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Characterization and *Invitro* Cytotoxicity of T-2 Toxin Isolated from Corn

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ABSTRACT

Aim: Main objective of the study is to characterize and evaluate *invitro* cytotoxicity of T-2 toxin isolated from corn.

Methodology: The fungal strain was isolated by standard blotter method; morphological identification was done using scanning electron microscope (SEM). The fungal strain was identified as *F. sporotrichioides* based on its cultural and morphological characteristics and internal transcribed spacer (ITS/ITS4) primers. The toxin was characterized using thin layer chromatography (TLC), HPLC and Fourier Transform Infrared Spectroscopy (FTIR)

Result: 100ml of culture filtrate yielded 10.126µg of toxin. The functional groups of toxin were identified by (FTIR) method. *Invitro* cytotoxicity of T-2 toxin was evaluated on human keratinocytes, hepatoma and neuron cell lines. HaCaT and HepG2 cells were found to be more sensitive than SHSY5Y to T-2 toxin exposure at 50-100ng and 100-200ng.

Discussion and conclusion: T-2 toxin was extracted and characterized from corn samples, by analytical and molecular methods in addition FTIR analysis verifies the functional groups of T-2 toxin. Further *invitro* cytotoxicity was evaluated using different human cell lines. Comparably skin and liver cell lines found more sensitive to T-2 toxin exposure. Sensitivity of the cell lines to the toxin at ng may be due to the purity of the toxinKey Words: Plasma Fibrinogen, Haemodialysis, Diabetes.

INTRODUCTION

T-2 mycotoxin, a low molecular weight (250-500 Daltons) non-volatile compound produced by fungi of the genera *Fusarium*, *Myrothecium*, and *Stachybotrys*[1]. Predominantly *F. sporotrichioides* is responsible for the production of T-2 toxin and HT-2 toxin. The most important factors that influence T-2 toxin production are weather conditions and moisture content. T-2 toxin is produced under a wide temperature range (0 to 32 °C), with maximum production at temperatures below 15 °C [2, 3]. T-2 toxin is tremendously heat stable and is the only mycotoxin known to have been used as a bio threat weapon delivered via food or water sources, as well as, by means of droplets, aerosols, or smoke from various scattering systems and exploding munitions [4, 5].

Chemically, T-2 toxin is a tetra cyclic sesquiterpenoid with 12, 13- epoxytrichothec-9-ene ring system[6] with hydroxyl group at the C-3 position, acetyloxy groups at C-4 and C-15 positions, atom of hydrogen at C-7 position and an ester-linked isovaleryl group at the C-8 position [7]. The toxicity can be reduced by the cleavage of esters [8, 9]. T-2 toxin is a well known inhibitor of protein, DNA and RNA syntheses and also it is known to interfere with the metabolism of membrane phospholipids and increase the level of liver lipid peroxides [10, 11].

The T-2 toxin is well-known to cause acute and chronic toxicity, as well as alimentary toxic aleukia (ATA) and Kashin-Beck Disease (KBD) in humans and animals. It has been shown to cause alteration of blood–brain barrier (BBB)[12-15]. T-2 toxin toxicological effects have been summarized in Food and Agricultural Organization (FAO), World Health Organization (WHO), Council for Agricultural Science and Technology and the Expert Committee on Food Additives [16, 17]. European Union states; T-2 toxin as a common contaminate to cereals and cereal based products and there are sev-

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eral reports from different regions of the world associating damage of agricultural field by T-2 toxin and its producing organisms and it extends to humans and animals [18,19]. Sensitive and precise analytical methods have been developed for measuring mycotoxins in cereals and cereal based products in order to gauge the risk of animal /human exposure [1-3].

T-2 toxin toxicity is the most severe compare to other trichothecenes. Major target organs include skin, liver, nerves etc. In the present study, the fungal species growing on corn was isolated and identified as T-2 Toxin producing organism by molecular characterization and T-2 Toxin identified by FTIR analysis and toxicity of the toxin produced was evaluated in human keratinocytes, hepatoma, and neuron cells *in vitro*.

MATERIALS AND METHOD

Potato dextrose agar, Potato dextrose broth, glutaraldehyde(Himedia Bangalore), methanol, ethyl acetate, ethanol, silica gel, (SRL, Bangalore), T-2 toxin (Chempure Mumbai), ethidium bromide (Sigma Banglore) were used.

