed gradual reduction in feed intake, water intake, diarrhoea and loss of body weight. Animals were weak and depressed. Diarrhoea was more prominent in II and III groups.

Table no. 3: Effect of mixed fungal culture filtrates at different doses on bodyweight (g) in rats during repeated dose 28 day oral toxicity study.

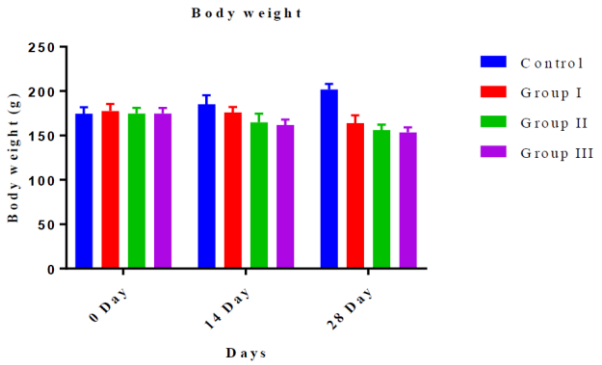
Groups	Body weight (g)		
	0 Day	14 Day	28 Day
Control	174.5 ± 2.30	185.2 ± 3.20	201.7 ± 2.03
Group I (Low dose)	177.6 ± 2.51	175.9 ± 1.96*	163.8 ± 2.83***
Group II (Medium dose)	174.6 ± 2.07	164.7 ± 3.07***	156.2 ± 1.92***
Group III (High dose)	174 ± 1.94	161.8 ± 1.98***	153.4 ± 1.82***

Values are mean ± SEM, n = 10, * P < 0.05, ** P < 0.01, *** P < 0.001,

Body weight of all the treated groups on day 0, was not statistically significant (P > 0.05) compared to control group.

On day 14, body weight (g) was significantly decreased (P < 0.05) in group I (low dose), and (P < 0.001) in group II (medium dose) and group III (high dose) compared to control group.

On day 28, body weight (g) was significantly decreased (P < 0.001) in group I (low dose), group II (medium dose) and group III (high dose) compared to control group.



a. Biochemical parameters

Table no. 4: Serum Alanine Aminotransferase (ALT).

Groups	ALT (µ/L)		
	0 Day	14 Day	28 Day
Control	36.17 ± 1.18	37.19 ± 1.08	39.23 ± 0.85
Group I (Low dose)	35.79 ± 1.14	39.32 ± 1.51	45.09 ± 1.88**

Group II (Medium dose)	36.45 ± 1.28	42.56 ± 1.48*	47.22 ± 0.86***
Group III (High dose)	35.40 ± 1.14	44.35 ± 1.01***	48.74 ± 1.52***

Values are mean ± SEM, n = 10, * P < 0.05, ** P < 0.01, *** P < 0.001,

The serum ALT concentrations of all the treated groups on day 0 was not statistically significant (P > 0.05) compared to control group.

On day 14, mean serum ALT concentration was significantly increased (P < 0.05) in group II (medium dose) and (P < 0.001) in group III (high dose) compared to control group.

On day 28, mean serum ALT was significantly increased (P < 0.01) in group I (low dose), and (P < 0.001) in group II (medium dose) and group III (high dose) compared to control group.

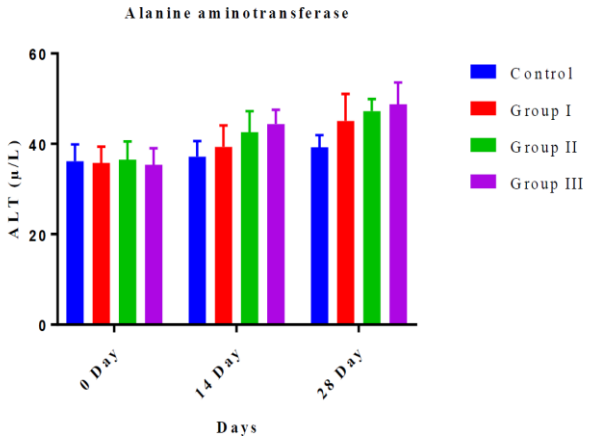


Table no. 5: Serum aspartate aminotransferase (AST).

Groups	AST (µ/L)		
	0 Day	14 Day	28 Day
Control	105.75 ± 2.19	105.68 ± 1.60	109.55 ± 2.19
Group I (Low dose)	104.87 ± 2.52	111.60 ± 1.69***	126.83 ± 2.17***
Group II (Medium dose)	103.56 ± 1.72	123.72 ± 1.81***	147.66 ± 1.66***
Group III (High dose)	107.42 ± 2.32	136.81 ± 1.8***	158.29 ± 2.14***

Values are mean ± SEM, n = 10, * P < 0.05, ** P < 0.01, *** P < 0.001,

The serum AST concentrations of all the treated groups on day 0 was not statistically significant (P > 0.05) compared to control group.

On day 14, mean serum AST concentration was significantly increased (P < 0.001) in group I (low dose), group II (medium dose) and group III (high dose) compared to control group.

On day 28, mean serum AST concentration was significantly increased (P < 0.001) in group I (low dose), group II (medium dose) and group III (high dose) compared to control group.

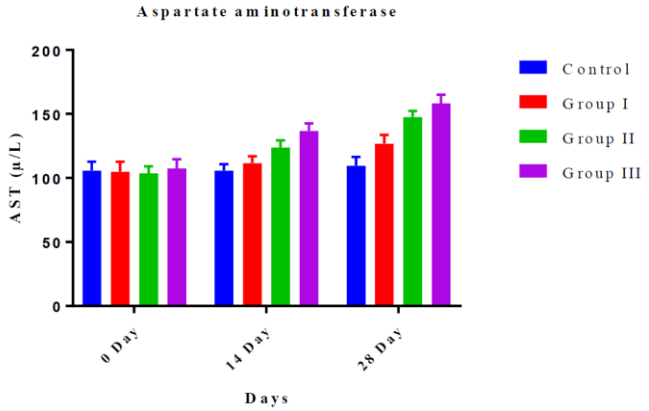


Table no. 6: Blood urea nitrogen.

Groups	BUN (mg/dl)		
	0 Day	14 Day	28 Day
Control	23.15 ± 0.95	23.46 ± 0.61	23.83 ± 0.49
Group I (Low dose)	22.96 ± 0.61	24.13 ± 1.28	28.36 ± 1.08**
Group II (Medium dose)	23.22 ± 0.81	27.64 ± 0.92**	29.17 ± 0.72***
Group III (High dose)	22.88 ± 0.56	27.41 ± 0.92**	31.16 ± 0.92***

Values are mean ± SEM, n = 10, * P < 0.05, ** P < 0.01, *** P < 0.001,

The blood urea nitrogen concentrations of all the treated groups on day 0 was not statistically significant (P > 0.05) compared to control group.

On day 14, blood urea nitrogen concentration was significantly increased (P < 0.01) in group II (medium dose) and group III (high dose) compared to control group.

On day 28, blood urea nitrogen concentration was significantly increased (P < 0.01) in group I (low dose) and (P < 0.001) in group II (medium dose) and group III (high dose) compared to control group.

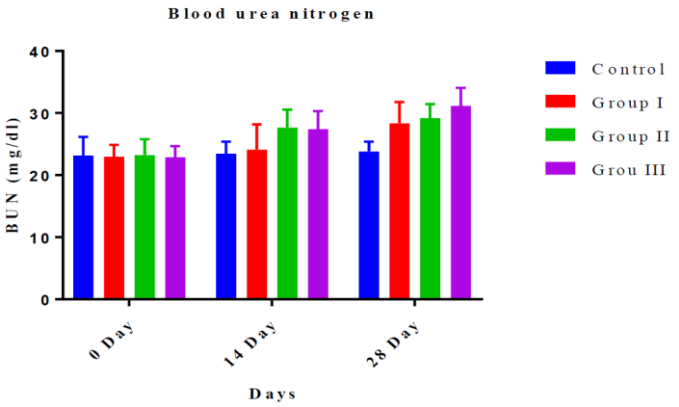


Table no. 7: Serum creatinine.

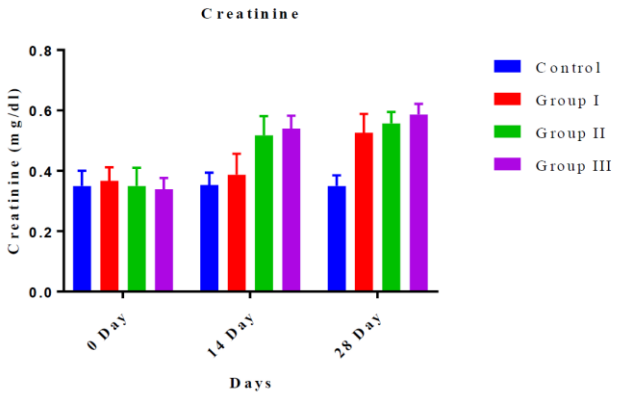
Groups	Creatinine (mg/dl)		
	0 Day	14 Day	28 Day
Control	0.35 ± 0.02	0.35 ± 0.01	0.35 ± 0.01
Group I (Low dose)	0.37 ± 0.01	0.39 ± 0.02	0.53 ± 0.02***
Group II (Medium dose)	0.35 ± 0.02	0.52 ± 0.02***	0.56 ± 0.01***
Group III (High dose)	0.34 ± 0.01	0.54 ± 0.01***	0.59 ± 0.01***

Values are mean ± SEM, n = 10, * P < 0.05, ** P < 0.01, *** P < 0.001,

The serum creatinine concentrations of all the treated groups on day 0 was not statistically significant (P > 0.05) compared to control group.

