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CYTOPROTECTION STUDY OF CURCUMIN ON HELA CELLS AGAINST AFLATOXIN INDUCED TOXICITY

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ABSTRACT

Curcumin (diferuloylmethane or 1,7-bis (4-hydroxy-3-methoxyphenol)-1,6-heptadiene-3,5-dione) is a natural compound present in turmeric, a rhizome of the plant *Curcuma longa* Linn. Curcumin also has been shown to possess various other biological activities, such antioxidant, antimutagenic and anti-carcinogenic activities. The purpose of this study was to evaluate the concentration of curcumin for cytoprotection study on HeLa cells. Aflatoxin was a model-toxin and is obtained from a strain of *Aspergillus flavus*. Cytoprotective activities of curcumin was conducted on aflatoxin toxicity. They have been reported to be extremely carcinogenic, teratogenic, and hepatotoxic to both humans and animals. For further experiment cell inoculum seeded in 96 well plate incubated overnight were doused with 10-fold dilution of curcumin. After 48 hrs incubation the neutral red (NR) assay procedure for cell survival/viability was used to evaluate cytoprotective concentration of curcumin. Here the preliminary result shows that HeLa cells at relatively low concentrations of 10µg/mL curcumin was found to protect significantly against aflatoxin B[1].

KEYWORDS: Aflatoxins, Cytoprotection, Curcumin, Aspergillus flavus, HeLa cells, The HeLa carcinoma cell line has been widely used as a model system to evaluate toxic effects of various substances

The moulds that are major producers of aflatoxins are *Aspergillus flavus* and *Aspergillus parasiticus* (Bankole *et al.*, 2004). *Aspergillus flavus* (Samajpati, 1979) is a major producer of aflatoxins B_2, whereas aflatoxin B and G are produced by *Aspergillus parasiticus* species. Aflatoxins (Figure 1) have more limited distribution reported by Garcia-Villanova *et al*. in 2004. Black olive is one of the substrates for *Aspergillus parasiticus* growth and aflatoxin B_1 production as detected by Leontopoulos *et al.*, in 2003. Aflatoxin B_1 is particularly important since it is the most toxic and potent hepatocarcinogenic natural compound ever characterized (Bennett and Klich, 2003). Aflatoxin toxicity is due to the oxidation of its 8.9 vinyl bond to yield the biologically active aflatoxin B1-8-9 epoxide (Lee *et al.*, 2005). Much progress has been made in elucidating the biochemical and molecular mechanisms that are involved in aflatoxin carcinogenesis. Evidence suggests that multiple CYP450 isoenzymes contribute to AFB1 epoxidation in rats (Klein *et al.*, 2003).

Curcumin (Figure 1) was first identified in 1910 by Lampe and Milobedzka, which is the primary active constituent of turmeric and is responsible for its vibrant yellow colour (Lal J., 2012). Curcumin, the diferuloylmethane inhibited the formation of the aflatoxin B_1 reductase product, aflatoxicol, by chicken liver cytosol. Soni *et al*., (1997) studied the in-*vitro* effects of certain food additives on aflatoxin production by *Aspergillus parasiticus*. They also observed that aflatoxin production was inhibited (more than 90%) by extracts of turmeric, garlic as well as asafoetida at concentrations of 5-10 mg/mL, whereas curcumin has been attributed various pharmacological activities such as antioxidant, antimicrobial and cytotoxic activities (Lal *et al.*, 2012; 2013; Singh and Lal, 2020). Direct action of curcumin for inhibiting carcinogenesis has been shown to inhibit cell proliferation and induce apoptosis in hepatic cancer cells (Yoysungnoen *et al.*, 2008). Previous study has reported that curcumin is a potent inducer of detoxifying enzymes and thereby prevents the toxicity induced by a chemical carcinogen (Singletary *et al.*, 1998).

