3. MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Equipments:

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Major Equipments</th>
<th>Make</th>
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<tbody>
<tr>
<td>1.</td>
<td>Biohazard safety cabinet</td>
<td>ESCO</td>
</tr>
<tr>
<td>2.</td>
<td>Electronic Balance</td>
<td>Denver Instrument, India</td>
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<tr>
<td>3.</td>
<td>Refrigerator</td>
<td>LG (India)</td>
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<tr>
<td>4.</td>
<td>Moisture analyser</td>
<td>AD MS-70</td>
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<tr>
<td>5.</td>
<td>Microscope</td>
<td>Zeiss</td>
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<td>6.</td>
<td>Autoclave</td>
<td>NSW, India</td>
</tr>
<tr>
<td>7.</td>
<td>FTIR</td>
<td>Axiovision, India</td>
</tr>
<tr>
<td>8.</td>
<td>HPLC</td>
<td>Waters</td>
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<td>9.</td>
<td>4ºC Cold Chamber</td>
<td>Cryo Scientific, India</td>
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<td>10.</td>
<td>Vertical Autoclave</td>
<td>NSW, India</td>
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<tr>
<td>11.</td>
<td>pH meter</td>
<td>Esico, India</td>
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<tr>
<td>12.</td>
<td>PCR</td>
<td>Perkin-Elmer Applied Biosystems, India</td>
</tr>
<tr>
<td>13.</td>
<td>Microwave</td>
<td>National, India</td>
</tr>
<tr>
<td>14.</td>
<td>UV spectroscope</td>
<td>Scinco</td>
</tr>
<tr>
<td>15.</td>
<td>Hot Air Oven</td>
<td>Osworld, India</td>
</tr>
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### 3.1.2 Glassware & plastic ware:

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Glassware &amp; Plastic ware</th>
<th>Make</th>
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<tbody>
<tr>
<td>1.</td>
<td>Beaker</td>
<td>Borosil, India</td>
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<td>2.</td>
<td>Cell culture plates</td>
<td>Borosil, India</td>
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<tr>
<td>3.</td>
<td>Measuring cylinder</td>
<td>Borosil, India</td>
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<td>4.</td>
<td>Flask</td>
<td>Schott Duran, India</td>
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<td>5.</td>
<td>Test tubes</td>
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<tr>
<td>6.</td>
<td>Micro pipette tips</td>
<td>Tarsons, India</td>
</tr>
<tr>
<td>7.</td>
<td>Micro centrifuge tubes</td>
<td>Tarsons, India</td>
</tr>
<tr>
<td>8.</td>
<td>Micro Pipette</td>
<td>Eppandorff, India</td>
</tr>
<tr>
<td>9.</td>
<td>Glass vails</td>
<td>Borosil, India</td>
</tr>
<tr>
<td>10.</td>
<td>Centrifuge tubes</td>
<td>Tarsons, India</td>
</tr>
</tbody>
</table>

### 3.1.3 Consumables:

1. Parafilm,
2. Tissue Paper,
3. Filter Paper,
4. Cello Tape,
5. Aluminium Foil,
6. TLC plates, (Merck)
### 3.1.4 Chemicals:

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Reagent</th>
<th>Make</th>
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<tbody>
<tr>
<td>1.</td>
<td>Sodium bicarbonate</td>
<td>S.D. fine chem. Limited (India)</td>
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<tr>
<td>2.</td>
<td>Glycerol</td>
<td>Rankem (India)</td>
</tr>
<tr>
<td>3.</td>
<td>Acetone</td>
<td>S.D. fine chem. Limited (India)</td>
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<tr>
<td>4.</td>
<td>Rectified spirit</td>
<td>Rankem (India)</td>
</tr>
<tr>
<td>5.</td>
<td>Absolute Alcohol</td>
<td>JiangsuHuaxi International (India)</td>
</tr>
<tr>
<td>6.</td>
<td>Chloroform</td>
<td>Rankem (India)</td>
</tr>
<tr>
<td>7.</td>
<td>Methanol</td>
<td>Rankem (India)</td>
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### 3.1.5 Media