Isolation of *Fusarium species* from corn sample

Isolation of *Fusarium*was carried out by standard blotter method. Corn samples were collected from different localities. Corn seeds were incubated for 3-5 days under moist condition on petriplates. As the spore of fungi developed into mycelia, it was transferred aseptically to sub-culture on potato dextrose agar to develop pure colonies, and the colony morphology was examined under microscope.

Scanning electron microscopy

After observation under microscope the sample subjected to scaning electron microscopy (SEM). The mycelia were fixed in glutaraldehyde in 0.1M PBS for 30 min., washed with 0.1M PBS and dehydrated by immersing in ice cold ethanol for 10min. The dehydrated samples were smeared on silver stub like a thin film, and were coated by cathodic spraying (Polaron gold). The SEM observations were made using a ZEISS Instrument (EHT=15.00 kv, signal A=VPSE G3).

Fusarium species DNA Extraction

7 days old mycelium mat was collected and frozen at -80^{0}C and lyophilized. Lyophilized sample were homogenizedinlysis buffer and extraction was carried by phenol chloroform method. DNA was quantified in nanodrop spectrophotometer, PCR was performed to amplify DNA [20].

Polymerase Chain Reaction

The PCR of fusarium was performed using universal primers (ITS/ITS4 primers). Standard protocol was followed to amplify the DNA, after successful amplication products were inspected by agarosegel electrophoresis containing (0.01%) ethidium bromide and observed under UV(Ultra violet) light. Gels were documented using Gbox (GE Health Care, Mumbai).

Fusarium culture for toxin production

Culture preparation

F. sporotrichioides MTCC 1894 Venkataramana et al. [21]was used as the test organism and was obtained from Department of Microbiology, Defence Food Research Laboratory, Mysuru. The fungus was purified and harvested at room temperature on potato dextrose broth (PDB).

Toxin extraction and purification

The *F. sporotrichioides* was inoculated to PDB broth and incubated at for 3 weeks incubation method was followed according to Busman et al., [22] with slight modification. After incubation the broth was filtered and the filtrate was collected and mixed with equal volume of ethanol and incubated for 24 hrs, then it was concentrated using flash evaporator. The extracted toxin was stored in amber vial for further characterization.

Thin Layer Chromatography (TLC)

T-2 toxin was extracted and detected by thin layer chromatographic method[23].The plates were prepared by adding 50 ml of distilled water to 25g silica gel powder and mixed uniformly to get homogenous mixture (slurry) and then drawn on glass plates of 20 x 20cm using TLC applicator with thickness of 2mm and allowed to dry. The coated plates were activated by drying in forced draft hot air oven at 110^{0}C for one to two hours. The activated plates were then cooled to room temperature. Extracted toxin (crude) was spotted on TLC plate with capillary tube and allowed to run using toluene: ethyl acetate: formic acid (6:3:1) as mobile phase. After that the plate were dried, viewed under UV light (366nm) and calculated R_f value.

High performance liquid chromatography (HPLC)

Reverse phase HPLC with C-18 column was used. The extracted toxin dissolved in methanol was passed through membrane filters, Methanol: water (70:30) mixture was used as mobile phase. 20 µl of the toxin was injected to HPLC column and made to run through the column for 20 min. The flow rate of the column was adjusted to 0.7 ml and wavelength to 228nm. Toxin was detected in UV detector system. Standard toxin was also injected to determine the appropriate peak and concentration of toxin extracted[23].

Fourier transformed infrared spectroscopy

The functional groups of extracted toxin were anlyzed using

FTIR (Nicolet 380 Thermo) based on the peak values in the region of IR radiation. The toxin was passed into the FTIR, the functional groups of the compounds were separated based on their peaks ratio. The FTIR spectra were recorded in the absorption range between 4000 and 500cm^{-}1.