On day 14, serum creatinine concentrations was significantly increased (P < 0.001) in group II (medium dose) and group III (high dose) compared to control group.

On day 28, serum creatinine concentrations was significantly increased (P < 0.001) in group I (low dose), group II (medium dose) and group III (high dose) compared to control group.



b. Haematological parameters

Table no. 8: Total leucocyte count (TLC).

Groups	TLC (10^3 cells/mm^3)		
	0 Day	14 Day	28 Day
Control	8.67 ± 0.27	8.49 ± 0.16	8.45 ± 0.14
Group I (Low dose)	8.66 ± 0.17	7.75 ± 0.16	7.56 ± 0.20*
Group II (Medium dose)	8.67 ± 0.14	7.57 ± 0.24*	7.68 ± 0.17*
Group III (High dose)	8.77 ± 0.18	7.67 ± 0.27*	7.46 ± 0.28**

Values are mean ± SEM, n = 10, * P < 0.05, ** P < 0.01, *** P < 0.001

There was no statistical (P > 0.05) change in the Total Leukocyte count (TLC) of all the treated groups on day 0 compared to control group values.

On day 14, significantly decreased (P < 0.05) total leucocyte count (TLC) in group II (medium dose) and group III (high dose) compared to control group observed.

On day 28, significantly decreased (P < 0.05) total leucocyte count (TLC) in group I (low dose), group II (medium dose) and (P < 0.01) group III (high dose) compared to control group observed.

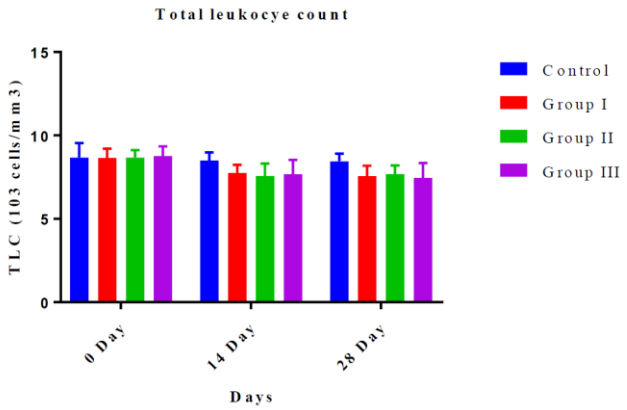


Table no. 9: Total erythrocyte count (TEC).

Groups	TEC (10^6 cells/mm^3)		
	0 Day	14 Day	28 Day
Control	8.25 ± 0.24	8.41 ± 0.16	8.55 ± 0.16
Group I (Low dose)	8.15 ± 0.17	7.8 ± 0.19	7.55 ± 0.20**
Group II (Medium dose)	8.27 ± 0.25	7.66 ± 0.16*	7.47 ± 0.14***
Group III (High dose)	8.18 ± 0.30	7.53 ± 0.18*	7.34 ± 0.11***

Values are mean ± SEM, n = 10, * P < 0.05, ** P < 0.01, *** P < 0.001,

There was no statistical (P > 0.05) change in the Total erythrocyte count (TEC) of all the

treated groups on day 0 compared to control group values.

On day 14, total erythrocyte count (TEC) was significantly decreased ($P < 0.05$) in group II (medium dose) and group III (high dose) compared to control group.

On day 28, total erythrocyte count (TEC) was significantly decreased ($P < 0.01$) in group I (low dose) and ($P < 0.001$) group II (medium dose) and group III (high dose) compared to control group.

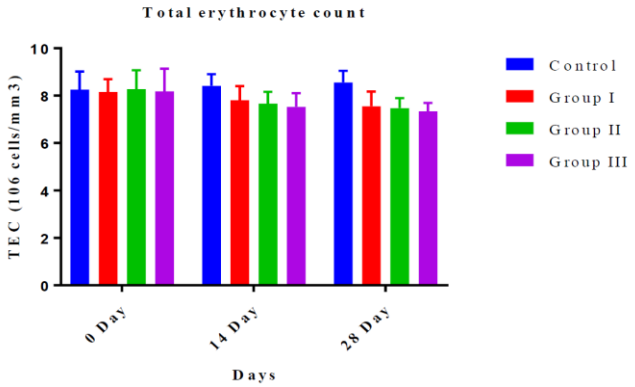


Table no. 10: Hemoglobin (Hb).

Groups	Hb (%)		
	0 Day	**14 Day**	**28 Day**
Control	14.27 ± 0.35	14.62 ± 0.34	15.08 ± 0.24
Group I (Low dose)	14.32 ± 0.37	14.13 ± 0.34	13.52 ± 0.51*
Group II (Medium dose)	14.3 ± 0.29	13.29 ± 0.42*	13.19 ± 0.38**
Group III (High dose)	14.33 ± 0.37	13.2 ± 0.34*	12.04 ± 0.28***

Values are mean ± SEM, n = 10, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

There was no statistical ($P > 0.05$) change in the hemoglobin concentration of all the treated groups on day 0 compared to control group values.

On day 14, hemoglobin concentration was significantly decreased ($P < 0.05$) in group II (medium dose) and group III (high dose) compared to control group.

On day 28, hemoglobin concentration was significantly decreased ($P < 0.05$) in group I (low dose), ($P < 0.01$) in group II (medium dose) and ($P < 0.001$) in group III (high dose) compared to control group.

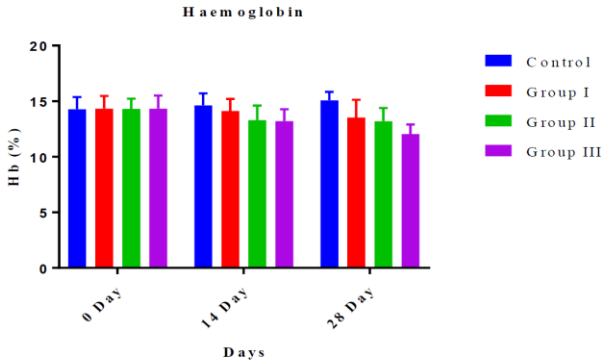


Table no. 11: Packed cell volume (PCV).

Groups	PCV (%)		
	0 Day	14 Day	28 Day
Control	43.8 ± 1.22	44.71 ± 1.75	45.55 ± 1.46
Group I (Low dose)	43.74 ± 1.08	41.29 ± 2.08	38.42 ± 1.67**
Group II (Medium dose)	43.6 ± 1.37	38.98 ± 1.63*	36.72 ± 1.42***
Group III (High dose)	43.45 ±1.56	37.37 ± 1.74**	34.46 ± 0.83***

Values are mean ± SEM, n = 10, * P < 0.05, ** P < 0.01, *** P < 0.001,

There was no statistical (P > 0.05) change in the packed cell volume (PCV) of all the treated groups on day 0 compared to control group values.

On day 14, packed cell volume (PCV) concentration was significantly decreased (P < 0.05) in group II (medium dose) and (P < 0.01) in group III (high dose) compared to control group.

On day 28, packed cell volume (PCV) concentration was significantly decreased (P < 0.01) in group I (low dose), (P < 0.001) in group II (medium dose) and group III (high dose) compared to control group.

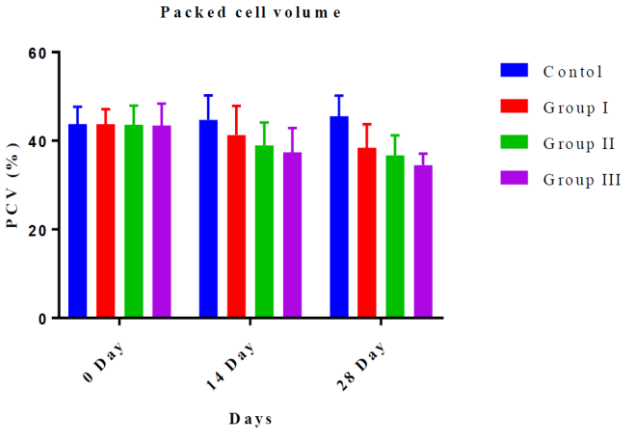


Table no. 12: Blood clotting time in rats.

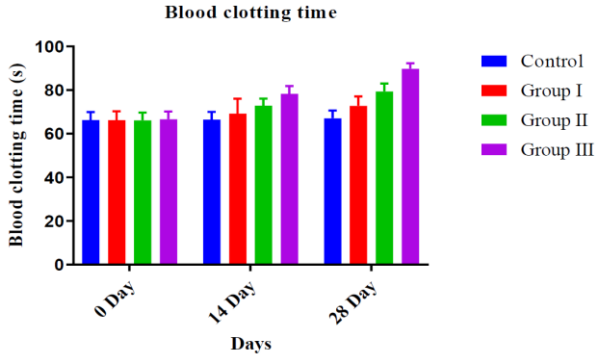
Groups	Blood clotting time (s)		
	0 Day	14 Day	28 Day
Control	66.31 ± 1.16	66.42 ± 1.14	67.01 ±1.13
Group I (Low dose)	66.23 ± 1.28	69.17 ± 2.17	72.82 ± 1.36**
Group II (Medium dose)	66.09 ± 1.16	72.88 ± 1.02**	79.39 ± 1.15***
Group III (High dose)	66.62 ± 1.13	78.32 ± 1.13***	89.82 ± 0.79***

Values are mean ± SEM, n = 10, * P < 0.05, ** P < 0.01, *** P < 0.001.

