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COMPARATIVE CYTOTOXIC ANALYSIS THROUGH MTT ASSAY OF VARIOUS FUNGI ISOLATED FROM RICE STRAW FEEDINGS OF DEGNALA DISEASE AFFECTED ANIMALS

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ABSTRACT

The objective of current study was to investigate the comparative cytotoxic effects of various fungi suspected as the etiological agent(s) of Degnala disease. Several toxigenic potential containing candidate fungi extracted from the rice straw feedings of Degnala affected animals were subjected for comparative cytotoxic analysis through MTT assay on vero cell line. There were five different species / genera of candidate fungi including *Aspergillus flavus, A. niger, A. terrus, Fusarium* and *Penicillium* all with variable toxigenic ability. Mycotoxin containing extracts of all these fungi were added in the DMEM+F-12 (with 5% FBS) media used for the exponential growth of vero cell line on 96 well cell culture plate. After an incubation period of 24 hours all the experimental groups were compared with the control on the basis of optical density (OD) value. *Fusarium* showed a very significant (p-value = 0.006) decrease in the number of live cells, then was the *A. flavus* (p-value = 0.039) as compared to the control. The current findings revealed that various toxigenic fungal isolates are not having equal cytotoxic potential. This study can be helpful in locating the actual cause of Degnala disease in bovine.

Keywords: MTT assay, Degnala disease, Cytotoxic, Mycotoxin, Toxigenic fungi.

INTRODUCTION

Rapidly increasing world population with gradually decreasing resources of food in the under developed or less developed countries of the world is creating an alarming situation (Smith, 2013). Spoilage of the food ingredients during storage period is a serious problem which not only worsens the quality of food nutrients but also adversely affects the health status of animals and the human beings (Hashmi *et al.* 2006; Gallo *et al.* 2015).

In scarcity season farmers have to move towards the utilization of valuable cash crops or the by-products of cereal crops (Riaz *et al.* 2008). Among these by-products is the feeding of wheat straw and rice straw for fulfilling the appetite of large ruminants. Especially during the season of drought and winter, feeding of rice straw to cattle and buffaloes is commonly practiced in the rice production areas of Punjab, Pakistan. During the period of draught and low temperature in winter season, when humidity is increased due to rain fall, storage of rice straw near water logged areas, flooded conditions or storage immediately after harvesting, may lead to the production of fungal colonies on stored rice straw (Kalra and Bhatia, 1990). Feeding of this fungal affected rice straw can lead to the production of Degnala disease (Irfan, 1971; Maqbool *et al*. 1998; Jadhav *et al.* 2003; Dandapat *et al*. 2011; Karki *et al*. 2012; Kumar, 2016).

These fungi produce their affects through their secondary metabolites called mycotoxins which have ill effects on animals, humans and agricultural products resulting in diseases and economic losses. Some fungi are capable of generating more than one type of mycotoxins while some mycotoxins are created by more than one kind of fungi (Pandya and Arade, 2016). According to Sinha *et al.* (2001) mycotoxin and mycotoxigenic fungi were found in straw samples of paddy, maize and wheat. In stored straw of different cereal grains, the common occurrence of the ochratoxin A, zearalenone and citrinin was observed from four mycotoxigenic fungal isolates including *Aspergillus, Fusarium* and *Penicillium.* The nature of substrates, relative methods of storage and prevailing environmental conditions govern the association of fungal organisms as well as their incidence. Both toxigenic and non-toxigenic isolates diverge in the pattern of the metabolism, although they have same morphology and same growth rate (Sinha *et al*. 2001). Ruminants occupy a wide agricultural niche and hence are exposed to a variety of mycotoxins in variable quantities and conditions, creating tough challenges for veterinarians in making diagnostic interpretations on contaminated forages, grains and feed stuffs (Mostrom *et al.* 2011).

At present it is known that different genera of fungi have been extracted from rice straw feedings of Degnala affected animals as reported by Maqbool *et al*.