Cell Culture Studies

The Human skin cell line HaCaT, human liver cancer cell line HepG2 and human neuroblastoma cell line SHSY5Y was procured from National Center for Cell Sciences, Pune, India. The SHSY5Y and HaCaT cell lines were cultured in DMEM/F-12 mixture and HepG2 cell line were cultured in MEM/F-12 mixture supplemented with 10% FBS, anti-mycotic solution (Sigma, St.Louis, MO, USA). Cells were maintained and incubated at 37°C in a humidified atmosphere and 5% CO_2 atmosphere 95% air at 37^{0}C. The three cell lines were treated with different concentration of T-2 toxin (ng) for 24 hr.

Cell viability assay

To asses the changes in the metabolic activity of the cell MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was used. The Tetrazolium dye used to measure the cell proliferation and cell viability. The HaCaT, HepG2 and SHSY5Y cell were seeded in different 96 well plates and treated with T-2 toxin at different concentration. After treatment incubated with MTT (0.5 mg/ml) at 37^{0}C for 4hrs and the formed formazan crystals dissolved in DMSO. The absorbance measured at 540nm (VERSA max Hidex plate chameleon TM V (Finland)). The cell viability expressed as percent of control.

Lactate dehydrogenase (LDH) leakage assay

Lactate dehydrogenase (LDH), leakage a biomarker of cellular cytotoxicity and cytolysis. Cell injury was assessed by measuring the LDH efflux to the media after cells has been treated for 24 hr. The protocol following the manufacturers' instructions (LDH-estimation kit (Agappe-11407002, Mysore, India)). The HaCaT, HepG2 and SHSY5Y cell were seeded in different 24 well plates and cell count set as 1X10^4 cell/well. The cells were subjected to different concentration of T-2 Toxin. After the treatment the cells were centrifugedat 2500Xg for 5min at 4^{0}C and supernatant was collected and measured the LDH activity estimated.

Propidium iodide staining

The HaCaT, HepG2 and SHSY5Y cells were seeded at 1X10^{4}cell/well into a six well plate. At 90% confluence, the cells were treated with T-2 toxin and incubated for 24 hrs. After incubation the cells were washed with PBS and fixed with 100% ethanol. The fixed cells stained with 100ng/ml propidium iodide for 20 min and observed under fluorescent microscope.

Statistical analysis

The data were represented as the mean ± SD. Experiments were analyzed using one-way ANOVA followed by Tukey post hoc test. Statistical significance is indicated *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$.

RESULTS

Morphological observation of isolated sample

Morphological identifications of the fungal isolates were made using the criteria of [24-26]. The isolated sample on PDA formed cottony colony brownish, thread like filaments the hyphae and each branching contains aerial mycelia, clusters of hyphae, called sporodochia. These species have irregular shaped globular microconidia, that are measured 5-7 µm in diameter (Figure 1).

Microscopic observation of isolated hyphae

The microscopic observation was done in inverted microscope and scanning electron microscope (40 x magnification).The isolated hyphae were in tubular shape and conidia was identified with branches. The mycelia were measured in inverted microscope found to be 3.5µm and in scanning electron microscopy measured around 1-5 µm (Figure 2).

Molecular Identification

The morphological identifications were further confirmed by molecular method (PCR) (Figure 3). A single band of 500bp was obtained from the isolate and that was compared with standard culture of *Fusarium sporotrichioides* and amplified with ITS/ITS4 primers.

Thin Layer Chromatography (TLC)

The samples showed sky blue colour bands under UV transilluminator at 365nm. $R_f$valueof T-2 toxin on silica gel found to be 0.48mm, comparable to standard T-2 toxin (Figure 4).

High performance liquid chromatography (HPLC)

High performance liquid chromatography was used to quantify the toxin in the extract with UV detector. The amount of toxin was found to be 10.126 µg from 100ml broth culture (Figure 5).

Fourier transformed infrared spectroscopy

FTIR is qualitative and quantitative analytical technique for identification of functional groups of organic, inorganic samples and chemical compounds. The extracted sample revealed the presence of alkanes, alkenes, esters, alcohols and carboxylic acid, compared with standard (Figure 6 and Table 1 and 2).

Cell viability assay

Cell viability was determined by Tetrazolium dye for T-2 toxin with increasing concentration on three different cell lines: HaCaT, HepG2 and SHSY5Y. The results showed that, after 24 hr exposure T-2 toxin decreased cell viability in a dose dependent manner. 50-75ng T-2 toxin was sufficient to reduce viability of HaCaT cells is 50-70% for HepG2 cells 100-150ng of toxin was required and for SHSY5Y 200-250ng was required (Figure 7). Compare to HaCaT and HepG2 cells SHSY5Y showed increased cell viability. The HaCaT cell line more sensitive to T-2 toxin.