There was no statistical (P > 0.05) change in the blood clotting time of all the treatment groups on day 0 compared to control group values.

On day 14, blood clotting time was significantly increased (P < 0.01) in group II (medium dose) and (P < 0.001) in group III (high dose) compared to control group.

On day 28, blood clotting time was significantly increased (P < 0.01) in group I (low dose), and (P < 0.001) in group II (medium dose) and group III (high dose) compared to control group.



c. Pathology

I. Gross pathology

In all the treated group animals, there were congestive changes in heart, lungs, liver, spleen, intestine and kidney. Group I, II and III animals showed intestinal haemorrhages. spleenomegaly and hepatomegaly. Remaining organs were normal in appearance and no significant gross pathological lesions were observed (Plate No. 6.1-6.6).

II. Histopathology

A. Control group

Histopathology of control group animals revealed normal architecture in all organs. Heart,

kidney and intestine of all treated groups didn't reveal any observable changes.

B. Group I (Low dose group)

Liver: Microscopically, lesions in the liver included mild congestion, acute cellular swelling in hepatocytes with granular cytoplasm, portal and sinusoidal congestion, portal hepatitis, focal parenchymal necrosis and focal biliary sclerosis (Plate 7.1).

Kidney: The microscopic lesions noticed were slight congestive changes in cortex and medulla, glomerular degeneration with focal nephritis, cellular swelling, hydropic and vacuolar degeneration (Plate 7.2).

Intestine: Revealed desquamation of epithelial cells, degeneration and necrosis of the intestinal epithelium and increased goblet cell activity. Congested capillary bed at lamina propria was also noticed (Plate 7.3).

Heart: Microscopically, epicardial congestion and oedema along with mild degree of degenerative changes were noticed (Plate 7.4).

Spleen: Mild degree of congestion with lymphocytic depletion was noticed (Plate 7.5).

Lungs: Revealed mild congestion with widening of interstitium and focal haemorrhagic lesions (Plate 7.6).

Stomach, brain and genital organs were apparently normal.

C. Group II (Medium dose group)

Liver: Microscopic lesions seen in the liver were, portal hepatitis, central and portal venular congestion, sinusoidal congestion, multifocal parenchymal hepatitis. Necrosis with pyknotic nucleus, central and portal vascular sclerosis, multiple bile ducts formation, focal areas of fatty changes along with inflammatory cells around portal veins, were also seen (Plate 8.1).

Kidney: The microscopic lesions were degenerative changes, glomerulonephritis with ballooning of tubules and glomerular necrosis (Plate 8.2).

Intestine: Revealed loss of villus epithelial cells, degeneration and necrosis, loss of crypts and catarrhal inflammation with goblet cell proliferation (Plate 8.3).

Heart: Microscopically, edema was evident with epicardial & myocardial congestion along with haemorrhage, and disruption of cardiac muscle fibres were also identified (Plate 8.4).

Spleen: Haemorrhages, red pulp congestion, trabecular vascular congestion and lymphoid proliferation and aggregation were noticed (Plate 8.5).

Lungs: Revealed mild to moderate degree of congestive changes (Plate 8.6). Stomach, brain and genital organs were apparently normal.

D. Group III (High dose group)

Liver: Microscopic lesions noticed in the liver included multiple bile duct formation, portal hepatitis, focal central and portal vascular sclerosis, central & portal vascular congestion along with sinusoidal congestion. Focal areas of acute cellular swelling, fatty degeneration and haemorrhages were also observed. Inflammatory cell deposition around bile duct, multifocal portal hepatitis, hepatic parenchymal degeneration, central venous sclerosis, pyknotic nucleus, hyaline bodies and focal area of fibrosis were also noticed (Plate 9.1).

Kidney: Lesions seen in kidney were, congestive changes in cortex and medulla, degenerative changes in tubular areas, glomerular degeneration, loss of glomerular tufts, ballooning degeneration in distal convoluted tubules and proximal convoluted tubules. Eosinophilic precipitates/deposition in tubules & glomerulus, focal haemorrhagic lesions and interstitial nephritis was also recorded (Plate 9.2).

Intestine: Microscopically, intestine revealed desquamative changes, necrotic changes, loss of villi and crypts, lymphocytic infiltration in lamina propria. Goblet cell hyperplasia in crypts, congested capillaries, and degenerative changes leading to deposition of necrotic debris in the lumen was also observed (Plate 9.3).

Heart: Microscopically, heart revealed epicardial and myocardial vascular congestion, separation of myocardial fibres, wavy pattern of myofibrils and mild to moderate degenerative changes (Plate 9.4).

Spleen: Congestive changes in red pulp, trabecular vascular congestion, lymphoid aggregation around central arteriole, focal areas of lymphoid depletion and diffuse haemosiderosis was observed (Plate 9.5).

Lungs: Microscopical lesions documented in the lungs were, congestive changes, oedematous changes, inflammatory cell deposition around the bronchiole and focal hemorrhagic lesions (Plate 9.6).

Plate 6: Gross pathology of Group III (High dose group) rats.

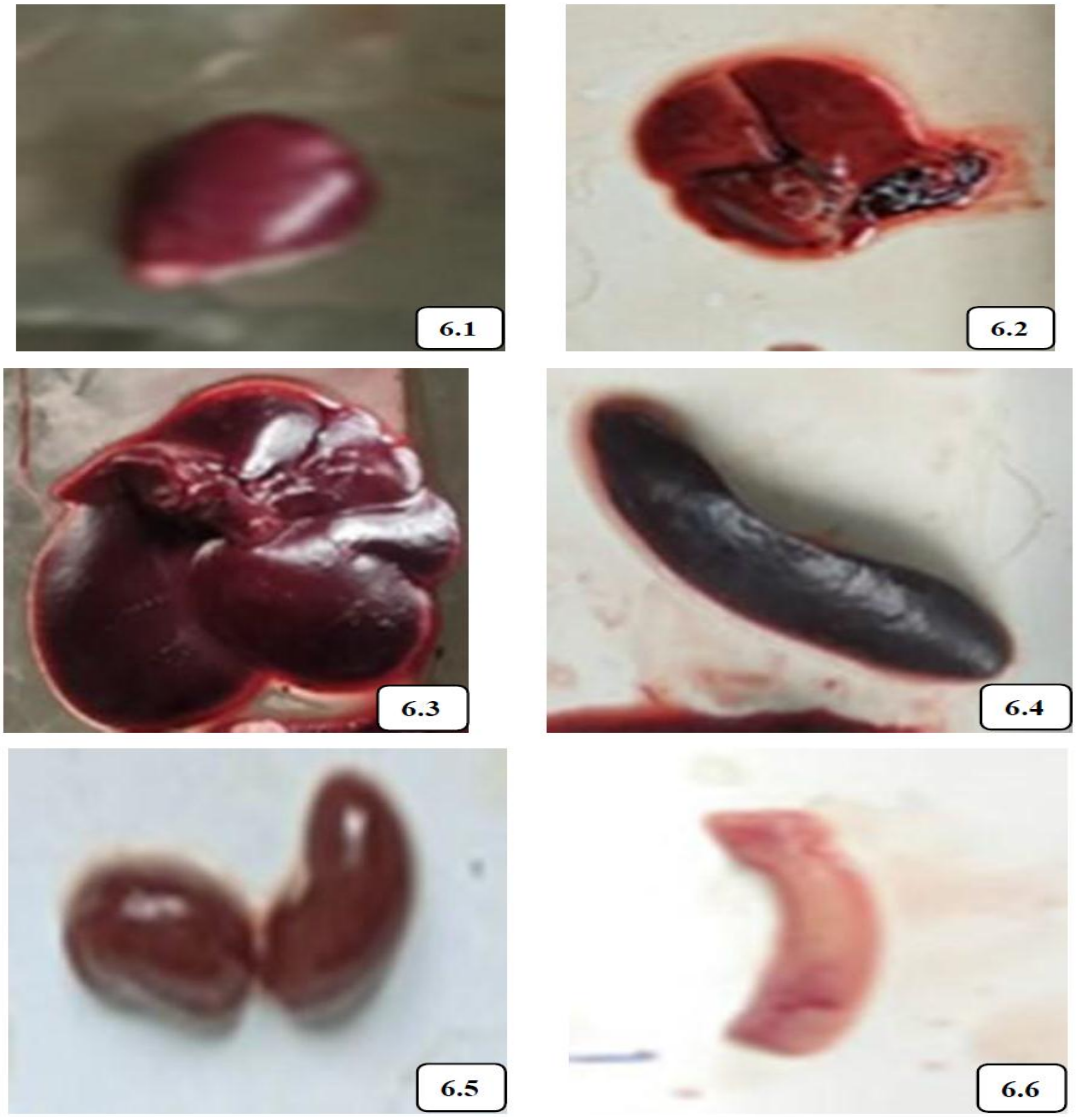
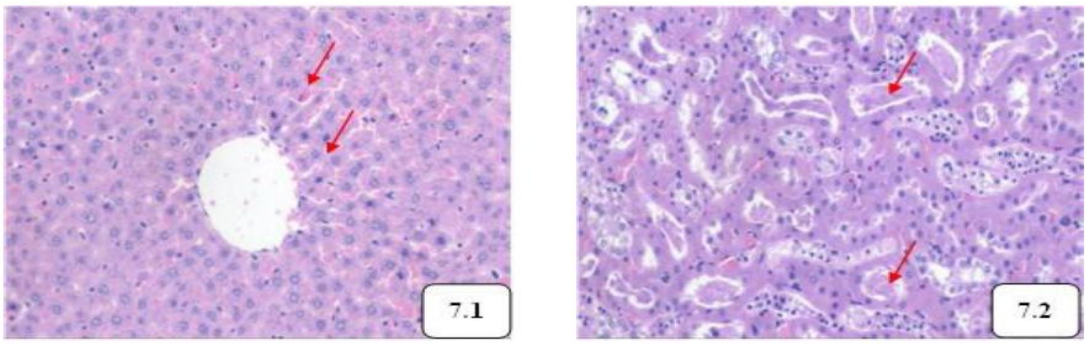