<table>
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<tr>
<th>S.No.</th>
<th>Media</th>
<th>Make</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Potato dextrose Broth (PDB)</td>
<td>Himedia, India</td>
</tr>
<tr>
<td>2.</td>
<td>Saboraud dextrose Broth (SDB)</td>
<td>Himedia, India</td>
</tr>
<tr>
<td>3.</td>
<td>Malt Extract Broth (MEB)</td>
<td>Himedia, India</td>
</tr>
<tr>
<td>4.</td>
<td>Yeast Extract Broth (YEB)</td>
<td>Himedia, India</td>
</tr>
<tr>
<td>5.</td>
<td>Nutrient Broth (NB)</td>
<td>Himedia, India</td>
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</tbody>
</table>
3.2 METHODOLOGY

This study was done in research laboratory of Biotechnology Division at Defence Research and Development Establishment, Gwalior. Aspergillus flavus was isolated from crop samples collected from Gwalior and the study was carried out using advance materials & methodology.

3.2.1 Sample collection

Food crop samples were collected from the Gwalior region of Madhya Pradesh. Samples of food crop containing 100g of wheat, rice, maize gram, groundnut and soya bean were randomly collected and placed in sterilized plastic bags under aseptic condition for the study of Aspergillus infestation and aflatoxin production.

3.2.2 Isolation of fungal species from the food crops sample

The 10g of crop samples were mashed and serially diluted in sterilized saline as described by Warcup (1960) with slight modification. The dilution were shaken vigorously on the magnetic shaker for 20 – 30 min. to obtain uniform suspension. Four 15ml sterile tarson tubes filled with 9ml saline were taken and labeled as 1(10⁻¹), 2(10⁻²), 3(10⁻³), 4(10⁻⁴). One ml of previously prepared sample was taken to prepare 10ml suspension of serial dilutions as 10⁻¹, 10⁻², 10⁻³ and 10⁻⁴. Fresh potato dextrose agar (PDA) plates were prepared aseptically with the help of sterile pipette. Dilution platting was done by transferring 100μl suspension of each dilution to the centre of the PDA plate separately. The culture was spread with the help of the sterile glass spreader by sweeping movement of the spreader. The plates were continuously rotated at regular intervals so as to permit the even distribution of the culture throughout the plate.
3.2.2.1 Preparation of spore suspension

Spore suspension was prepared in a tissue culture flask having proper growth of *Aspergillus flavus* culture by adding 20 ml of 10% glycerol (V/V) solution. The spore suspension obtained was filtered through muslin cloth in a sterile plastic vials. The spores in filtered suspension was counted by Neubauer’s chamber and cryo-preserved for further study of aflatoxin production.

3.2.2.2 Characterization of fungi

Isolated fungal species were characterized by morphologically and biochemically (Carbohydrate assimilation test). General identification of the *Aspergillus* species was based on the morphological characteristics of the colony and microscopic examinations of culture (McClenny, 2005). Although molecular methods continue to improve and become more rapidly available, microscopic observation and culture morphology are commonly used and essential tools for identification of *Aspergillus* species. In 2003 American Society for Microbiology (ASM) survey documented that 89% of laboratories performing mycological examinations (morphology based), 16% of them use serological tests and fewer than 5% use molecular tests for identification of microbial pathogens (Warris et al., 2001). Isolation of culture and phenotypic identification of common clinical isolates of *Aspergillus* species is usually quick and easy.

3.2.2.3 Morphological studies

For microscopic examination, slides were prepared by placing a drop of 10% glycerol on a clean grease free slide with the help of dropper and a small tuft of the fungus was transferred on a slide preferably with spores and spore bearing structure, onto the drop, using sterilized needle and gently tease material by using the two mounted needles and mix it gently then place a coverslip with care to avoid trapping air bubbles in the smear during preparation of slide. Standard strain
and isolated fungal species were observed on glass slide under optical microscope (AXIOSCOP 2 MAT) and identified as *Aspergillus flavus* according to Hedayati *et al.*, (2007).