(1998). Some authors correlated different specific fungi like Penicillium and Fusarium as the causative agent of Degnala disease. They claimed such findings by simply extracting some specific fungi from affected animals or through fungal contaminated feeding trials (Dandapat *et al*. 2011; Karki *et al*. 2012). According to Sinha *et al.* (2001) different variety of mycotoxins was extracted from rice straw storages indicating toxigenic nature of different fungi contaminating this feed commodity. In this work we have tried to apply another scientific technique of MTT assay to converge the number of many fungi to a single, most cytotoxic one for a more precise analysis of the etiological agent of Degnala disease.

MATERIALS AND METHODS

Screening for Toxigenic Potential: Different fungi (n=85) isolated from rice straw samples (n=40) collected (Thrusfield, 2005) in winter season taken from the feedings of Degnala affected animals were identified on the basis of macroscopic and microscopic characteristics. All these isolates were cultured for heavy toxin production on Sabouraud's Dextrose Broth (SDB) to evaluate their toxigenic potential. In each flat bottom flask having 150 mL SDB, 10^6 spores of each separate fungus were added, incubated at 28C^o for 45 days in dark conditions, shaked vigorously daily for 2-3 times to avoid from sporulation.

Mycotoxins were extracted by standard extraction method described by *Association of Official Analytical Chemists* (AOAC, 1995a). Shortly, after 45 days each SDB flask was autoclaved to stop any further metabolic activity, well mixed by automatic shaker and then out of this, fungus cultured solution of 12.5 mL was taken into another 250 mL flat bottom conical flask. For mycotoxin extraction, 45 mL chloroform, 5 mL methanol, 5 mL distilled water and 2.5 g Sodium chloride was added. The mixture was shaken for 30 min on a shaker at 150-160 rpm. Mixture was filtered through whattman #4 filter paper and filtrate was collected in a glass beaker. Beaker was placed in a water bath at 60°C to evaporate the filtrate. The residue (TLC stock solution) was reconstituted in 2 mL chloroform, transferred in eppendorf tubes and processed further or stored at 4°C. This stock solution was used to perform TLC and HPLC for determining and quantification of toxigenic strains respectively.

High Performance Liquid Chromatography (HPLC): Mycotoxin producing fungal species were confirmed and comparatively quantified through HPLC by taking standard solutions of Aflatoxins B1 (25 ng/mL) and B2 (7.5 ng/mL) prepared in the acetonitrile solution as described by AOAC, (1995b). TLC stock solution (1 mL) was taken in a 50 mL beaker containing 9 mL of acetonitrile, 1 mL of distilled water along with 0.25 g of NaCl, shaked well at a rate of 120 rpm for one hour. This solution was filtered through whattman #41 filter paper and the filtrate was collected in a glass beaker, evaporated through water bath at 60^{o}C till 1 mL solution was remained which was loaded into specific vials used to keep in the "solution up-taking HPLC loader assembly". HPLC was performed with the help of Agilent-1100-Series^{TM},USA, using fluorescent detector at 360nm (Excitation) and 440nm (Emission). Height, area and time of appearance of the peaks related to each sample were compared with the loaded standards and concentration of mycotoxins in the solution of each fungal isolate was ultimately determined.

Screening for Cytotoxic Potential: Comparative cytotoxicity was analyzed of only those fungal isolates which were positive for mycotoxin production ability (toxigenic strains). It was performed by Methyl thiazol tetrazolium assay (MTT assay) on vero cell lines as mentioned by Muller *et al*. (2017) with some minor modifications as mentioned below.

MTT stock solutions: One candidate from each of the separate fungal genera/species origin was added in this comparison study. Stock solutions of all the respective candidate fungi were prepared from TLC stock solution. One mL of the TLC stock solution was taken, Chloroform was evaporated and addition of 1 mL of Dulbecco Modified Eagle Medium (DMEM)+F-12 media (containing L-Glutamine and inactivated Fetal Bovine Serum) was carried out and this solution (called as MTT stock solution) was stored at 4^{o}C till further use.