Plate 7: Histopathology of Group I (Low dose group) rats.



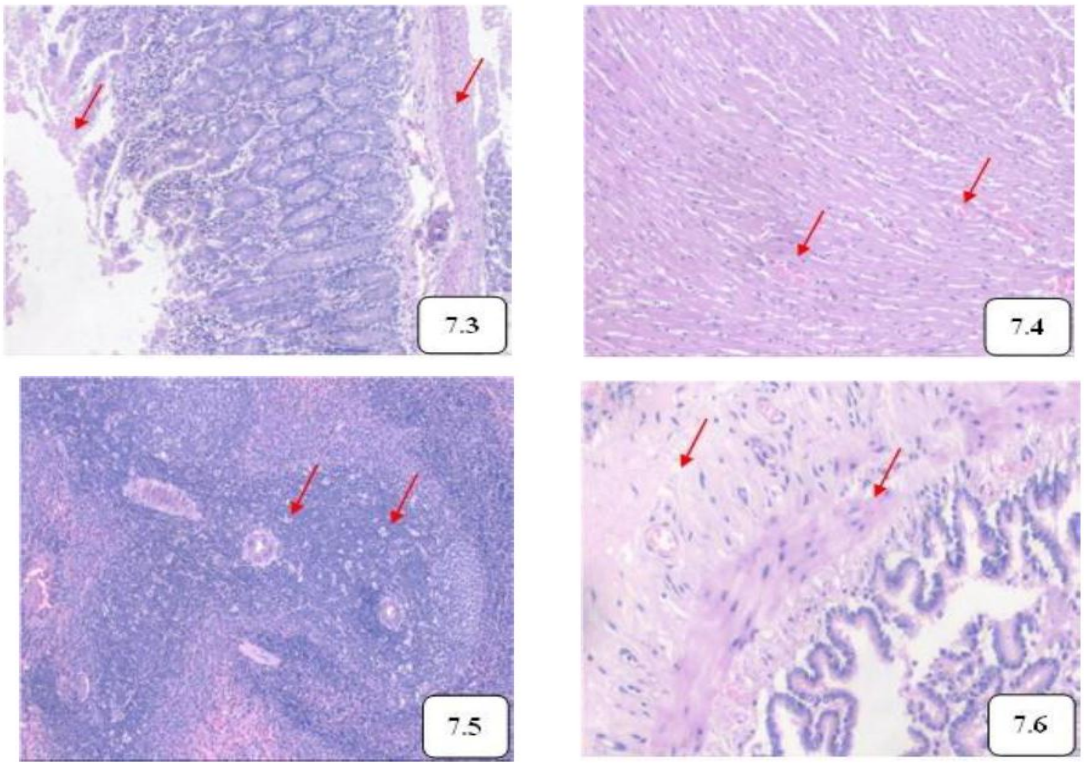
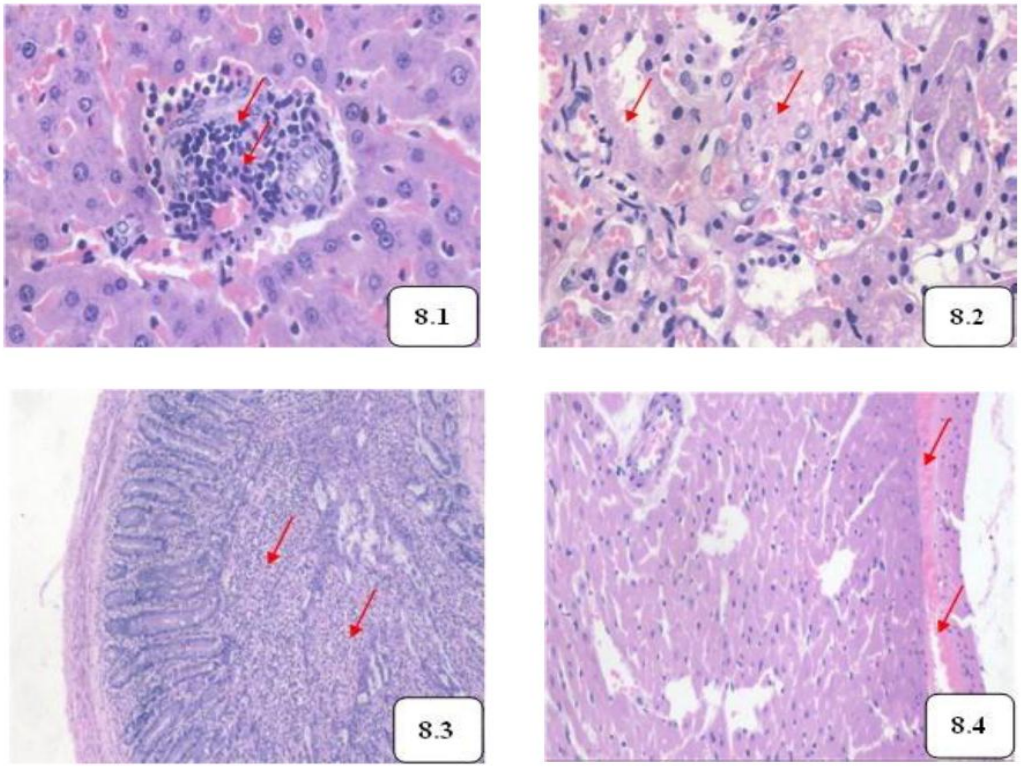


Plate 8: Histopathology of Group II (Medium dose group) rats.



