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MOLECULAR CHARACTERIZATION AND SUBCHRONIC TOXICITY STUDY OF *FUSARIUM SPOROTRICHOIDES* FUNGUS IN RATS ISOLATED FROM GROUNDNUT HAY

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Abstract: The present study was conducted to characterize the fungus by molecular method that caused obscure disease in cattle after consuming contaminated groundnut hay and was correlated with the sub chronic toxicity study in rats. Ailing cattle were showing the clinical signs of colic, tenesmus, bleeding from natural orifices, central nervous system abnormality symptoms, anorexia, chronic recumbancy and death after few days. Fungus was isolated from the groundnut hay, identified, characterized by molecular methods. The fungus contaminated material was subjected to multimycotoxin analysis using LC-MS/MS method. Apicidin and beauvericin mycotoxins found in high concentrations. To confirm further, toxicity studies were conducted in rats. Clinical signs of toxicity were observed. The present study shown the toxic feature of the culture filtrates isolated from ground nut hay in rats.
Keywords: Sub chronic, Fungal isolate, Beauvericin, Apicidin

INTRODUCTION

The cattle of the south Karnataka region, especially Sira taluka of Tumkur district and surrounding areas were suffering from peculiar type of disease. Cattle were showing the clinical signs toxicity, especially soon after monsoon. Clinical signs observed were colic, tenesmus, ruminal atony, anorexia, bleeding from nostrils, rectum and fly bite site. After few days, cattle were unable to get up, chronic recumbancy observed and ultimately end up with death of cattle. During screening of the suspected material, mould growth was very much prominent and preliminary investigation revealed monsoon harvested groundnut hay was susceptible for fungal contamination due to improper drying and humid environmental conditions. Fungal contaminants identified from infected hay were *Macrophomina, Puccinia, Cercospora, Fusarium, Aspergillus* and *Rhizopus* species (Vinay *et al.,* 2007). Usually fungi

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produce a wide variety of secondary metabolites of low molecular weight like mycotoxins, which results in greatest monetary loss to the livestock industry (Bennet and Klitch, 2003).

Fungal growth is typically triggered by warm and wet conditions. Sufficient high levels of fungal metabolites in the feeds can have toxic effects .Thus, the present study was designed to characterize the fungus isolated from groundnut hay by molecular method and toxicity study was conducted in rats to confirm the same.

MATERIALS AND METHODS

Fungi contaminated groundnut hay was collected from the affected region, where the toxicity was observed. In the present study, the isolation of the fungi from the fungal contaminated groundnut hay was carried out in the Mycology laboratory of the Department of Pharmacology and Toxicology, Veterinary College, Bangalore. Potato dextrose agar (PDA) and potato dextrose broth were the medium of choice used to culture the fungi.

Fungal isolation and identification of the fungi: For the isolation, bits of fungal contaminated groundnut hay were surface sterilized for 1 min with 1 per cent sodium hypochlorite solution, rinsed twice in sterile distilled water, dried in a laminar air flow cabinet and inoculated on to solidified PDA petri plates. The inoculated plates were incubated at room temperature for 3-5 days. Upon sporulation, the fungus was tentatively identified and pure culture of the fungus was raised by hyphal tip culture from the apparently pure culture colonies. The pure isolate of the fungus thus obtained was maintained on PDA slants.

Identification of the isolated fungus, both microscopic and by molecular method using PCR technology up to genus level was done by Fungus Identification Service, Mycology and Plant Pathology Group, Agharkar Research Institute, Pune.

Design of the experiment: Acute toxicity study was conducted to determine the dose required. Sub chronic oral toxicity study was conducted as per OECD guidelines 408, to determine the toxicity of the fungal culture filtrate in rats. Four groups of rats were made as follows.

Group I - *Fusarium sporotrichoides* (A) - 2 ml broth; Group II - *Fusarium sporotrichoides* (B)- 1 ml broth; Group III- *Fusarium sporotrichoides* (C)- 0.5 ml and Group IV- Control - 2 ml PD Broth were administered orally. Toxicity study was conducted by using apparently healthy young Wistar albino rat. The rats were grouped (n=20) and housed in polypropylene rat cages during the experiment. General clinical observations were made thrice a day. Daily all the animals were observed for morbidity and mortality. Necropsy was done and organs were collected for histopathological studies.

Haematology and biochemistry: Blood samples were collected on 0, 45 and 90th day during the sub chronic toxicity study period. Blood clotting time was estimated, during blood collection itself. For every 30 seconds, capillary tube was broken. Time taken by the blood to form thread like structure while breaking the capillary tube was noted in seconds. Using Automatic Blood Cell Counter (ERMA INC, Model PCE-210, Tokyo, Japan) and commercially available kits hematology parameters like Total erythrocyte count (TEC), White blood cell (WBC) count, Hemoglobin (Hb) concentration and Packed cell volume (PCV) were estimated: Clinical biochemistry study was done to investigate the toxic effects. Using Microlab 300 (Vitalab Scientific, The Netherlands) and commercially available diagnostic kits from Merck (Ecoline®, Merck Specialties Limited, Goa, India), biochemical parameters like Alanine aminotransferase (ALT) activity, Aspartate aminotransferase (AST) activity, Creatinine (CRT) concentration and Urea nitrogen concentration were estimated.