Lactate dehydrogenase (LDH) leakage assay

The cytotoxicity of T-2 toxin further carried out by assuring LDH activity in the culture media. The LDH a cytosolic enzyme leakes into the surrounding medium if the plasma membrane gets damaged and is used to asses membrane damage with T-2 Toxin challenge of HaCaT, HepG2 and SHSY5Y cells in dose dependent manner. As cell viability showed that HaCaT is more sensitive to T-2 Toxin. The highest LDH release was found in HaCat cells 50-75ng, followed by HepG2 (100-150ng) and SHSY5Y (200-250). As shown in Figure 7(C), The shape of T-2 Toxin treated cells become irregular and destruction of monolayer was observed and compared with control cells.

Propidium iodide staining

To confirm the cytotoxic effects of T-2 toxin on cell lines, nuclear condensation was studied by Propidium iodide staining. The Propidium iodide does not pass through viable cell membrane however reach the nucleus and it can pass through the damaged membranes. In Control cells had minimal propidium iodide stained cells, while treated groups had increased number of propidium iodide stained cells (Figure 8).

DISCUSSION

T-2 toxin is a type A trichothecene mycotoxin produced by different fusarium species, including *F. sporotrichioides, F. poae and F. acuminatum*[27-29]. These species grow on a variety of cereals, grains, particularly in oats, barley, rye,wheat, maize, rice, beans, soybean and in some cereal-based products especially in cold climate region or wet storage condition [30]. Several surveys have revealed *F. sporotrichioides* causes root, stem and ear rot, with severe reductions in crop yield. In addition *F. sporotrichioides*is capable of producing T-2 toxin which can infect pre harvest plants or stored grains[31,32.]

There are an array of studies on fusarium isolation, its characterization and inhibition of toxin producing species. The present study focused on molecular and analytical characterization of T-2 toxin and T-2 toxin producing organism. FTIR characterization was unaccustomed method for exploration of functional groups in microbiology. Fusarium mycotoxin can be produced on every part of the plant, hence the need for accurate identification at the species level. Fusarium isolation was carried out by standard blotter method. However identification was based on morphological features and molecular method. In the bright field microscope, hyphae and conidia measured 5-7 µm in diameter. *Identification of fusarium and other species* followed through the SEM for morphological investigation, microconidia were with a smooth cell wall and maintained membrane structure, diameters measured around 1-5µm[33,34].

Many studies have been discovered ITS/ITS4 primers established recognition of fusarium[35-38]. Taking into consideration all these available datawe aimed to confirm the rDNA region by providing ITS/ITS4 target sequence for molecular detection of *F. sporotrichioides.* PCR method with above primers successfully tested against isolates which exhibited greatest homology in the target.

T-2 toxin a metabolite of *F. sporotrichioides* was extracted in ethanol and characterized using TLC, HPLC and FTIR.T-2 toxin visualized as bluish spot on the TLC plates and compared with standard toxin R_f value found to be 0.48 mm. In our study quantification of T-2 toxin was done as per the method of [19]with slight modification using UV detector investigated 10.126 µg toxin.

According to previous reports T-2 toxin is more toxic than other mycotoxin. In the present study, we tested T-2 toxin cytotoxicity by MTT and LDH leakage assays. The data showed human skin cell line and liver cell line are the more sensitive of all the cell lines tested, whereas neuronal cell is the least cytotoxic to T-2 toxin. Albarenque et al. [39]showed T-2 toxin decreased the cell viability at 25 µg in rat keratinocyte primary cultures. In the present study HaCaT cell viability decreased at 50-75ng. According to Bouaziz et al.[40] T-2 toxin induced maximum cell death in HepG2 compare to zearalenone and ochratoxin A at 60nM with increasing concentration, this report proves that cell viability of Hepg2 with T-2 toxin causes toxicity (100-150ng) comparably same concentration. Therefore, these results suggest T-2 toxin are potent inducer of hepatotoxicity.