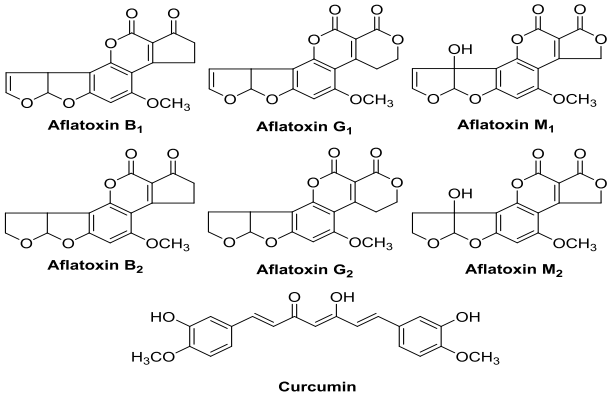

Figure 1: Chemical structure of various aflatoxins and curcumin

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Continuing over interest on aflatoxin studies (Khare *et al*., 2018), in the present communication, study was done to evaluate the concentration of curcumin for cytoprotection on HeLa cells against aflatoxin B_1 inducing toxicity after 48 hrs incubation the neutral red (NR) assay procedure for cell survival/viability.

Experimental

Aflatoxin B_1, curcumin and neutral red dye were purchased from Himedia chemical company. Eagle's Minimum Essential Medium (EMEM) and Foetal Bovine Serum (FBS) were purchased from Thermo Fisher Scientific, Dimethyl sulphoxide, (DMSO) were purchased from Merck and used as received. HeLa cells were procured from National Centre for Cell Science, Pune, India, the procured cells were free from any kind of bacterial and fungal contamination.

Isolation of Aflatoxin

After incubation culture was filtered using Whatman filter paper grade 1, filtrate was concentrated to 37 °C using rotatory evaporator and in the concentrated solution about 20 mL of chloroform was added and refluxed for about 40-60 °C using water condenser for 20-30 min. The mixture was taken in separating funnel and separate chloroform layer containing crude toxins. The concentrated filtrate was shaken repeatedly with 150 mL of chloroform and the extraction was repeated 2-3 times and the toxin was extracted by shaking it several times with 0.5 M sodium bicarbonate solution. All the lipid materials were removed by filtration after keeping the sodium bicarbonate extract over-night in a separating funnel. The extracts were pooled and concentrated; thus, the crude toxin was isolated. Purification of crude toxin was done by column chromatography it includes column preparation, capturing of the aflatoxins and washing was done following the Aflaprep® (R-Biopharm) procedure purified aflatoxins (Scheme 1).

RESULTS AND DISCUSSION

Primarily, isolated aflatoxin was detected by thin layer chromatography (TLC). Thus, TLC plates obtained were examined and compared with standard samples of aflatoxins. As reported in literature, the presence of aflatoxins may be detected by observing the TLC plate under strong UV illumination and it fluorescence as bluish green spots (Lal *et al.*, 2012).