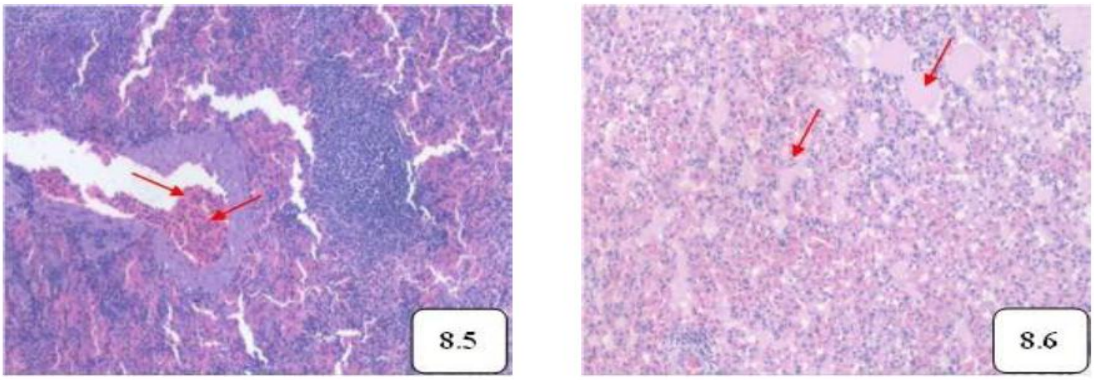
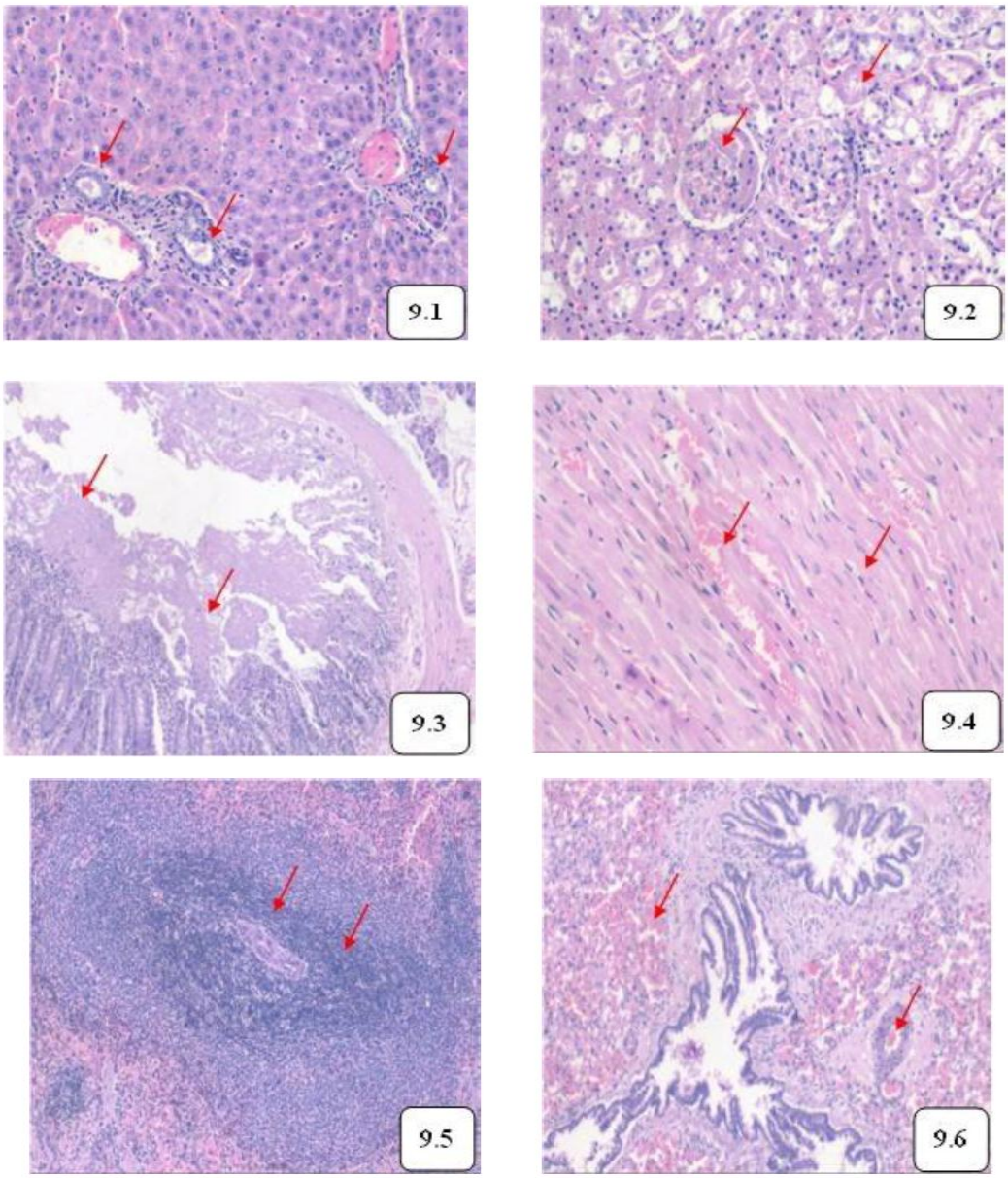


Plate 9: Histopathology of Group III (High dose group) rats.



4. DISCUSSION

In the present study, toxicity potential of fungal isolates from the brewer's spent grain was investigated in rats. During the disease investigation process and discussions with veterinarians working near brewing industries, at Kechhenahalli and B Durga of Davanagere district, Kabbenur of Dharwad district and Kumbharahalla of Bagalkot district veterinarians informed that farmers were feeding brewery waste as concentrate feed tocattle and they were suffering from sub-acute rumen acidosis (SARA), ruminal atony, ruminal impaction, anorexia, weakness, often uncontrolled coughing, dyspnea, abortions in pregnant cows, poor milk quality, decreased milk production, gradual weight loss and infertility. Apart from this, the serious threat to the cattle was there when this food stuff was infected by the different fungi and was fed to the cattle. The cattle suffered from different clinical signs of anorexia, unthriftiness, dullness, depression, diarrhoea, convulsions and many other non-specific clinical signs which may be attributed to the different mycotoxins present in the fungal infected brewer's spent grain.

On observations, feeding material was brewer's spent grain, a beer brewing industrial by-product and had mould growth on it. Hence suspecting that fungalgrowths on brewery waste might be the reason for animal ailments and it was decided to take up the study on toxicity of fungal isolates from Brewer's spent grain (BSG).

In Northern Karnataka, farmers fed the fungal contaminated sorghum fodder of the previous years to cattle and buffaloes. Ingesting such stale sorghum fodder, animals exhibited the clinical signs of paraplegia, salivation, staggering gait and dyspnea with an increase in serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) values. Similar type of toxicity episodes in cattle were reported which had consumed stale sorghum fodder, paddy straw, maize hulls, groundnut hay etc which exhibited various clinical signs.[22&23]

In the present study rats were gavaged with mixed different fungal culture filtrates to produce toxicity, after isolating the distinct fungus from the fungal infected brewer's spent grain gathered from the regions where cattle were affected, the goal of this experiment was to confirm that the toxins generated by the fungi that are responsible for producing toxicity in cattle using rat as a model. The inquiry was carried out to determine the toxic nature of the fungi that caused toxicity in cattle, as there was no reference in the literature for the clinical signs displayed by cattle after ingesting stale fungal-infected brewer's spent grain.

Similar type of toxicity studies using fungal species isolated from stale fodder were conducted by gavaging the isolated fungal culture filtrates of various fungi which had caused mycotoxicosis in cattle were done such as with sorghum by[24] groundnut hay by[25] with paddy straw by[26] and[27] with ground nut hay by,[28] usingmaize hulls by[29] and maize stalks by[30] which affected the cattle and goats.

4.1. Fungal isolation

In the present study, the species of fungi isolated and identified from the fungal contaminated brewer's spent grain were *Rhizopus oryzae, Aspergillus flavus* and *Aspergillus nomius***.** Spoilage of spent brewers' grains can happen as soon as five to seven days after the bag or silo is opened, resulting in increased mould growth, lower moisture content, and poor palatability.[18]

In a study conducted by[31] in malted barley and brewer's spent grains, toxigenic organisms from the genera *Fusarium*, *Aspergillus*, *Penicillium*, *Cladosporium*, *Geotrichum*, and *Alternaria* were found, the detected*Fusarium* spp, accounted for 30% of contamination, with Aspergillus spp, accounting for 27.3% while *Mucorales* accounted for 30% of brewer's grain samples respectively.[32] in his study of microflora analysis in 80 brewer's grain samples revealed *Aspergillus* spp. as the most frequent (42.5%) genus isolated, followed by *Mucor* spp. (32.5%), *Rhizopus* spp. (32.5%), *Penicillium* spp. (7.5%), and *Fusarium* spp. (2.5%). *Cladosporium* spp. was detected in only one sample also reported.

4.2. Screening for the presence of various mycotoxin

The method developed herein for the quantification of mycotoxins complies with the common methods found in the scientific literature detailing the procedures utilized for the specific detection and quantification of multiple mycotoxins for each individual sample analyzed by means of liquid chromatography coupled toelectrospray ionization tandem mass spectrometry.

The fungal contaminated brewer's spent grain samples, *Rhizopus oryzae, Aspergillus flavus,* and *Aspergillus nomius* inoculated culture filtrate broths were analysed for the presence different mycotoxins by using High Performance Liquid Chromatography with Fluorescence Detection and Ultra-Performance Liquid Chromatography (HPLC-FLD) method.

HPLC-FLD is one of the standard techniques for quantitative analysis of mycotoxins from

food and feed ingredients affected with fungi.[33] For the detection of aflatoxins, HPLC with a fluorescence detector (FLD) provides a relatively fast, efficient, sensitive, specific, and global technique. As a result, the HPLC-FLD system is a very adaptable separation/detection system for chemical component identification and it is used universally to detect the multi mycotoxins present in feed or its ingredients.[34]

Fungal culture filtrate of PD broth inoculated with *Aspergillus flavus* isolate obtained from brewer's spent grain showed the significant concentrations of AFB1and AFB2 (Fig. 5).

Fungal culture filtrate of PD broth inoculated with *Aspergillus nomius* isolate obtained from brewer's spent grain showed the significant concentration of AFB1. (Fig. 6) Significant concentration of AFB1 was detected in fungal contaminated brewer's spent grain samples (Fig. 7).[32] reported analysis of mycotoxins in brewer's grain revealed contamination with aflatoxins, while no ochratoxins were detected. Twenty-seven (33.75%) of the 80 samples analysed were positive for aflatoxins (B1+B2+ G1+ G2), with levels ranging from 1 to 3 µg/kg.

Aflatoxins are one of the highly toxic secondary metabolites derived from polyketides produced by fungal species such as *Aspergillus flavus, A. parasiticus*,and *A. nomius.*[35]

4.3. Repeated dose 28-day oral toxicity study

In the present study, the fungal culture filtrate was administered to induce the toxicity in rats since it was appropriate method to administer the desired dose of the broth culture filtrate containing major secondary metabolites or mycotoxins. The dose selected in rat and mice was based on the maximum allowable dose to be administered to these animals as per the standard protocols.[28,30&36]

The mixed fungal culture filtrates of various fungi was made based on the hypothesis that, in field conditions, the animals might have consumed the brewer's spent grain contaminated with *R.oryzae, A. flavus* and *A.nomius* may be in equal or different proportions. In order to know the interaction of the mycotoxins in inducing the toxicity and also to mimic the toxicity observed in field condition in rats. In mixedfungal culture filtrates *Rhizopus oryzae* (25%) + *Aspergillus flavus* (50%) + *Aspergillus nomius* (25%) was used. *A. flavus* culture filtrate at 50% was used because the fungus was rapidly growing and ubiquitous in nature.