Weidner et al [41] worked on T-2 toxin induced neurotoxicity with human astrocytes primary culture and shown that T-2 toxin disturb the blood-brain barrier (BBB) *in vitro*. They reported cytotoxicity at 10 nM and disturbance of barrier at 75nM. In our study using SHSY5Y cell line to the toxicity was noticeable at 200 -250mg.In the present study we could grow the T-2 toxin producing fusarium species on corn, isolate and characterize the species upto molecular level. T-2 Toxin was extracted and characterized. And toxicity of the toxin at cellular level was carried out using three different

cell lines, A liver cell line (HepG2), a skin cell line (HaCaT) and neuroblastoma (SHSY5Y) cell line. The results obtained are qualitatively similar to the once reported by others. Sensitivity of the cell lines to the toxin at ng may be due to the purity of the toxin.

CONCLUSION

F. sporotrichioides was isolated from corn sample and T-2 Toxin was extracted, characterization of species done by molecular method and T-2 toxin was quantified using analytical techniques. Further different dosage of T-2 toxin in different cell lines evaluated cytotoxicity

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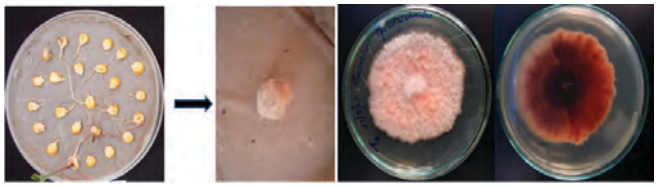

Figure 1: Isolation of fusarium from corn sample by standard blotter method.

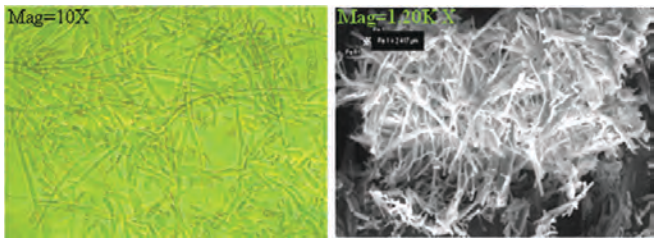

Figure 2: Inverted microscopic image and Scanning electron microscopic image (Mag=Magnification).

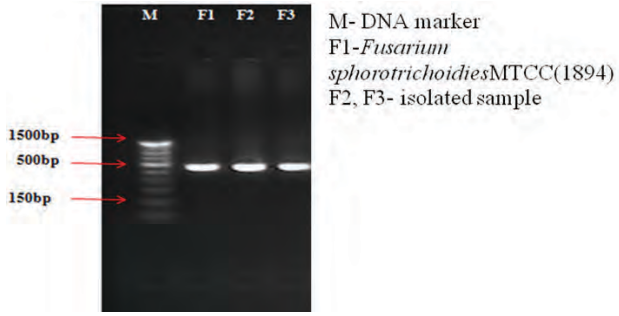

Figure 3: Agarose gel electrophoresis PCR products amplified with ITS/ITS4 primers.

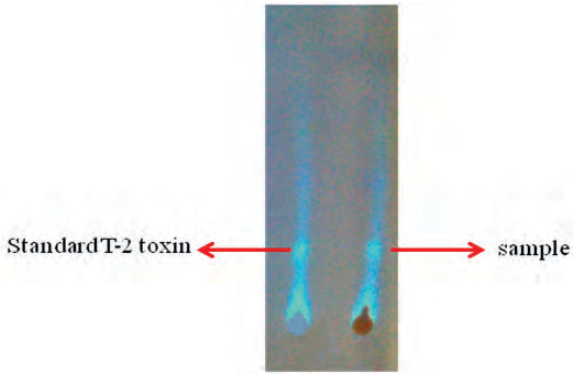

Figure 4: Thin Layer chromatography (Blue colour spot showing Standard T-2 Toxin),Toluene: ethyl acetate: formic acid (6:3:1) as mobile phase.