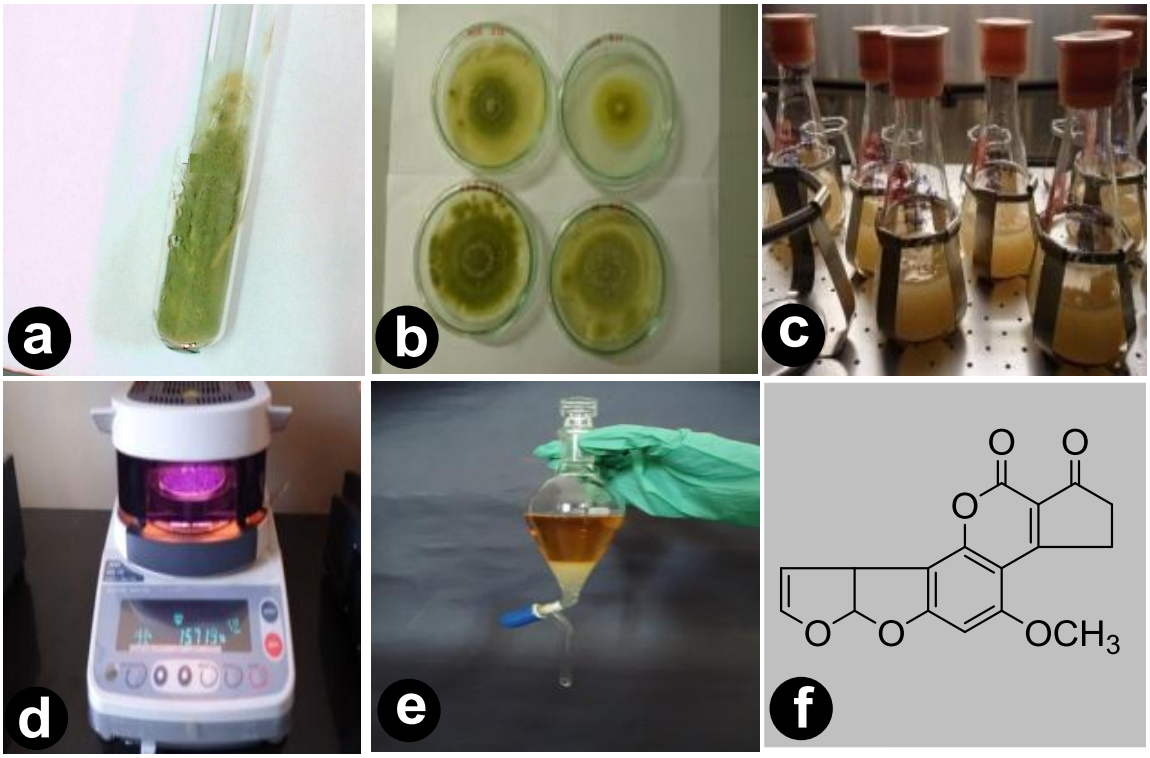

Figure 2: (a) Culture of *A. flavus* was isolated from soil sample, (b) Screening of pure culture of *A. flavus* on different media, (c) Optimization of growth of fungi on SD broth has been inoculated by spore suspension of *A. flavus* in baffle flask and kept in shaker incubator for 5-24 days at 28 ºC, (d) After incubation, culture media was filtered and dried weight of biomass was measured with the help of moisture analyzer, (e) Extraction of aflatoxin in separating funnel in $CHCl_3$, (f) Structural formula of aflatoxin B_1