Chemicals and Cell culture availability: MTT Solution was prepared by adding 1 mg MTT dye in 1 mL of DMEM (having FBS) solution and stored at 4^{o}C for further use. Vero Cells culture was obtained from cell culture bank of Microbiology department, University of Veterinary and Animal Sciences, Lahore. Streptomycin, Ciprofloxacine, Penicillin and Amphoterecin B were added at the rate of 100µg/mL, 50µg/ml, 100IU/mL and 50µg/mL respectively in the DMEM+F-12 media.
The growth medium was prepared by adding 5 % fetal bovine serum [FBS (Capricorn Scientific GmbH, Ebsdorfergrund, Germany)] in DMEM+F-12 at the time of use while the maintenance medium was prepared by adding 1 % FBS in DMEM+F-12. MTT dye was purchased from MP Biomedicals, LLC, Germany and used in the trial.

Cell culture growth and Toxin addition: Each cell culture plate was maintained at 37°C temperature in a humidified incubator (Sanyo, USA) containing 5% CO_2 and 95% air. Hemocytometer was used to count cells to be added in each well. Suspension of cells in exponential growth period was prepared and was seeded at the rate of 10^6 cells per well in a 96 well plate having 90 µL of cell

suspension solution in each well. Cells were allowed to attach and grow for 24 hr before next treatment with mycotoxin containing solution from different fungal isolates.

Addition of 10 µL of MTT stock solution after an incubation time of 24 hr with ten-fold dilutions were carried out in a descending order starting from the first well till 12th well for each separate isolate to be compared with other ungal groups and the negative control group.

Cell viability / OD value analysis: After an exposure of the combined effect of mycotoxins for 24 hr, the cell culture solution was discarded. Then 100 µL of MTT solution (was added in each well and again incubated at 37°C for 4 hr. After discarding the supernatants of the reaction, 100 µL of DMSO (dimethyl sulfoxide) was added in each well and mixed vigorously to dissolve the intracellular formazan product. The absorbance (OD value) of each sample was spectrophotometrically measured at 595 nm using a micro-plate ELISA reader.

Statistical Analysis: Data on OD value obtained from all the serial dilutions of each individual fungal isolate were statistically compared through one way ANOVA using SPSS software and probability (p) value <0.05 was considered significant (Steel *et al*., 1997). The results thus obtained were used to rank all the isolates in a descending cytotoxic order.

RESULTS

Toxigenic Isolates: There were 11 fungal isolates related from 5 different groups (species/genera of fungi) having toxigenic ability with following combination.

Aspergillus flavus (6 isolates of blue fluorescence)**:** ***A. niger*** (1 isolate of blue fluorescence)

A. terrus (2 isolates of blue fluorescence)**:** ***Penicillium*** (1 isolate of green fluorescence)

Fusarium (1 isolate each, of green fluorescence).

Toxigenicity Confirmation and Quantification: Mycotoxin producing fungal species analyzed by TLC, were confirmed and quantified comparatively with the help of HPLC by comparing the standards of AflatoxinsB1 andB2. It was noted that all of these isolates (n=11) were toxigenic with variable quantities of mycotoxins. From all of the 06 isolates of *Aspergillus flavus* and out of 02 isolates of *Aspergillus terrus,* sample having highest quantity of Aflatoxin B1 were S 8.5 (Sample I.D) and G11.2 (Sample I.D) respectively. Indicating that all the isolates of a single species are not equally toxigenic. Regarding *Aspergillus niger*, *Penicillium* and *Fusarium* only one isolate was determined to be toxigenic as analysed through TLC and HPLC from their different isolates separately (Figure. 1-2).

Cytotoxic Potential: It was performed for comparative cytotoxic potential (lethal for the live cells) analysis. Five isolates out of all the eleven toxigenic fungi (one from each genera/species identified) were subjected for this comparison through MTT assay.