The remarkable macromorphological features in species identification were reverse and surface colouration of colonies, presence of exudates, presence of pigment, colony diameter and colony texture while the micromorphological characteristics for the identification were conidial heads, stips, color and length, vesicles shape and serration, metula covering, conidia size, shape and roughness and also colony features including diameter after 6 days of incubation.

### 3.2.2.4 Carbohydrate assimilation test

The basal medium consisted of 8.4mM NH₄H₂PO₄, 2.7mM KCl, and 0.8mM MgSO₄.7H₂O, supplemented with 0.035mM ZnSO₄.7H₂O and 0.02mM CuSO₄.5H₂O was used in the biochemical test. Brom cresol purple was added as an indicator at a concentration of 50mg/L, and the medium was autoclaved at 121°C for 10min. Filter –sterilized carbohydrates were then added to the medium in a final concentration of 1% (w/v). The pH of medium was adjusted to 5.4 by adding NaOH or HCl, and 2ml portions of the medium was dispensed into 10ml test tubes. The tubes were inoculated with fungal mycelia and control tubes for each fungus and carbohydrates (+fungus/carbohydrates and –fungus/ +carbohydrates) were also prepared. All tubes were incubated at 20°C for 14 days. A changes in the color of the medium to orange or yellow was taken as positive result and to pink or purple as negative result as shown in figure17 (Kitancharoen *et al.*, 1998).
3.2.3 Identification of Aflatoxin producing *Aspergillus* species by PCR based method

In our studies, the PCR reaction was targeted against aflatoxin synthesis regulatory gene (aflR). The fungal isolates were maintained on potato dextrose agar media. Cultures were sub-cultured periodically and 5-day-old slant cultures were used in these studies. For the detection of aflatoxigenic species, DNA was isolated by following procedure.

3.2.3.1 The Genomic DNA extraction was isolated using DNeasy Plant Mini kit (Qiagen)

The Genomic DNA extraction, from pure fungal cultures was done by washing the mycelial pellets twice with 1 ml of distilled water. The mycelial clump was slightly agitated with a mortar pestle, immersed in liquid nitrogen for freezing and immediately ground into fine powder. Further process were carried out according to manufacturer’s instructions given in DNeasy Plant Mini kit (Qiagen) as follows:

- The tissue powder under and liquid nitrogen was transferred in tube and allowed the sample to thaw.
- In tube 400 µl buffer AP1 and 4µl RNase A stock solution (100 mg/ml) were added to a maximum of 1g (wet) or 20 mg (dry) disrupted fungal tissue and vortexed vigorously.
- The mixture incubated for 10 min at 65°C and mixed 2 or 3 times during incubation by inverting tube.
- Buffer AP2 (130 µl) was added to the lysate, mixed and incubated for 5 min on ice.
- The lysate was, Centrifuged for 5 min at 20,000 x g.
• The lysate was poured into the QIAshredder Mini column (lilac) placed in a 2 ml collection tube, then centrifuged for 2 min at 20,000 x g.
• The flow through was transferred in a new tube.
• Buffer AP3/E (1.5 volumes) was added to the cleared lysate (flow through), and mixed by pipet.
• Sample mixture (650 µl) including any precipitate that may have formed, was loaded into the DNeasy Mini spin column placed in a 2 ml collection tube.
• Above step was repeated with remaining sample. Flow through was discarded.
• DNeasy Mini spin column was placed in a 2ml collection tube, 500µl Buffer AW was added and centrifuged for 1 min at ≥ 6000xg. The flow through was discarded.
• The 500 µl Buffer AW was added to the DNeasy Mini spin column, and centrifuged for 2 min at 20,000 x g to dry the membrane.
• The DNeasy Mini spin column was transferred to a 1.5ml or 2ml microcentrifuge tube, and pipetted 100 µl Buffer AE directly onto the DNeasy membrane. Incubated for 5 min at room temperature (15-25°C) and then centrifuged for 1 min at ≥ 6000xg to elute.