The different proportions of two identified fungi were used to learn about the interaction of

mycotoxins in inducing toxicity and to mimic the toxicity observed in field conditions in rats. Similar studies were also done by many earlier workers as[37] who used fungal culture filtrate isolated from citrus soils for anti-nematodal activity against the *Tylenchulus semipenetrans* which is a plant pathogenic nematode.

Clinical observation

The rats gavaged with mixed fungal culture filtrates of *R.oryzae A,flavus* and *A.nomius* showed clinical signs of depression, weakness, reduced feed intake, water intake diarrhoea, and reduced body weight which might be attributed to toxic nature of fungal filtrates. Similar findings were reported by.[38&39]

In a study of aflatoxicosis in cattle, clinical findings and biochemical alterations conducted by[40] noticed that cattle were suffered fromaflatoxicosis exhibited clinical signs of depression and inappetence predominantly.

Study of aflatoxins hazards in grain/aflatoxicosis and livestock, feed refusal, reduced growth rate and decreased feed efficiency are the predominant signs ofchronic aflatoxin poisoning. In addition, listlessness, weight loss, rough hair coat and mild diarrhoea may occur.

4.4. Body weight of rats

Body weight values are given in result section Table No. 3 and Fig. 8

On day 14, body weight (g) was significantly decreased ($P < 0.05$) in group I (low dose), and ($P < 0.001$) in group II (medium dose) and group III (high dose) compared to control group.

On day 28, body weight (g) was significantly decreased ($P < 0.001$) in group I (low dose), group II (medium dose) and group III (high dose) compared to control group.

Aflatoxin B1 induced carcinogenicity study in Wistar rats conducted by[41] demonstrated higher mortality rate, reduction in growth rate andrelative body weight were recorded in adult males than adult female.

In a study of effect of aflatoxin ingestion in feed on body weight gain and tissue residues in rabbits, a significant weight loss equivalent to 13 % mortality was found in the aflatoxin-fed rabbits.[42]

The contamination with aflatoxin caused a dose-related decrease in weight gain,

modifications in some blood parameters, and alterations of the immune response, in particular of cytokine expression in piglets.[43]

The decrease in body weight (g) of rats might be attributed to reduced feed intake due to the toxic content in culture filtrates, the decreased body weight in rats treated with mixed *R. oryzae*, *A. flavus and A nomius* fungal culture filtrates correlated with the findings of[27&30] who alsoreported the decrease in body weight in the sub-acute toxicity study in rats fed with fungal culture filtrates of *Aspergillus niger* isolated from maize and paddy straw respectively.

Hence it was evident from the above findings that, the mycotoxins cause marked decrease in the body weight and also the histopahologic lesions of loss of villiand crypts, in the intestine, might made rats devoid of absorptive surfaces for feed and essential nutrients leading to malabsorption causing weakness and loss of weight in the fungal culture filtrate gavaged group rats.

4.5. Biochemical parameters

Serum ALT concentration values are given in result section Table No. 4 and Fig. 9

On day 14, mean serum ALT concentration was significantly increased ($P< 0.05$) in group II (medium dose) and ($P < 0.001$) in group III (high dose) compared to control group.

On day 28, mean serum ALT was significantly increased ($P < 0.01$) in group I (low dose), and ($P < 0.001$) in group II (medium dose) and group III (high dose) compared to control group.

More conclusive evidence of aflatoxin involvement in disease includes acute to chronic liver disease with concomitant increases in specific liver enzymes in the serum. The effect of *A. flavus* on the liver of experimental rats administered with antiretroviral drugs, immune suppression caused by antiretroviral drugs allowed infiltration of *A. flavus* to liver of the rats causing hepatotoxicity leading to significant rise in ALT and AST and ALP.[44&45]

Increased serum ALT levels in the fungal-culture filtrates gavaged groups indicate that the toxins present in the culture filtrates have caused significant damage to liver tissue, as confirmed by histopathological findings such as severe congestion, focal necrosis, vacuolar degeneration, biliary hyperplasia, fibrotic change in periportal areas, and karyomegaly, pyknotic nucleus in some hepatocytes as observed in the present study.[46]

Serum AST values are given in result section Table No. 5 and Fig. 10

On day 14, mean serum AST concentration was significantly increased (P< 0.001) in group I (low dose), group II (medium dose) and group III (high dose) compared to control group.

On day 28, mean serum AST concentration was significantly increased (P < 0.001) in group I (low dose), group II (medium dose) and group III (high dose) compared to control group.

The significant increase (P<0.05) in AST in rats fed with the *A. niger* and *P. resticulosm* fungal culture filtrate, and in mice fed with the culture filtrate of the fungi *A. glaucus*, *A. ornatus*, *P. aurantiogriseum* and *P. resticulosm* isolated from the paddy straw.[25]

The significant increase (P<0.05) in AST in rats fed with the fungal culture filtrate of *A. clavatus* and *A. flavus* isolated from maize hulls. The rise in AST levels can be traced back to liver injury and muscular degeneration by muscle wasting evidenced by decrease in body weight, where the toxin might have changed permeability and caused enzyme leakage. Cell necrosis, which leads to the release of intracellular enzymes into the bloodstream, is another probable source of increased enzyme concentrations. Gross and histopathological lesions in liver showed severe congestion, localised necrosis, vacuolar degeneration, and biliary hyperplasia in the fungal-culture filtrates gavaged groups further supports the liver damage.[29]

Blood urea nitrogen (BUN) values are given in result section Table No. 6 and Fig. 11.

On day 14, blood urea nitrogen concentration was significantly increased (P < 0.01) in group II (medium dose) and group III (high dose) compared to control group.

On day 28, blood urea nitrogen concentration was significantly increased (P < 0.01) in group I (low dose) and (P < 0.001) in group II (medium dose) and group III (high dose) compared to control group.

The elevated serum urea nitrogen concentration in comparison to control suggests the possible role of the toxins in causing kidney damage. This was further supported by histopathological observations like, congestion along with vacuolar degeneration, necrosis ballooning of the some of the tubules and fibrosis in theinterstitium.

The significant increase in BUN values in most of the albino mice treated with metabolites

of *Aspergillus japonicus, A. nidulans, A. niger, Aspergillus sp., A. terreus, Cladosporium sp., Fusarium sp., Mycelia sterilia, Paecilomyces sp., Penicillium sp.,* and *Syncephalastrum sp.*[46][26] also found similar findings of significant increased BUN values in rats and mice gavaged with fungal filtrate of *Aspergillus terreus*, *Penicilium digitatum* and *Cladosporium oxysporum.*[47] revealed in study of l-proline alleviates kidney injury caused by AFB1 and AFM1 through regulating excessive apoptosis of kidney cells, that apoptosis of kidney cells lead to higher levels of creatinine (CRE), urea (UREA), uric acid (UA) in the AFB1 and AFM1 treatment groups of mice than the control.

Serum creatinine values are given in result section Table No. 7 and Fig. 12

On day 14, serum creatinine concentrations was significantly increased ($P<0.001$) in group II (medium dose) and group III (high dose) compared to control group.

On day 28, serum creatinine concentrations was significantly increased ($P < 0.001$) in group I (low dose), group II (medium dose) and group III (high dose) compared to control group.

The elevated serum creatinine concentration in treatment group comparison to control suggested the possible role of the toxins in causing kidney damage which was further evidenced by histopathological observations like, glomerulitis, interstistial nephritis, hyaline change, tubular degeneration, and eosinophilic deposition in the glomeruli of the some of the tubules and fibrosis in the interstitium of nephrons and also possibility of creatinine release from the wasting muscles due to loss of body weight in the fungal-culture filtrates gavaged groups. Increased creatinine is anindicator of kidney damage.[48]

Further the findings are also supported by the observation of[49] who stated that there will be increased serum creatinine concentration in the rats treated with the fungal culture filtrate of *Trichoderma harzianum, Penicillium citrinum* and *Aspergillus versicolor.* The increased serum creatinine concentration in the rats treated with mixed fungal culture filtrate of *Aspergillus niger, Aspergillus terreus, Rhizoctonia bataticola* and *Rhizopus stolonifera* was attributed to the kidney damage.

Total leukocyte count values are given in result section Table No. 8 and Fig. 13

On day 14, significantly decreased ($P < 0.05$) total leucocyte count (TLC) in group II

(medium dose) and group III (high dose) compared to control group observed.

On day 28, significantly decreased ($P < 0.05$) total leucocyte count (TLC) in group I (low dose), group II (medium dose) and ($P < 0.01$) group III (high dose) compared to control group observed.