Cell Culture Analysis: After an incubation period of 24 hr with fungal solution in each of the separate row, microscopic visualization was performed before the addition of MTT solution. It was seen that in different competing fungi most of the 1st two to four wells were with less number of attached cells compared to the control (Figure 3). It was the first indication about the expected outcome of the toxin containing solution addition in the cell culture assembly.

MTT Assay: By taking optical density (OD) value of each well in all the five rows selected for separate fungal isolate of different origin along with negative control (6th row), one way analysis of variance (one way ANOVA) was performed taking probability (*p*-value) value to be significant when <0.05, to access most lethal fungal solution in all the five isolates compared to the negative control. It was shown that there was a significant difference (0.012) between the mentioned groups related to their OD value. Significant value (*p*-value), standard error (SE) and 95% confidence interval values taken by the statistical analysis through one way ANOVA by calculating the OD value obtained after MTT Assay in different fungal groups are mentioned below (Table. 1; 3.2and Fig. 4; 5). It was calculated that *Fusarium* (S 8.1 isolate) was the most lethal with S 8.5 (*Aspergillus flavus*) on the second number with a probability value (*p* value) of (0.006) and (0.039) respectively.

Final Conclusion: Finally it was concluded that out of all the 85 suspected fungal isolates obtained from the rice straw feedings of Degnala affected animals ***Fusarium*** was the most cytotoxic isolate.