3.2.3.2 Agrose Gel Electrophoresis and quantification of DNA:

The agarose gel electrophoresis was carried out to visualize the DNA by UV transilluminator. Agrose (0.8) was prepared in 1X TAE buffer (Appendix). It was dissolved by boiling in microwave oven and allowed to cool about (50°C) before adding ethidium bromide to a final concentration of 0.5 µg/ml. The gel was mixed thoroughly before casting on the plate containing an appropriate comb. The gel was allowed to set completely for 35-45 min. at room temperature.
It was mounted on the electrophoresis tank containing 1X TAE buffer as running buffer before loading the DNA sample.

DNA sample was mixed with 0.2 % volume of 6X loading dye (Appendix). The samples were loaded slowly in the well. DNA was separated by applying a constant voltage of 100V and after migration of dye to sufficient distance, the current was put off gel was examined by UV transilluminator.

Size of DNA was estimated by comparison of mobility of standard DNA ladder (100 bp) containing molecules of known sizes. DNA was quantified by reading absorbance at 260 nm using UV- Visible spectrophotometer (Specord 200). Concentration of DNA was determined using a calibration curve generated by different amounts of lambda DNA. Purity of DNA preparation was determined by observing $\text{OD}_{260\text{nm}} / \text{OD}_{280\text{nm}}$ ratio. A value of near 1.8 was taken as standard for purity.
3.2.3.3 Polymerase chain reaction (PCR)

PCR was performed using (Perkin-Elmer Applied Biosystems). Thermal Cycler, using template DNA isolated by the method described earlier. Primers used for amplification of target gene are shown below.

**Primer sequence:**
- **aflR F** (50- AACC GCATCCACAATCTCAT-30) and
- **aflR R** (50-AGTGCAGTTCGCTAGAAC- 30)

In a sterile 0.2ml thin walled PCR tube, reagents were added in the following order:

**Reaction mixture:**
- 10X amplified buffer: 2.5 µl
- Solution of four dNTPs: 5 µl
- MgCl₂ (25mM): 1-2 µl (1.2-2.0mM)
- Forward primers: 1 µl (20pmol)
- Reverse primers: 1 µl (20pmol)
- Taq DNA polymerase: 1-5 µl
- Distilled water: 13-18 µl
- Total volume: 25 µl

The polymerase chain reaction (PCR) was performed in 25 ml reaction mixtures containing 100ng of genomic DNA. 0.025nM each of deoxyribonucleoside triphosphates, primers at 4nM each and reaction buffer (10 mM Tris– HCl, pH 8.0, 50 mM KCl, 1.5 mM MgCl₂, 0.1 % Triton X-100 and 0.2 mg gelatin per ml). Each reaction mixture was heated to 95.8°C for 10 min before adding 0.3 units of Taq DNA polymerase. A total of 30 PCR cycles, each cycle at 0.3 min at 94.8°C for denaturation, 0.45 min at 50.8°C for annealing, 1.15 min at 72.8°C for extension and
a 10 min final extension at 72.8°C was run on a programmable DNA thermal cycler, model (Perkin-Elmer Applied Biosystems). The PCR products were analysed by electrophoresis on a 1.2 % agarose gel in 1x TAE buffer (50 mM Tris–acetate, 1mM EDTA, pH 8.0) stained in 1 mg/ml ethidium bromide as a reference of Mateo et al., (2011).

### 3.2.4 Screening of *Aspergillus* species for high yield of Aflatoxin.

*Aspergillus* cultures was screened for aflatoxin production potential in a four different liquid medium:

a. Potatodextrose broth (PDB)

b. Sabouraud dextrose broth (SDB)

c. Malt extract broth (MEB) and

d. Yeast extract sucrose broth (YESPB) (see Appendix for media composition).

The flask containing sterile media was cooled inoculated with 1ml of spore suspension of *Aspergillus* species and incubated at 28±2°C for 8-10 days in submerged fermentation for obtaining high yield of aflatoxin. The procedure of Koehler *et al.*, (1975) was followed with some modification.
3.2.4.1 Optimization of culture conditions for aflatoxins production

Screening of all seven *A. flavus* strains and aflatoxin production was done in four different media i.e. PDA, SDB, MEB and YESPB the best growth and high yield of aflatoxin was observed in YESP broth medium. Therefore YESPB was taken as basal media for optimization.