Decrease in total leukocyte count (TLC) is due to immunosuppression known to be caused by aflatoxins and also microscopic histopathologic lesions of lymphoid depletion in spleen might supports the decrease in total leukocyte count.[50] studied immunotoxicity of aflatoxin B1 in rats and its effects on lymphocytes and the inflammatory response in a chronic intermittent dosing and concluded that AFB1 effects on the immune system can be either stimulatory or suppressive dependent on a critical exposure window of dose and time.[51] observed in study of evaluation of acute immunotoxicity of aerosolized aflatoxin B1 in female C57BL/6N mice, decrease in the mean white blood cell count of treated v/s. naive mice at all dose levels.

Haematological parameters[27&30]

Reported decreased **total erythrocyte count** (TEC) in the sub-acute toxicity study in rats fed with fungal culture filtrates of *Aspergillus niger* which was isolated from maize and paddy straw respectively.

In study of effects of aflatoxin on some haematological parameters and protective effectiveness of esterified glucomannan in Merino rams by[52] resulted in decreased erythrocyte, leukocyte count, hemoglobin, and hematocrit levels. Aflatoxicosis caused the lymphocytopenia and monocytopenia but increased percentage of neutrophil counts in aflatoxin treated group compared with the other groups.[53] in his study of effect of dietary aflatoxins (AFB1) on hematological and biochemical indices of male buffaloes, showed the total erythrocyte count (TEC) at day 14 was lowest in growing male buffaloes fed with aflatoxin added concentrate feed.

The mechanism by which AFB1 aggravated pathogenesis of anemia could involve down-regulation of erythropoietin activity. Down-regulation of erythropoietin has been contributed to reduction in erythropiosis in bone marrow and faster rate of destruction of peripheral RBC in spleen resulting in decreased circulating TEC.

Haemoglobin (Hb) values are given in result section Table No. 10 and Fig. 15 On day 14,

hemoglobin concentration was significantly decreased (P < 0.05) in group II (medium dose) and group III (high dose) compared to control group.

On day 28, hemoglobin concentration was significantly decreased (P < 0.05) in group I (low dose), (P < 0.01) in group II (medium dose) and (P < 0.001) in group III (high dose) compared to control group.[54] while studying effects of long-term feeding of ammoniated, aflatoxin-contaminated corn to rats noted the signs of chronic toxicosisin rats fed aflatoxin-contaminated corn included increased mortality, decreased hematocrit and hemoglobin levels, elevated serum alkaline phosphatase activities, and a 100% incidence of liver neoplasia.[55] observed that, hemoglobin, packed cell volume, and erythrocyte count were reduced significantly in chickens fed with aflatoxin feed causing haemolytic anaemia. The biochemical and histopathological analysis of aflatoxicosis in growing hens fed with commercial poultry feed showed the decreased haemoglobin in test group than the control group.

Decrease in Hb can be related with reduction in size of RBC, impaired biosynthesis of heam in bone marrow or due to reduction in rate of formation of TEC.

Packed cell values (PCV) values are given in result section Table No. 11 and Fig. 16

On day 14, packed cell volume (PCV) concentration was significantly decreased (P < 0.05) in group II (medium dose) and (P < 0.01) in group III (high dose) compared to control group.

On day 28, packed cell volume (PCV) concentration was significantly decreased (P < 0.01) in group I (low dose), (P < 0.001) in group II (medium dose) andgroup III (high dose) compared to control group.[56] in their study of oxidative stress in extrahepatic tissues of rats co-exposed to aflatoxin B1 and low protein diet, significantly decreased body weight gain and PCV in rats treated with aflatoxin.[57] in their study of hematological and hemostatic changes in aflatoxin, curcumin plus aflatoxin and curcumin treated rat, observed rats administrated aflatoxin showed macrocytic hypochromic anemia which was confirmed by reduction in RBCs count, Hb, PCV, MCH and MCHC with increase in MCV.

4.5.1.Blood clotting time of rats

Blood clotting time values are given in result section Table No. 12 and Fig. 17

On day 14, blood clotting time was significantly increased (P < 0.01) in group II (medium

dose) and ($P < 0.001$) in group III (high dose) compared to control group.

On day 28, blood clotting time was significantly increased ($P < 0.01$) in groupI (low dose), and ($P < 0.001$) in group II (medium dose) and group III (high dose) compared to control group.[58] reported feeding of diet containing *A. flavus* caused increased blood clotting time in rats. Effects of various treatments on induced chronic aflatoxicosis in rabbits study by[59] prolongation of whole blood clotting time has been reported inrabbits induced with chronic aflatoxicosis resulted in lengthened prothrombin and activated partial thromboplastin times and also decreased plasma fibrinogen concentration.[27&30] reported the increase in blood clotting time in the sub-acute toxicity study in rats fed with fungal culture filtrates of *Aspergillus niger* which was isolated from maize and paddy straw respectively.

4.6. Histopathology

4.6.1.Liver

In low dose group (Group I), lesions in the liver included mild congestion, acute cellular swelling in hepatocytes with granular cytoplasm, portal and sinusoidal congestion, portal hepatitis, focal parenchymal necrosis and focal biliary sclerosis (Plate 7.1), where as in medium dose group (Group II), lesions seen in the liver were, portal hepatitis, central and portal venular congestion, sinusoidal congestion, multifocal parenchymal hepatitis. Necrosis with pyknotic nucleus, central and portal vascular sclerosis, multiple bile ducts formation, focal areas of fatty changes along with inflammatory cells around portal veins, were also seen (Plate 8.1).

In high dose group (Group III), the microscopic lesions noticed in the liver included multiple bile duct formation, portal hepatitis, focal central and portal vascular sclerosis, central & portal vascular congestion along with sinusoidal congestion. Focal areas of acute cellular swelling, fatty degeneration and haemorrhages were also observed. Inflammatory cell deposition around bile duct, multifocal portal hepatitis, hepatic parenchymal degeneration, central venous sclerosis, pyknotic nucleus, hyaline bodies and focal area of fibrosis were also noticed(Plate 9.1).[60]

Observed marked biliary proliferation with necrotic debris and parenchymal hepatitis in rats in acute and chronic effects of aflatoxin on the liver of domestic and laboratory animals.[61]

Recorded long-term effects of feeding aflatoxin- contaminated market peanut oil to Sprague-

Dawley rats and observed parenchymal liver damage with varying degrees of fatty change. They also noticed swollen cells with granular cytoplasm and cells having increase in the nuclear/cytoplasmic ratio, hyperchromatic nuclei and multiple nucleoli.[62]

In histopathological and biochemical investigations of protective role of honey in rats with experimental aflatoxicosis recorded hydropic degeneration, necrotic changes and dysplastic changes in hepatocytes predominantly. Also noticed cloudy swelling or vacuolar-hydropic degenerative hepatocytes particularly in the peri acinar and intermediate regions of the liver lobules, characterized with large foci of hepatocytes with granulated cytoplasm. Moderate to severe cytoplasmic vacuolation, indicating fatty change in some hepatocytes in the periportal regions, focal necrosis, hyperemia in some veins and sinusoids, increase in the number peri-sinusoidal cells and intrahepatic cholestasis were also noticed. Likewise, epithelial hyperplasia in the bile ducts and an increase in the number of bile ducts and focal mononuclear cell infiltration in the portal area were also recorded.

4.6.2. Kidney

The microscopic lesions noticed were slight congestive changes in cortex and medulla, glomerular degeneration with focal nephritis, cellular swelling, hydropic and vacuolar degeneration (Plate 7.2) in low dose group (Group I) followed by lesions with degenerative changes, glomerulonephritis with ballooning of tubules and glomerular necrosis (Plate 8.2) in medium dose group (Group II) where as in high dose group (Group III), the lesions in the kidney were, congestive changes in cortex and medulla, degenerative changes in tubular areas, glomerular degeneration, loss of glomerular tufts, ballooning degeneration in distal convoluted tubules and proximal convoluted tubules. Eosinophilic precipitates/deposition in tubules & glomerulus, focal haemorrhagic lesions and interstitial nephritis was also recorded (Plate 9.2).[63]

Observed hydropic and vacuolar degeneration, coagulative necrosis associated with degeneration of tubular architecture, moderate parenchymal tubular degeneration, manifested by tubal obstruction, epithelial swelling and blabbing, and fine granular appearance of the cytoplasm in the effects of diosmin on aflatoxin-induced liver and kidney damage in aflatoxin-treated rats.[64]

Recorded moderate parenchymatous tubular degeneration, predominantly of the distal tubules, manifested by epithelial swelling and fine granular appearance of cytoplasm in

aflatoxin treated rats. In the kidneys of experimental animals, they also observed hydropic and vacuolardegeneration with desquamation of epithelial tubular cells.[62]

Noticed degeneration and coagulative necrosis of proximal tubule epithelial cells, hyperemia in arterioles, glomerular and interstitial capillaries and hyaline casts in some of the tubule lumens in histopathological and biochemical investigations of protective role of honey in rats with experimental aflatoxicosis in kidney. Furthermore, megalocytic cells were also seen in tubular epithelium in aflatoxin treated group.