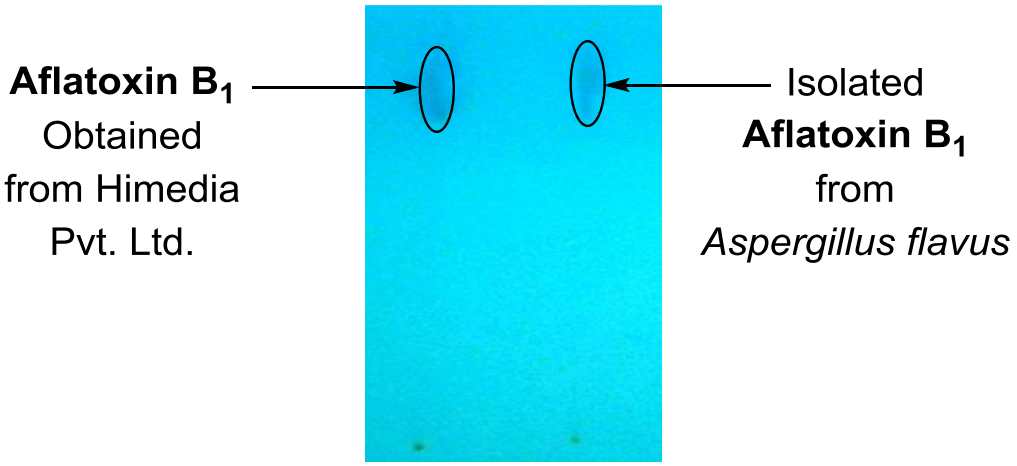

Figure 3: TLC plate showing aflatoxin B_1 observed under UV light

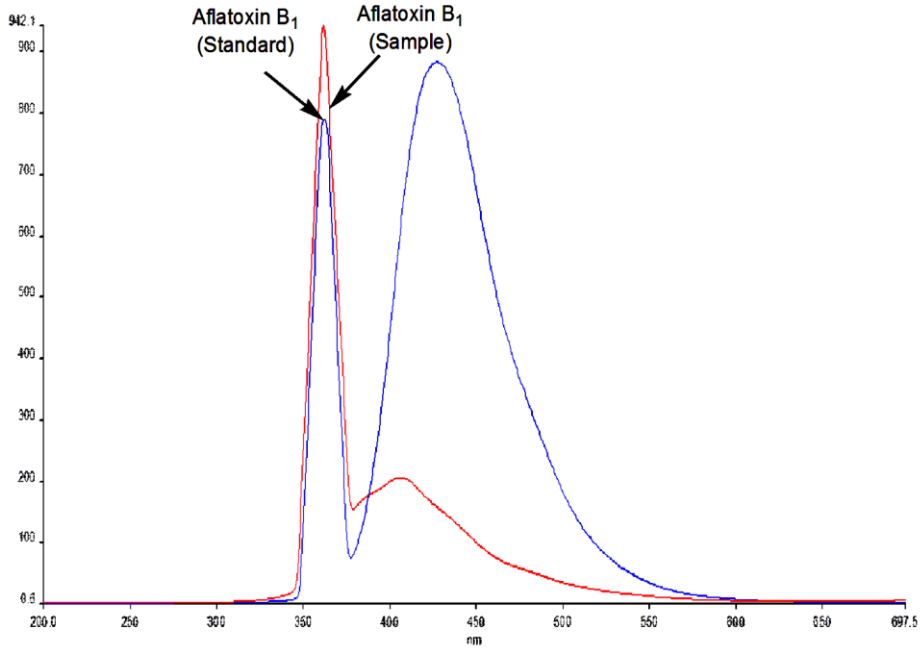

Figure 4: UV-visible spectra of standard and isolated aflatoxin B_1

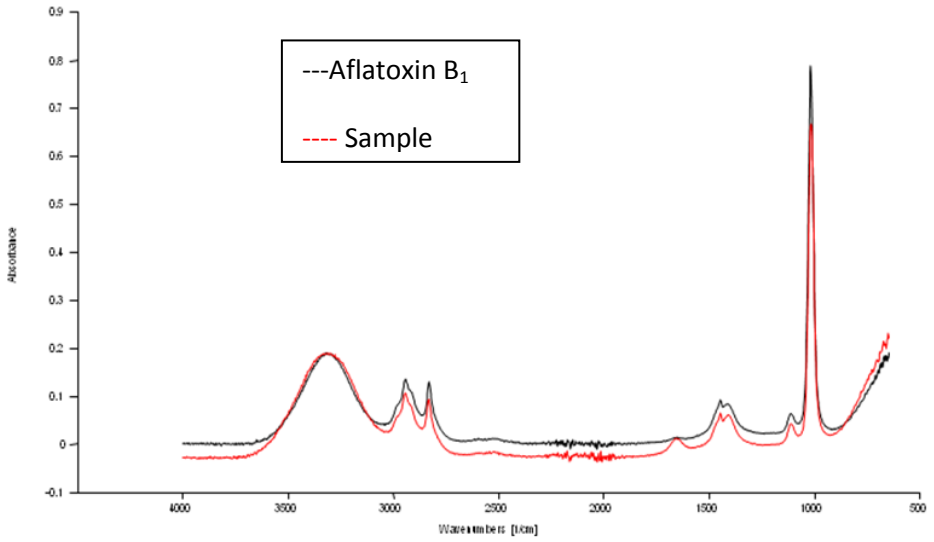

Figure 5: FT-IR spectra of standard and isolated aflatoxin B_1

HeLa cells were cultivated in Eagle's minimum essential medium at 37 °C in an incubator containing 90% humidity and 5% carbon dioxide. The culture medium is the most important single factor in culturing cells. Its main function is to provide the physical conditions of pH and osmotic pressure required for the survival of the cells. Eagle's minimum essential medium (EMEM), is a commercially prepared growth medium that contains essential amino acids, vitamins and salts. One of the most important nutrients required is glutamine which provides energy to the cells and carbon source (Freshney R.I., 1983). Additional proteins are required to enhance cell growth. Various types of animal sera are used to supplement culture media preparations for cell growth. The serum contains important proteins such as albumin, globulins and fetuin which act as carriers for minerals, fatty acids and hormones and have been found to be beneficial, promoting cell attachment to the substrate. The serum used is normally from a non-human source, to exclude human antibodies which could be inhibitory to the subsequent isolation of human viruses. Foetal bovine serum (FBS) is usually used as an additional protein source. A complete culture medium (CCM) contains FBS, EMEM, glutamine and antibiotics (penicillin, streptomycin, fungizone) and therefore sustains continuous cell growth (Reubel *et al.*, 1987).