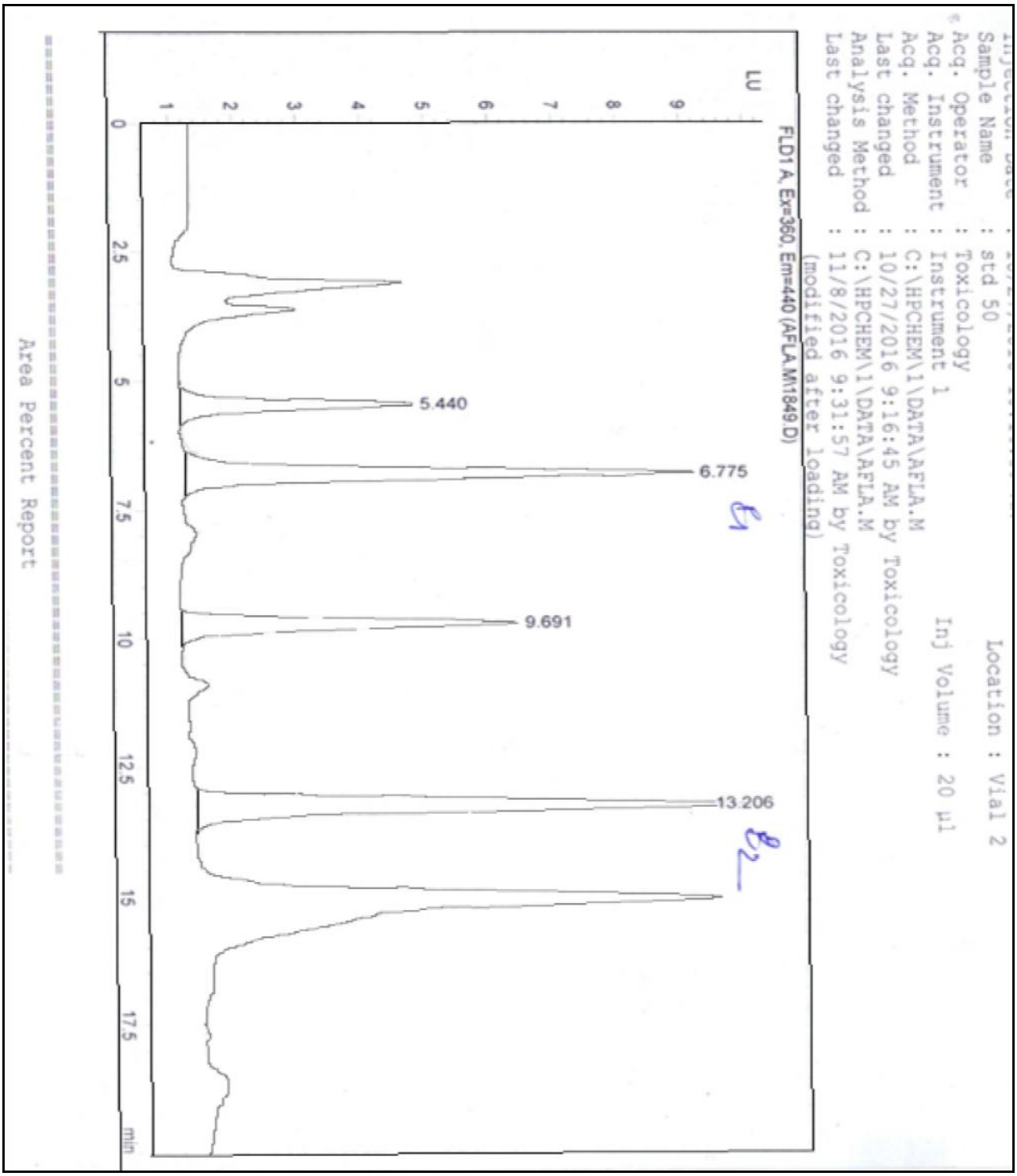

Figure 1: Columns of B1 and B2 standard solutions having their respective time verses concentration and area obtained curves in the HPLC graph.