4.6.3. Spleen

Mild degree of congestion with lymphocytic depletion was noticed (Plate 7.5) in low dose treated group (Group I) followed by medium dose group (Group II) with lesions of red pulp congestion, trabecular vascular congestion and lymphoid proliferation and aggregation were noticed (Plate 8.5) and in high dose group (Group III) showed congestive changes in red pulp, trabecular vascular congestion, lymphoid aggregation around central arteriole, focal areas of lymphoid depletion and diffuse haemosiderosis was observed (Plate 9.5).[65]

Noticed loss of the normal architecture, hypoplasia in some lymphatic nodules and depletion, represented by lymphocytopenia in spleen along with mild congestion and edema in red pulp in the study made on immunomodulatory effect of dietary turmeric against aflatoxins in mice : histological and immuno histochemical study.

The histopathological changes in the spleen of mice after exposure to Aflatoxin B1 purified from *Aspergillus flavus* in different doses revealed inflammatory cells particularly mononuclear cells infiltration in congested red pulp, fatty changes in addition to extensive apoptosis of lymphocytes in whitepulp characterized by cellular debris in multiple irregular spaces and depletion of white pulp with apoptosis of lymphocytes in white pulp.

4.6.4.Intestine

The lesions in intestine in low dose group (Group I) revealed desquamation of epithelial cells, degeneration and necrosis of the intestinal epithelium and increased goblet cell activity. Congested capillary bed at lamina propria was also noticed (Plate 7.3). In medium dose group (Group II), there were loss of villus epithelial cells, degeneration and necrosis, loss of crypts and catarrhal inflammation with goblet cell proliferation (Plate 8.3), followed by desquamative changes, necrotic changes, loss of villi and crypts, lymphocytic infiltration

in lamina propria. Goblet cell hyperplasia in crypts, congested capillaries, and degenerative changes leading to deposition of necrotic debris in the lumen was also observed in high dose group or Group III (Plate9.3).[66]

While studying in amelioration of Aflatoxin B1- induced gastrointestinal injuries by Eucalyptus oil in rats reported that the exposure to AfB1 alone caused the appearance of widespread hemorrhages in the mucosa and submucosa. The delineation between the mucosal, submucosal and muscularis layers were also less distinct in rats treated with AfB1, while a few congested vessels were evident in the gastric tissues in their study.[67]

In experimental aflatoxin B1 toxicosis in young rabbits-a clinical and patho-anatomical study observed the mucosa of the stomach and intestines initially showing vascular engorgement, focal areas of haemorrhages, thickening, hyperplastic mucus glands and a heterophilic inflammatory reaction whichwas followed by epithelial degeneration and desquamation up to the 40th day of toxicosis. On the 50th day, the superficial epithelium of the gastric and duodenal mucosae showing erosive lesions along with mononuclear cellular infiltrations was recorded.

Lungs

Lungs revealed mild congestion with widening of interstitium and focal haemorrhagic lesions (Plate 7.6) in low dose group (Group I). There were mild to moderate degree of congestive changes (Plate 8.6) in medium dose group (Group II) and the lesions in high dose group (Group III) were congestive changes, oedematous changes, inflammatory cell deposition around the bronchiole and focal hemorrhagic lesions (Plate 9.6).[68]

Observed large number of inflammatory cells and dilatedalveolar sac with exudates in studies made on toxic effect of Aflatoxin B1 on heart, lung and testis of male albino rats.

4.6.5.Heart

The lesions in low dose group (Group I) were epicardial congestion and oedema along with mild degree of degenerative changes were noticed (Plate 7.4) followed by edema was evident with epicardial & myocardial congestion along with haemorrhage, and disruption of cardiac muscle fibres were also identified (Plate 8.4)in medium dose group (Group II) and in high dose group (Group III) heart revealed epicardial and myocardial vascular congestion, separation of myocardial fibres, wavy pattern of myofibrils and mild to moderate

degenerative changes (Plate 9.4).[68]

Studied on toxic effect of Aflatoxin B1 on heart, lung, and testis of male albino rats and up on histopathological examination of heart recorded myocardial congestion & edema.

Present study's histopathological impressions in heart and lung are in accordance with the similar findings noticed by[68] indicating possible cardiotoxicity of mixed fungal culture filrate broth.

Based on the results of the present study, it can be concluded that:

- The culture filtrates of fungal isolates obtained from fungal contaminated brewer's spent grain were toxic to rats at the given dose and duration of treatment.
- The cardiotoxicity was also seen in Group I Group II and Group III rats gavaged with mixed fungal culture filtrates (*R.oryzae*, *A. flavus* and *A.nomius*) correlated with the biochemical and histopathological studies.
- The hepatotoxicity induced in Group I Group II and Group III rats gavaged with mixed fungal culture filtrates correlated with the biochemical andhistopathological studies.
- The spleen was congested in Group I Group II and Group III rats gavaged with mixed fungal culture filtrates showed sparse cellularity with lymphocytic proliferation in Group III (high dose) and lymphoid depletion in Group I (low dose group) and in some of the splenic corpuscles which is indicative of immunotoxic nature of the culture filtrates. Severe necrotic and desquamative enteritis was noticed in Group I Group II and Group III rats gavaged with mixed fungal culture filtrates.
- Nephrotoxicity was also seen in Group I Group II and Group III rats gavaged with mixed fungal culture filtrate which was evident through biochemical and histopathological studies.
- Additive effects were also noticed in Group I Group II and Group III rats gavaged with mixed fungal culture filtrate which was evident through biochemical and histopathological studies.
- Hence brewer's spent grain a by-product of beverage industry is potential non-conventional feed source to animals if its properly stored and used well before mold growth and other contamination.

Further study is needed to confirm the changes seen under natural disease process in large animals by considering many factors including dose, concentration of the fungal culture

extract, animal status and the form in which the test material is administered.

5. SUMMARY

The present study was undertaken to confirm a disease especially in cattle at the Kechhenahalli and B Durga of Davanagere district, Kabbenur of Dharwad district and Kumbharahalla of Bagalkot district in Karnataka state, after ingestion of the fungal contaminated brewer's spent grain. Animals were exhibiting the clinical signs of sub- acute rumen acidosis (SARA), ruminal atony, ruminal impaction, anorexia, weakness, diarrhoea, often uncontrolled coughing, dyspnea, poor milk quality, decreased milk production, gradual weight loss and infertility. All the causes of illness were ruled out and mycotoxicosis was suspected for the animals suffering based on the signs exhibitedby the animals.

The present study was undertaken to evaluate the toxicity of fungal contaminated brewer's spent grain in rats. The species of fungi isolated and identified from the fungal contaminated brewer's spent grain were *Rhizopus oryzae* (*R. oryzae), Aspergillus flavus* (*A. flavus)* and *Aspergillus nomius (A. nomius)*. The fungal contaminated brewer's spent grain was analyzed for the presence mycotoxins by HPLC-FLD method. The *R. oryzae* inoculated culture free filtrate PD broth materialwas screened for the presence of various mycotoxins and did not show any mycotoxins. The *A. flavus* inoculated culture free filtrate PD broth showed the presence of AFB1, AFB2, AFG1 and AFG2. The *A. nomius* inoculated culture free filtrate PD broth showed the presence of AFB1, AFB2, AFG1 and AFG2. Fungal contaminated brewer'sspent grain showed the presence of AFB1, AFB2, AFG1 and AFG2.

Repeated dose 28-day oral toxicity study was conducted in four groups consisting of ten rats of both sex. The mixed fungal culture filtrates of the three isolatedfungi, at *R. oryzae* 25%, *A. flavus* 50% and *A. nomius* 25% *ratio* was administered to induce the toxicity in rats in the dose range of 0.5ml/100g (low dose), 1ml/100g (medium dose), and 2 ml/ 100 g (high dose).

Biochemical parameters were studied to reflect the organ function and pathological studies to confirm the toxicity. The rats gavaged with mixed fungal culture filtrates of *R.oryzae*, *A. flavus* and *A.nomius* showed clinical signs of reduction in feed intake, water intake, diarrhoea and loss of body weight. Animals were weak, dull and depressed. Biochemical studies showed significant increase ($P < 0.05$) in the ALT and AST noticed in Group I (Low dose), Group II (Medium dose) and Group III (High dose) rats compared to control group rats.

Significant increase ($P < 0.05$) in blood urea nitrogen and serum creatinine seen in Group I (Low dose), Group II (Medium dose) and Group III (High dose) rats compared to control group rats. Hematological studies showed significant decrease ($P<0.05$) in total leukocytes count, total erythrocyte count, haemoglobin concentration and packed cell volume in Group I (Low dose), Group II (Medium dose) and Group III (High dose) rats compared to control group rats.

The gross and histopathological changes revealed cardiotoxicity, hepatotoxicity, immunotoxicity, and nephrotoxicity by the mixed fungal culture filtrates of *R.oryzae*, *A. flavus* and *A.nomius* in all the fungal culture filtrates gavaged groups. Thus, it is concluded from the observations made in the present study that the mixed fungal culture filtrate had shown toxicity in rats, attributed to the presence of mycotoxins/toxic metabolites. Further study is needed to confirm the changes seen under natural diseaseprocess in large animals by considering many factors including dose, concentration of the fungal culture extract, animal status and the form in which the test material is administered.

6. Acknowledgement

Department of Animal Husbandry and Veterinary Services, Government of Karnataka, Dr. Shridhar, N. B., Professor and Head, Department of Veterinary Pharmacology and Toxicology, Veterinary College, Shivamogga and chairman of my advisory committee. My advisory committee members, my collegues and juniors,

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