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

Screening for the presence of mycotoxins and Statistical analysis: Fungal infected material was screened for the presence of various mycotoxins by using LCMS-MS. Mean values and standard error of means were calculated and expressed as mean ± SEM. The data were analyzed by two-way ANOVA with Dunnett's post test using Graph Pad Prism Trial version 5.00 for Windows, Graph Pad Software, San Diego, California USA, www.graphpad.com.

RESULTS AND DISCUSSION

Identification of the fungi: The fungal species identified was *Fusarium sporotrichoides*.

Table 1: *F. sporotrichoides* nucleotide sequence alignment

<table>
<tr><td>Query 1</td><td>TCATTACCGAGTTTTCAATCCCCCAAACCCCTGTGAACATACCTTTATGTTGCCTCGGCG</td><td>60</td></tr>
<tr><td></td><td>|||||||||||||| ||| | |||||||||||||||||||||||||||||||||||||||</td><td></td></tr>
<tr><td>Sbjct 38</td><td>TCATTACCGAGTTTACAA-CTCCCAAACCCCTGTGAACATACCTTTATGTTGCCTCGGCG</td><td>96</td></tr>
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<tr><td>Query 61</td><td>GATCAGCCCGCGCCCCGTAAAACGGGACGGCCCGCCGCAGGAACCACAAAACTCTGATTT</td><td>120</td></tr>
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<tr><td>Sbjct 97</td><td>GATCAGCCCGCGCCCCGTAAAACGGGACGGCCCGCCGCAGGAACCACAAAACTCTGATTT</td><td>156</td></tr>
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<tr><td>Query 121</td><td>TAGTGTAACTTCTGAGTCTAAAAAACAAATAAATCAAAACTTTCAACAACGGATCTCTTG</td><td>180</td></tr>
<tr><td></td><td>||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||</td><td></td></tr>
<tr><td>Sbjct 157</td><td>TAGTGTAACTTCTGAGTCTAAAAAACAAATAAATCAAAACTTTCAACAACGGATCTCTTG</td><td>216</td></tr>
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<tr><td>Query 241</td><td>GTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTG</td><td>300</td></tr>
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<tr><td>Sbjct 515</td><td>ACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAA</td><td>571</td></tr>
</table>

Sequences producing significant alignments				
Accession	**Description**	**Max score**	**Query coverage**	**Max ident**
GQ505450.1	*Fusarium sp.* NRRL 34033	961	100%	99%
FN397345.1	Uncultured fungus clone H20_P_4_G4	957	99%	99%
EU479918.1	Uncultured soil fungus clone RS5M5c35P	957	99%	99%
JF300441.1	Uncultured fungus clone TH44	953	99%	99%
JF300424.1	Uncultured fungus clone TH49	953	99%	99%
EU479919.1	Uncultured soil fungus clone RS5M5c41P	952	99%	98%
GU932673.1	*Fusarium sp.* CB-1	944	98%	98%
DQ885388.1	*Fusarium sp.* FVS3	941	99%	98%

Macroscopic and microscopic morphology of fungus: Colonies of *Fusarium* on PDA grew slowly, filled the Petri plate and matured in 4-6 days. From the front, colour of colony was pink and as culture became old, pink colour faded slowly. Colonies were evenly distributed in periphery. Molecular method of identification by using polymerase chain reaction (PCR) confirmed the same. The nucleotide sequence alignment, BLAST, correlated with NCBI accession numbers, confirmed the fungus identification up to the genus level (Table I).

Toxicity studies in rats with fungal culture filtrates: Except control group of rats, all experimental rats were weak and depressed. Rats were diarrheic and exhibited severe arching of back. The rats lost of balance on hind limbs and sometimes on forelimbs. There was swollen forehead and conjunctival haemorrhage. Cutaneous haemorrhagic patches on back, scrotum, abdomen, ears and legs region were seen. Rats showed torticollis like signs. There was a significant change in the body weight, blood clotting time, biochemical parameters and hematological parameters in the treatment group of rats. Similar findings were also reported

by earlier workers, where reduced feed intake, diarrhoea, haemorrhages in the stomach and decreased body weight gain was observed in rats due to the presence of apicidin mycotoxin (Park *et al.,* 1999). The clinical signs of diarrhoea, weight loss were also reported in mice, when treated with apicidin derivative SD-2007 for 2 weeks (Kwack *et al.,* 2009).