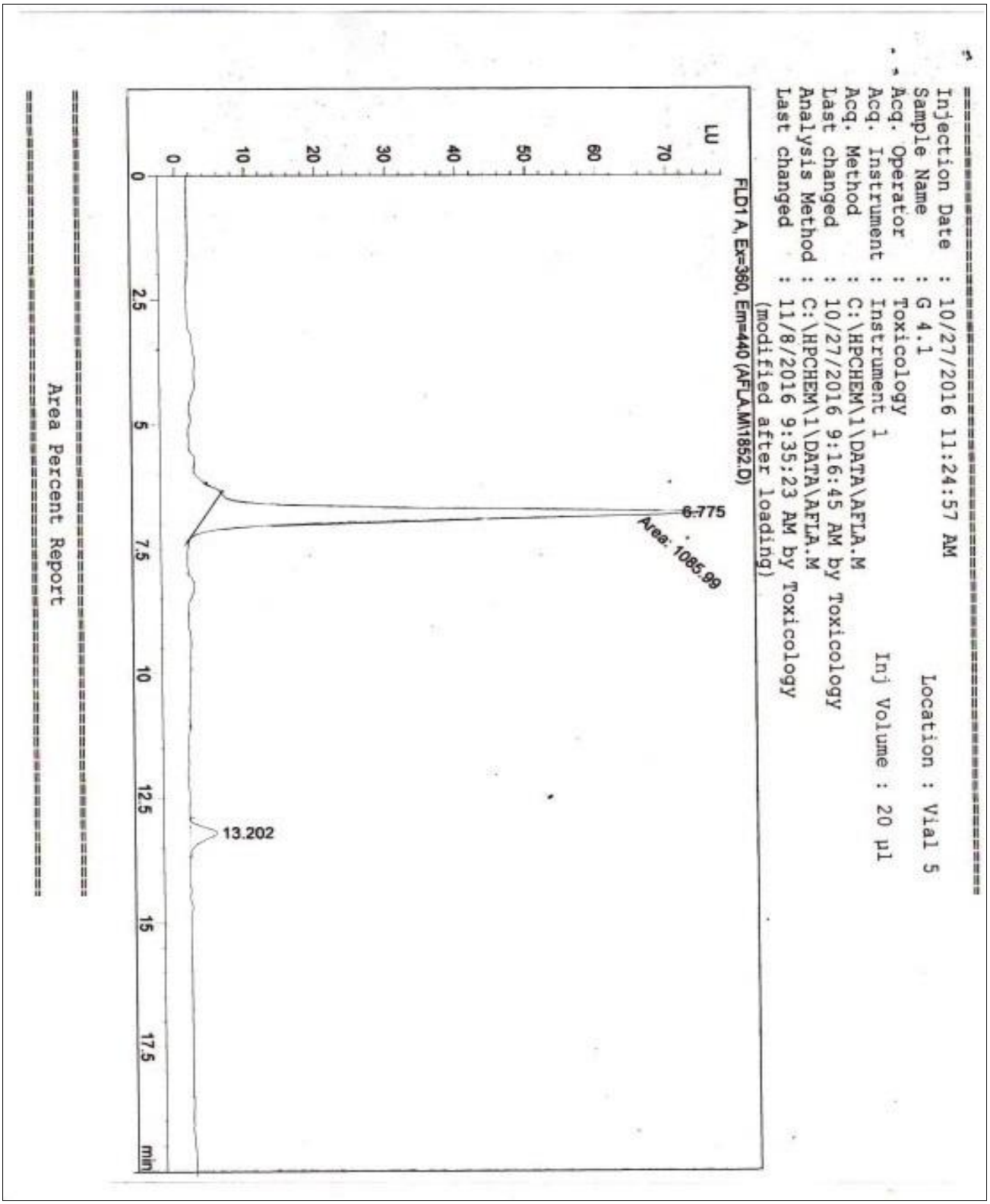

Figure 2: Time verses concentration curves compared with the standard curves in *A. Niger* showing high concentration of B1 Aflatoxin.

Table 1: Analysis of variance for different fungal isolate dilutions compared through MTT Assay on the basis of OD value

Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.306	5	.061	3.196	.012
Within Groups	1.265	66	.019		
Total	1.572	71			

Table 2: Evaluation of the significant difference (p<0.05) between control and experimental groupsfor OD value indicating comparative cytotoxicity.

Sr. No.	Isolate ID.	Fungal species	95% Confidence interval	SE +	*P*-Value
1=A	S 8.5	*A. Flavus*	-0.31720 to -0.02597	0.040794	0.039
2=B	G 11.2	*A. terreus*	-0.25420 to 0.03703	0.038281	0.399
3=C	G 4.1	*A. Niger*	-0.25520 to 0.03603	0.047251	0.389
4=D	S 8.1	*Fusarium*	-0.35503 to -0.06380	0.044654	0.006
5=E	G 12.1	*Penicillium*	-0.28236 to 0.00886	0.035404	0.165

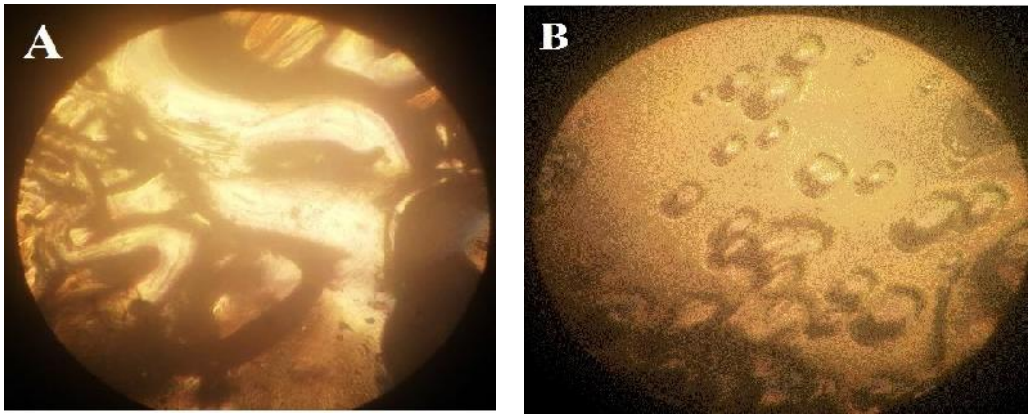

Figure 3: A is the Microscopic view of a single well from Negative control line indicating the presence of more number (darker colour) of live cells after an exposure time compared with mycotoxin containing solutions. B is the Microscopic view of 3rd well in the *Fusarium*,s 12 well line, showing less number of viable / attached cells (less dark area, cells with swellings, deshaped appearance as compared with the Control seen in (fig. 3 A) after incubation with MTT stock solution having specific quantity of relevant mycotoxin(s).