3.2.4.1a Effect of temperature

In order to study the effect of temperature on aflatoxins production and fungal growth experiment was carried out at 5°C intervals in the range of 20 to 50°C. The flasks were incubated in an incubator for stationary culture and in a rotatory shaker for shake culture at 150 rpm

3.2.4.1b Effect of pH

For the growth of fungi and aflatoxins production pH is an important factor. To determine optimum pH for aflatoxin production fungal culture grown at different pH ranges from 3 to 6 in YESP broth. The pH of the medium was adjusted by using 1N HCl or 1N NaOH.

At the end of incubation period, the content of each flask was filtered. Mycelium was separated from culture medium by gentle filtration and mycelia dry weight was determined by moisture analyser.

3.2.4.1c Effect of carbon source on aflatoxin production

It was previously reported that sucrose is the best carbon source for aflatoxin production by *A. flavus*. To optimize media different concentration (0, 5, 10, 15, 20, 25) % of sucrose was supplemented in the media. The flasks were incubated at 28±2 °C in an incubator for stationary culture and in a rotatory shaker for shake culture at 150 rpm.
3.2.4.1d Effect of nitrogen source on aflatoxin production

To detect the influence of nitrogen source for aflatoxin production by A. flavus (II), the medium was prepared by using different concentration (0, 1, 2, 3, 4, 5, 6) % of peptone. The flasks were incubated at 28±2 °C in an incubator and in a rotatory shaker at 150 rpm.

3.2.5 Purification, Detection and Characterization of Aflatoxin

3.2.5.1 Extraction:

After incubation, the mycelia were removed from the medium with the help of sterile needle and the liquid was filtered through Whatman No. 1 filter paper. The culture filtrate was concentrated under reduced pressure in a rotatory evaporator. The concentrated culture filtrate was shaken repeatedly with 150 ml volumes of chloroform and the extraction was repeated 2 or 3 times. The chloroform extracts obtained were mixed and filtered through Whatman No. 1 filter paper. From the filtered chloroform extracts, the toxin was extracted by shaking it several times with 0.5 molar 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 was pooled and concentrated, thus the crude toxin was isolated as per (Jayabarathi & Mohamudha 2010).
Fungal mycelia

+ 

Culture media

Residue

(Mycelia)

Filterate

Conc at 37°C

Concentrated solution

Add 150ml chloroform
refluxed at about 40°C

Separate the chloroform layer

Add 0.5 molar Sodium Bicarbonate

Distillate

Concentrate in rotatory evaporator

Aflatoxins

Scheme 1. Extraction of aflatoxins from *A. flavus* species
3.2.5.2 Purification:

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 as a reference standard with some modification. The key component of Aflaprep® is the immunoaffinity column which contains a gel suspension of monoclonal antibody covalently attached to a solid support (Candlish, 1998). The antibody is specific for aflatoxins B₁, B₂, G₁, and G₂. Following extraction of the toxins, the sample extract is passed through the immunoaffinity column. Any aflatoxin which is present in the sample is retained by the antibody within the gel suspension. The column is washed with water to remove extraneous non-specific material. The bound toxin is released by the antibody following elution from the column with 50 ml methanol. The elute is collected in a vial for analysis.

3.2.5.3 Detection:

Preliminary detection of aflatoxin was done by thin layer chromatography (TLC). The standard of aflatoxins (powder) was purchased from HiMedia (Mumbai) and reconstituted in 1 ml Benzene : Acetonitrile (98:2 v/v). In this process 5 µl of standard solution (1 mg/ml) of aflatoxin. Each sample of purified toxin extracted from fungal culture concentrated and redissolved in benzene & acetonitrile solution was spotted on TLC plate 5x10 cm (Merck). The plate was resolved in chloroform methanol (85:15) solvent system in a close chamber and run for 45 min at room temperature.

Further analysis was done by high performance liquid chromatography (HPLC). The HPLC system consisted of a pump (LC 600) and an UV detector (Model 486 Waters USA). To prepare the HPLC standard solution, the aflatoxin standard (1000 ng/ml) was diluted in methanol (1: 25
v/v) to give a 10 ng/ml aflatoxin B₁ solution. This standard solution double diluted in distilled water and 100 μl of this preparation was used in HPLC analysis.