Neurological disorders were also seen in experimental rats, which was due to the presence of beauvericin mycotoxin in very high concentration in *F. sporotrichoides* (5126.67 µg/kg) fungus infected wheat material and was supported by the earlier findings where beauvericin caused cytolysis (Wang and Xu, 2012). The clinical signs of haemorrhagic syndrome in rats were very much similar to that of the cattle observed under field conditions. Hence the presence of apicidin mycotoxin in in the *F. Sporotrichoides* culture filtrate/ fungal infected wheat material was attributed to such condition. This was further supported by the gross and histopathological lesions in the brain of the affected animals like severe congestion and infiltration of lymphocytes, degeneration, swelling of ependymal cells and congestion. This was correlated with the earlier findings (Kouti *et al.,* 2003).

Multimycotoxin Analysis: The fungus infected wheat materials were subjected to mycotoxins screening and quantification by using LC-MS/MS, in the Multimycotoxin Analysis Laboratory, Department of Pharmacology and Toxicology, Veterinary College, Bangalore. The wheat sample infected with *Fusarium sporotrichoides* had very high concentrations of beauvericin and apicidin mycotoxins *i.e.,* beauvericin 5126.67 µg/kg and apicidin 28.8 µg/kg of infected material.

Biochemical parameters (Table 2) and hematological parameters: biochemical parameters shown significant changes, but there were slight alterations observed in hematological parameters. The study concords with the earlier research conducted, where increased concentration of Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) was observed in mice after treating with apicidin's derivative SD- 2007 for 2 weeks (Kwack *et al.,* 2009). But these cultured materials had very high concentration of beauvericin, which might be responsible for causing hepatotoxicity, due to its inherent cytotoxic activity. The increase in creatinine concentration in these particular groups indicated possible role of toxins apicidin and beauvericin in causing kidney damage.

Gross Pathology and Histopathology: Experimental rats showed cutaneous haemorrhagic patches on back, scrotum, abdomen, ears and legs region. Haemorrhagic spots on eyes, nose and paws were the common finding observed in all the rats. In some rats, serosal surface of stomach, cecum had patchy haemorrhages. There was severe congestion of

heart, liver, lungs, stomach, spleen, testicles, cecum, kidneys and brain. Liver had petechial haemorrhages with necrotic points noticed.

The control group animals revealed normal architecture of all the organs histopathologically. The lesions in the brain of *F. sporotrichoides* culture filtrate administered animals comprised of congestion of blood vessels, edema, and vascular degeneration. Brain revealed severe congestion and perivascular cuffing, swelling of ependymal cells and congestion. Heart showed congestion and haemorrhage with presence of red blood cells in between muscle fibers. The lesions in liver were congestion, degeneration and necrosis of hepatocytes with infiltration of inflammatory cells and periportal biliary epithelial cells hyperplasia. Kidney showed severe congestion along with vacuolar degeneration and necrosis of tubular epithelial cells. The lesions in the intestine comprised of haemorrhages in the villi, villous epithelial cells desquamation, degenerating epithelial cells were present in the lumen with increased goblet cell activity.

CONCLUSION

Based on the present study it could be concluded that, the culture filtrates/ fungi infected material of fungal isolate obtained from mouldy groundnut hay was toxic to rats at the given dose and duration of treatment. The presences of very high concentration of beauvericin and apicidin mycotoxins were responsible for toxicity. Because of the similar clinical signs were exhibited by rats and were correlated with the biochemical, hematology parameters change and during histopathology also. Therefore toxicity observed in the cattle, during field conditions may be attributed to consumption of *F.sporotrichoides* infected mouldy groundnut hay.

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Table 2: The effect of *F.sporotrichoides* culture filtrates on various serum biochemical parameters in rats during sub chronic oral toxicity study

Biochemical parameter	**Days**	**Type of culture filtrate**			
ALT (U/L)		P D Broth (control) Group IV	*F.sporotrichoides* (A) Group I	*F. sporotrichoides* (B) Group II	*F. sporotrichoides* (C) Group III
	0	30.10±2.22	29.21±2.10	28.50±2.29	28.46±2.61
	45	29.83±2.18	69.50±3.01***	60.16±2.15***	44.00±1.87**
	90	32.15±2.94	78.34±5.53***	69.66±3.14***	50.00±4.00***
AST(U/L)	0	81.38±6.57	85.52±5.18	85.43±4.78	85.05±5.50
	45	101.66±5.99	165.83±2.54***	156.67±2.38***	118.50±6.80
	90	107.15±5.23	177.68±4.89***	161.15±5.66***	132.00±9.13**
Serum creatinine (mg/dl)	0	0.41±0.04	0.39±0.05	0.43±0.04	0.38±0.04
	45	0.38±0.03	0.76±0.04***	0.70±0.03***	0.650±0.01***
	90	0.36±0.03	0.94±0.09***	0.80±0.09***	0.68±0.07***
Serum urea nitrogen(mg /dl)	0	35.47±2.15	36.78±2.68	34.51±3.58	35.16±2.24
	45	38.00±3.16	59.72±3.12***	49.62±2.80	37.93±1.41
	90	39.22±1.73	69.78±4.38***	62.01±4.92***	54.50±4.06**

Values are mean ± SE, *** P < 0.001, ** P < 0.01, * P < 0.05, n = 20