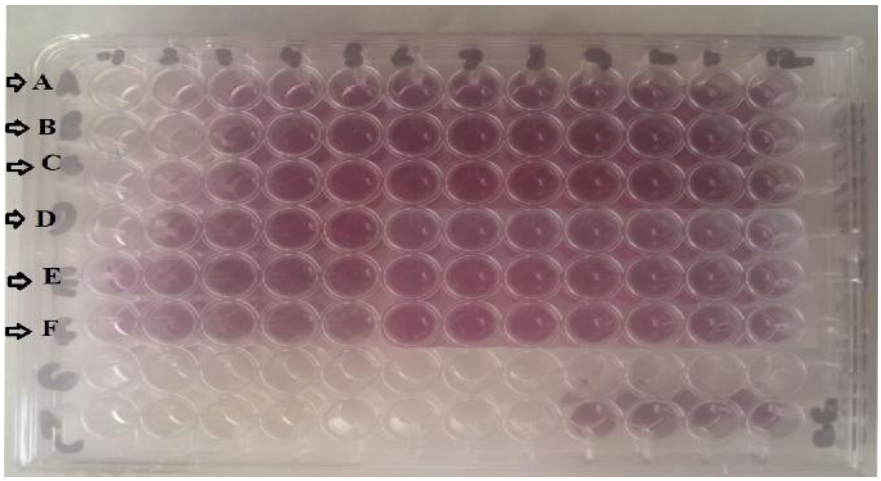

Figure 4: Purple coloured for mazan (more dark colour=more cell survival rate) in the 96 well plate processed after MTT assay. A= *A. flavus*, B= *A. terrus*, C= *A. niger*, D= *Fusarium*, E= *Penicillium* and F line is Negative Control (only DMEM media).

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.756	0.780	0.815	0.966	1.019	1.073	1.086	1.118	1.128	1.132	1.144	1.135
B	0.779	0.779	0.931	1.023	1.086	1.120	1.087	1.091	1.146	1.132	1.012	1.183
C	0.846	0.846	0.961	1.092	1.122	1.147	1.139	1.80	1.201	1.209	1.012	1.188
D	0.792	0.792	0.803	0.818	0.848	0.931	0.948	1.104	1.198	1.164	1.219	1.161
E	0.876	0.954	0.993	1.131	1.144	1.153	1.071	1.089	1.084	1.090	1.169	1.115
F	1.211	1.179	1.193	1.294	1.207	1.32	1.292	1.271	1.276	1.249	1.125	1.284

Figure 5: Computed results of 96 well plate after MTT assay showing the quantitative results related to cell survival in the form of OD value analysis through ELISA reader. A= *A. flavus*, B= *A. terrus*, C= *A. niger*, D= *Fusarium,* E= *Penicillium* and F line is negative control. Same value of *Fusarium* is noted at 7th well compared with 4th of *A. flavus* while in case of other isolates, at or before the 3rd well, this value was obtained showing a better cell survival rate compared to *Fusarium* and *A. Flavus*.

DISCUSSION

Basic theme of this work was to analyze the diagnosis of Degnala disease in large ruminants dwelling in the rice producing areas of Punjab, Pakistan. As till now the causative agent(s) of this ailment is not well defined and a little scientific work has been done towards its diagnosis.