These cells were revived in tissue culture flask containing 20% FBS, 10% DMSO and 70% MEM. For further experiment cell inoculum seeded in 24 well plates incubated overnight with 10 folds dilution of aflatoxin B_1. After 48 hrs incubation the neutral red (NR) assay procedure for cell survival/viability was used to evaluate cytotoxicity by determination of the IC_{50} (50% inhibiting concentration) as shown in figure 3.

Cytoprotection by curcumin determined similarly by reviving HeLa cells and treated with different dilution of curcumin. The result shows that 10µg concentration of curcumin shows healthy HeLa cells above this concentration shows toxicity. Therefore, HeLa cells treated simultaneously, pre-treated and post-treated with 10µg concentration of curcumin against IC_{50} of 10µg of aflatoxin B_1 and cell viability was measured by Neutral red assay procedure. NR is a weak cationic dye that readily penetrates the cell membrane and accumulates intracellularly in lysosomes (lysosomal pH < cytoplasmic pH), where it binds with anionic sites to the lysosomal matrix (Griffon *et al.*, 1995). Changes of the cell surface or the sensitive lysosomal membrane led to lysosomal fragility and other changes that gradually become irreversible. Such alterations brought about by the action of xenobiotics result in a decreased uptake and binding of NR.

Neutral Red Assay Procedure

1. Cultures were removed from incubator into laminar flow hood.
2. Neutral Red Solution (0.33%) was added in an amount equal to 10% of the culture medium volume.
3. Cultures were incubated for 2-4 hours depending on cell type and maximum cell density.
4. At the end of the incubation period, the medium is carefully removed and the cells quickly rinsed with 4.5% gluteraldehyde. Extended fixation times can result in leaching of the dye into the fixative solution.
5. The fixative or wash solution is removed, and the incorporated dye is then solubilised in a volume of destaining solution of acid alcohol (Ethanol, Acetic acid and water) equal to the original volume of culture medium. The cultures were allowed to stand for 10 min. at room temperature.
6. The quantity of dye incorporated into cells is measured by spectrometry at 570 nm, and is directly proportional to the number of cells with an intact membrane.

Table 1: Evaluation of Inhibitory Concentration (IC_{50}) of aflatoxin B_1 on HeLa cells

Entry	Concentration[a]	Optical Density[b]	% Viability	% Inhibition
1	Control	0.913	100	0
2	0.01	0.887	97.15	2.85
3	0.1	0.823	90.14	9.86
4	1	0.553	60.56	39.44
5	10	0.455	49.83	50.17
6	100	0.253	27.71	72.29

[a]OD was measure at 570nm. [b]µg/mL.

Statistical Analysis

The results from the cytotoxicity tests were analyzed using a Stats graphic Plus programme. Mean absorbances were expressed as % cleavage activity in comparison to cell controls (100%):

$$\% \text{ Cell viability} = \frac{\text{Mean absorbance of toxin treated cells}}{\text{Mean absorbance of control cells}}$$

Table 2: Evaluation of cytoprotection by curcumin

Entry	Curcumin treated HeLa cells	Optical Density[b]
1	Positive control	0.077
2	Pre-treatment	0.22
3	Simultaneous treatment	0.23
4	Post treatment	0.18

[a]OD was measure at 570nm.

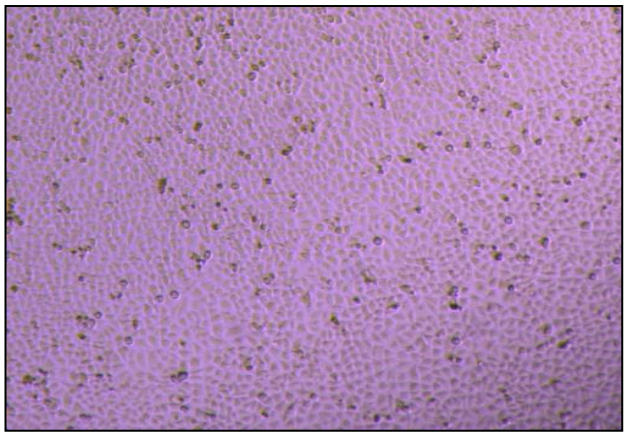

Figure 6: Healthy HeLa cells observed under fluorescence microscope.