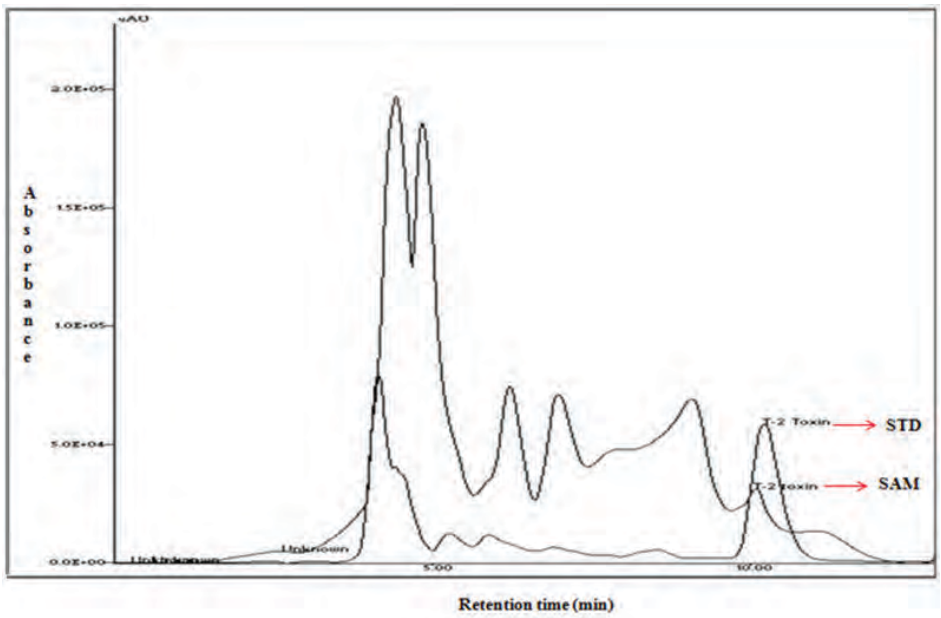

Figure 5: Quantification of T-2 toxin by High Performance liquid chromatography (STD= Standard T-2 Toxin, SAM= Extracted T-2 Toxin sample).