Appearance of the disease only in winter season gives a clear indication about the involvement of some biological factor(s) like fungi which need a specific temperature and other environmental factors including humidity (high water contents). This seasonal logic gives a contrary result compared to that of an idea that Selenium is involved in the etiology of Degnala disease as reported by many researchers (Dhillon and Dhillon, 1990; Datt *et al.* 2014). Feeding trials after contaminating rice straw with specific fungi like Fusarium, conducted by many other workers like Dandapat *et al.* (2011), this can be seen that if selenium is involved then there should be Degnala disease in all the animals which were given rice straw irrespective of rice straw origin and any fungal contamination. Toxicity of Selenium occurs mostly in arid ecological zones particularly in those areas where an annual rainfall mean is recorded to be less than 500 mm. On the other hand an excessive rainfall can promote a leaching out process of the present Selenium from soil (Sikdar *et al.* 2000). While in our study area an annual rain fall is very high as compared to those areas where rice cultivation is not practiced.

According to Sikdar *et al.* (2000) histopathological examination showed necrosis, eosinophilic infiltration in the subcutaneous connective tissues and loss of architectural details of the affected parts of the body but there was no growth of any fungal isolate from tissue scrapings from gangrene affected areas which strengthens this assumption that pathogenesis in Degnala disease involves mycotoxins instead of a local involvement of some saprophytic fungi.

In the present study 11 fungal isolates were reported to be toxigenic. These isolates were extracted from the rice straw being fed to cattle and buffaloes of rice growing areas of Punjab. The results of TLC and HPLC showed presence of mycotoxin producing present on the feed of the affected animals. This was the first study of such kind and there is no report indicating toxigenic strains in the diet of Degnala disease infected animals. Although Sinha *et al.* (2001) reported mycotoxins present in rice straw heaps present in an open environment but did not show any direct link with Degnala disease like our investigation.

MTT assay was the most turning-point related to the actual diagnosis of Degnala disease in our study work. As all the five fungi were of different origin (different species/genera) and they may produce a highly variable quantity and types of mycotoxins and this is practically impossible to see an overall combined effect of hundreds of different types of mycotoxins (Fink-gremmels, 2008). At the same time cytotoxic potential of non-fluorescing mycotoxins can-not be ignored (Chan *et al*. 2016). Combined effect of mycotoxins has also been studied by Sun *et al*. (2015) through MTT assay on various cell culture lines.

Involvement of *Fusarium* in the production of Degnala disease has been documented by various authors but they have concluded its etiological nature by different other approaches like feeding of fusarium contaminated rice straws to the bovine animals (Dandapat *et al.* 2011; Kathiriya *et al.* 2017). In the study of Irfan (1971) along

with isolation of different fungal species from rice straw being fed to Degnala affected animals, T-2 toxin was also reported to be extracted from the rice straw feeding. This toxin is produced by different strains of *Fusarium* during their growth on different feed based substances (Matejova*et al*. 2017). Due to the presence of T-2 toxin in contaminated rice straw feedings of Degnala affected animals different authors after this work postulated that *Fusarium* is the causative agent in Degnala disease as reported by (Kalra *et al.* 1997; Sikdar *et al*. 2000; Dandapat *et al*. 2011; Kumar, 2016). All of these groups were able to isolate *Fusarium* from the rice straw which was offered to the affected animals.

Dandapat *et al.* (2011) carried out a study in which he collected 12 *Fusarium* isolates from Degnala positive animal's feedings. They reproduced Degnala disease by feeding experimentally *Fusarium* infested rice straws to the buffalo calves. Although there have been production of experimental disease through this approach but this does not look practically possible that by collecting samples of rice straws from an open environmental situation through a vast area (four Indian states), there may be the production of only 12 fungal isolates and they were of only one genus.

This study is giving a more logical sequence towards the diagnosis of Degnala disease supported by some of the latest scientific techniques like HPLC and MTT assay on vero cell culture line. After all it can be postulated from our work and from the work of Kalra *et al.* (1997); Sikdar *et al.* (2000); Dandapat *et al.* (2011); Kumar (2016) and Kathiriya *et al.* (2017) that *Fusarium* is the causative agent in Degnala disease and this assumption can be traced back to the second ever reported work in Pakistan after Shirlaw (1939) by Irfan (1971) in a context of the T-2 toxin and our finding strengthens this assumption.

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