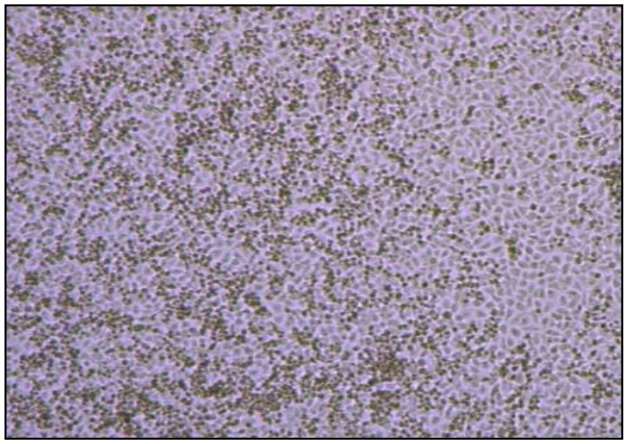

Figure 7: 10ug/mL aflatoxin B_1 24h post treatment showing progressive, rounding and inhibition of cell growth

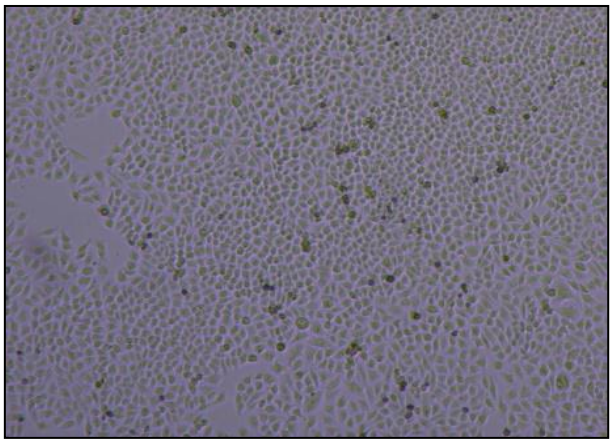

Figure 8: 10ug/mL curcumin 24 h pretreatment showing cytoprotection

Early evidence for the carcinogenicity of aflatoxins in humans came from epidemiological studies (a case-control study and descriptive studies) that correlated geographic variation in aflatoxin content of foods with geographic variation in the incidence of liver cancer (hepatocellular carcinoma, or primary liver-cell cancer). Hepatic carcinoma is one of the five most common cancers worldwide with a high prevalence in Asian countries due to endemic hepatitis B virus infection (Parkin *et al.*, 2003). Previous study has reported that curcumin is a potent inducer of detoxifying enzymes and thereby prevents the toxicity induced by a chemical carcinogen (Singletary *et al.*, 1998). Therefore, for the cytoprotection study against aflatoxin B_1 curcumin was used. Curcumin is a hydrophobic polyphenol derived from turmeric, the rhizome of the herb *Curcuma longa*. Extensive research conducted within the past two decades has revealed that cancer is a result of the disregulation of multiple cells signalling pathways. Curcumin is a highly pleriotropic molecule that modulates numerous targets. Cytochrome P450 (CYP) are phase-I enzymes involved in activation of carcinogens whose inhibition causes cellular protection against cancer. It was published previously that curcumin inhibits alkylation reaction of ethoxy resorufin methoxy-resorufin and pentoxy-resorufin catalysed by CYP1A1, 1A2 and 2B1 in rat liver by Thapliyal and Maru (Thapliyal and Maru, 2001). Similarly, curcumin inhibited aflatoxin-DNA adduct formation catalysed by the CYP system (Firozi *et al.*, 1996).

CONCLUSION

It is concluded that efficacy of curcumin against mycotoxins like aflatoxin suggest its possible use in minimizing the risk of mycotoxins. Present study on HeLa cells supports the previous studies that curcumin may be the future drug for the treatment of hepatocellular carcinoma caused by aflatoxin B_1.

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