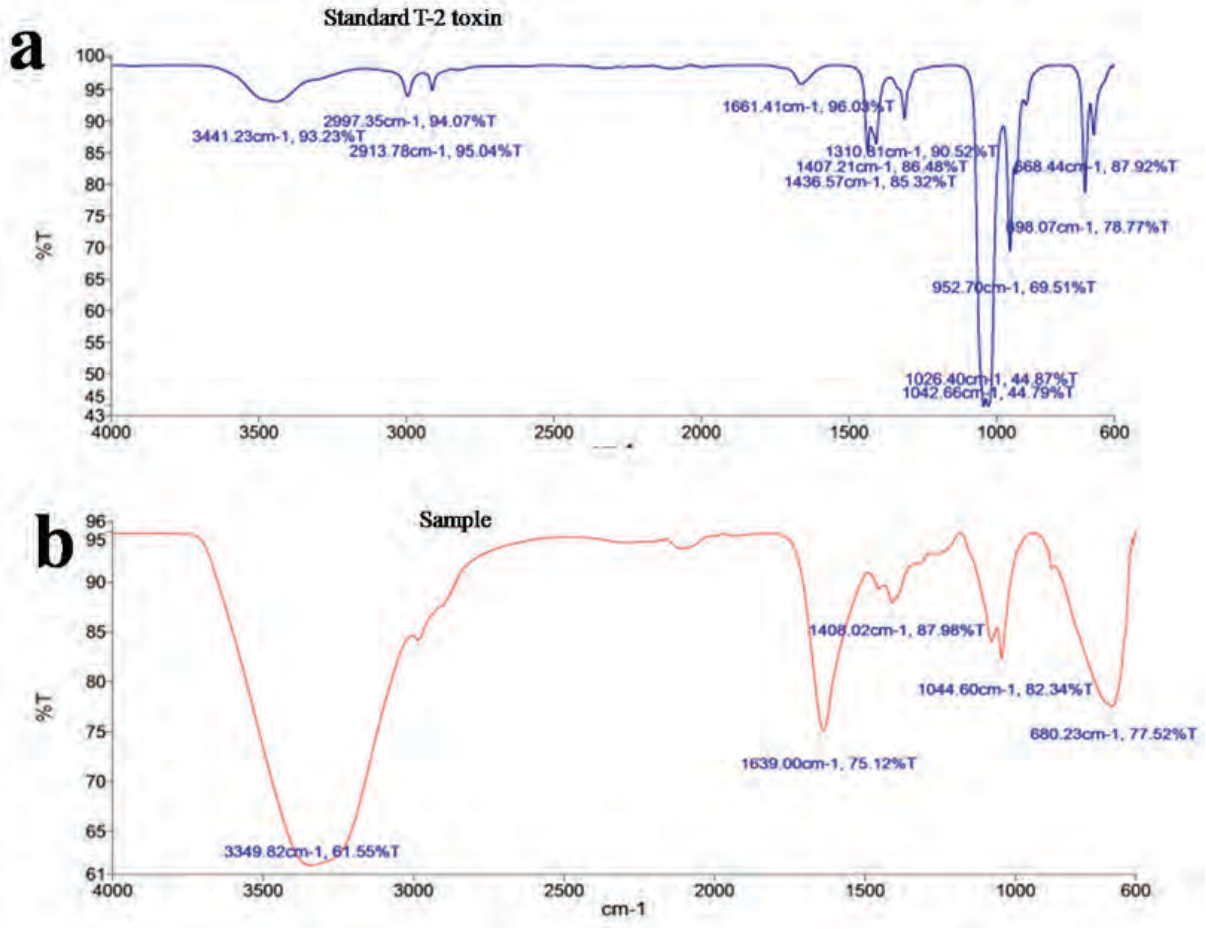

Figure 6: Fourier transform infrared spectroscopy (FTIR). Figure (a) showed standard T-2 Toxin peak and Figure (b) showed extracted T-2 Toxin sample peak.

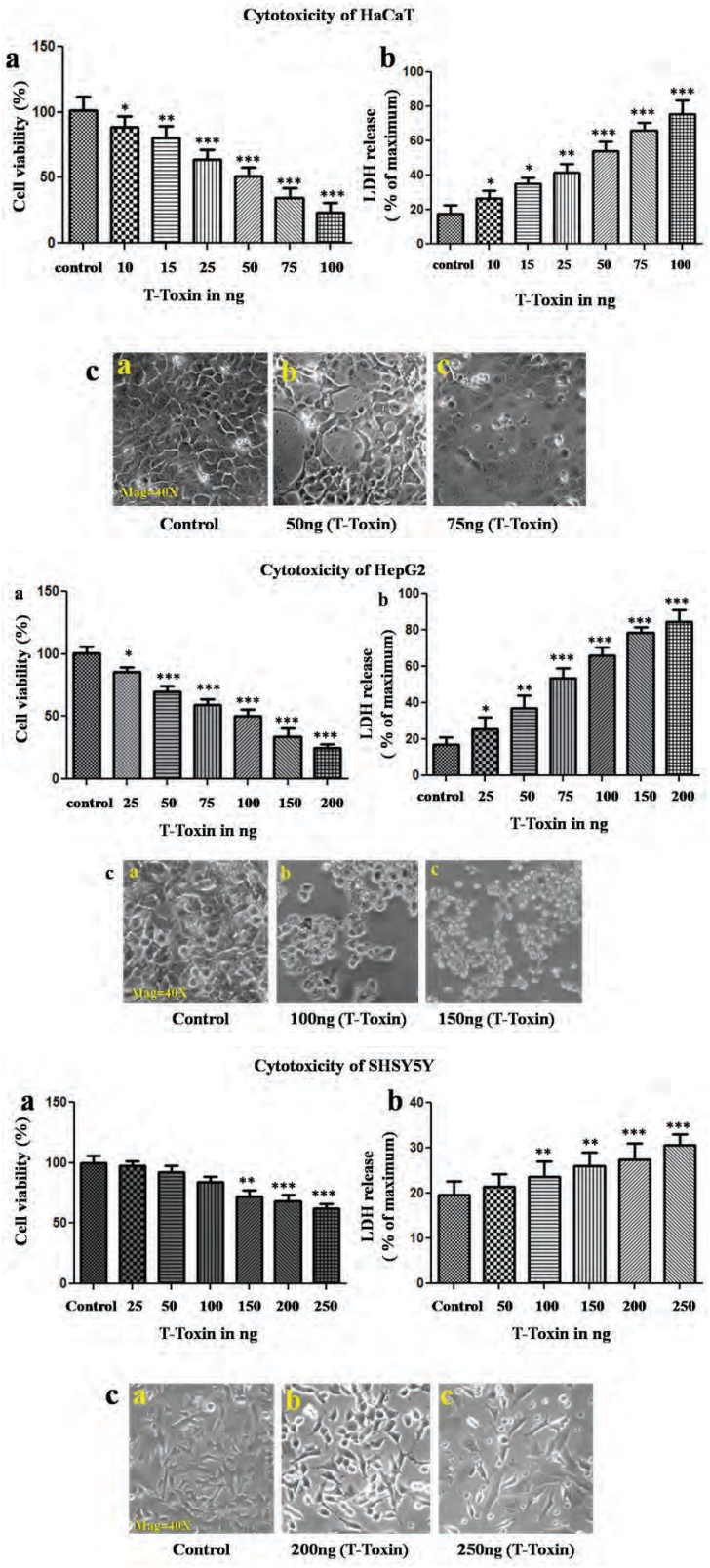

Figure 7: Cytotoxic effect of control and T-2 toxin treated cell lines for 24 h. a: Dose dependent T-2 Toxin induced cytotoxicity in HaCaT, HepG2 and SHSY5Y cells, the cell viabilitywas determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay. b:Cytotoxic effects of T-2 Toxin induced cytotoxicity in HaCaT, HepG2 and SHSY5Y cells analysed by lactate dehydrogenase(LDH) leakage assay. c: Effectsof T-2 Toxin induced morphological alterations inHaCaT, HepG2 and SHSY5Y cells by phase-contrast microscopy. The data are expressed as the mean ± SD of three independent experiments in triplicate. Significances are shown in comparison to control cells (**p* <0.05; ***p* <0.01; ****p* < 0.001) (Mag= Magnification 40X).

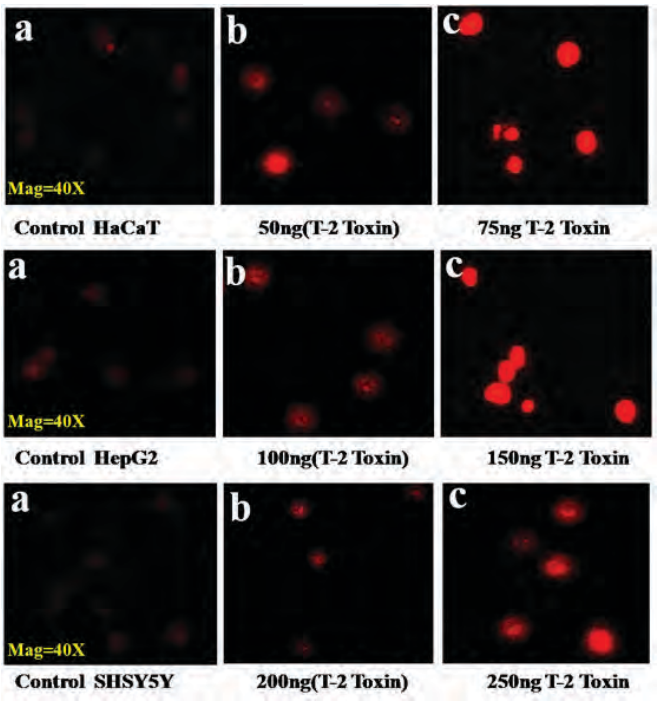

Figure 8: Nuclear morphological changes in control and T-2 Toxin treated cell lines for 24 h. a: Control cell lines incubated with media. banc c T-2 Toxin treated cell lines(Mag= Magnification 40X).

Table 1: FTIR analysis of Standard T-2 toxin

Sl. No	Peak values	Functional groups
1	3441.23	Alcohol
2	2997.35	Alkanes
3	2913.78	Alkanes
4	1661.41	Alkenes
5	1310.81	Esters
6	1407.21	Alkanes
7	1436.57	Alkanes
8	1026.40	Carboxylic acids
9	1042.66	Esters
10	952.70	Alkenes
11	698.07	Carboxylic acids
12	668.44	Cis- alkene

Table 2: FTIR analysis of extracted sample

Sl. No	Peak values	Functional groups
1	3349.82	Alcohol
2	1639.00	Alkenes
3	1408.02	Alkanes
4	1044.60	Esters
5	680.23	Carboxylic acids