**Table 3: Chromatographic Condition for HPLC**

<table>
<thead>
<tr>
<th>Column</th>
<th>C18,150mm×4.6 mm,5μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow rate</td>
<td>1.0 ml/min</td>
</tr>
<tr>
<td>Detection</td>
<td>UV at 365 nm</td>
</tr>
<tr>
<td>Injection volume</td>
<td>100 μl</td>
</tr>
<tr>
<td>Run time</td>
<td>20 min.</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>Water: Methanol (70:30v/v)</td>
</tr>
</tbody>
</table>
3.2.5.4 Characterization

**Ultraviolet Spectroscopy:**

Aflatoxin detected by the screening process was quantified by the method of Nabney and Nesbitt (1965). The methanolic fraction was evaporated to dryness using a rotary evaporator and the residue was dissolved in 1ml of chloroform. The ultraviolet absorption spectrum of the methanolic solution isolated and purified then recorded. The optical density of methanolic filtrate at 363 nm was determined. This was then divided by the extinction coefficient (21,800) of aflatoxin B₁, and the resulting figure was multiplied by the molecular weight of aflatoxin B₁ (312) to obtain the concentration of aflatoxin was (Mohammedi and Atik 2013) by the following formula:

$$\text{AFB}1 \text{ content (μg ml}^{-1}\text{)} = \left\{\left[\frac{(D \times M)}{(E \times L)}\right]\right\} \times 1000$$

Where, D: absorbance, M: molecular weight of aflatoxin (312), E: molar extinction coefficient of aflatoxin B₁ (21,800), and L: path length (1cm)

**Fourier Transform Infrared Spectroscopy (FTIR):**

A new analytical method was developed for the determination of aflatoxins by Fourier Transform Infrared (FTIR) Spectroscopy using horizontal attenuated total reflectance technique. All spectra were obtained using a Axiovision FT-IR spectrometer equipped with a ZnSe crystal and controlled by SPINWIN software. All spectra were collected by resolution of 4 cm⁻¹ in the range of 4000-650 cm⁻¹. FT-IR spectra of aflatoxin standards and purified toxins by methanol water (70:30, v/v) were obtained by placing only 20 μl of solution on the ATR cell.
3.2.6 Formulation of Aflatoxin with non-toxic natural compound and demonstration of its antimicrobial activity.

3.2.6.1 Antibacterial activity by Disc diffusion method:

**Inoculums preparation:**

The overnight grown culture of bacteria strains of *E.coli* and *staphylococcus aureus*, obtained from biotechnology division, DRDE, Gwalior was serially diluted in nutrient broth to obtain bacterial count approx. 7.5 x10^5 cfu/ml.

Freshly prepared nutrient agar plate was then flooded with the corresponding bacterial culture (100μl) of the test organism, dried for 3 mins at 37ºC and after drying of the flooded plate, four filter paper discs (Whatman no.1) of 6 mm diameter were soaked in the four different dilutions (25, 50, 100, 200 μg/ml) of the aflatoxin and aflatoxin and curcumin (1:1) placed at the specific locations on the surface of the flooded plate, marked as quadrants at the back of the plates. The same technique was repeated in the case of the remaining test organisms for both the extract. All flooded plates with corresponding filter paper discs were incubated at 37ºC for 24 hours. The diameters of the zones of inhibition were measured using HiAntibiotic Zone Scale™ (HiMedi.Mumbai) and compared accordingly.

3.2.6.3 Antifungal activity by agar well diffusion method:

**Preparation of fungal culture:**

Fungal cultures of *A.niger* and *Penicillium* species were grown in conical flasks of 250 ml capacity containing 100 ml sterile broth, plugged with cotton wool and autoclaved at 121ºC for 48-120 hrs at 28ºC. Seeded agar plates of each test fungal organism were prepared. With sterile
borer, 6mm wells were prepared in which purified aflatoxin and aflatoxin and curcumin (1:1) with a concentration of 25 µg, 50 µg, 100 µg, and 200 µg/ml in methanol was added. The plates were then kept for incubation for 3 days at 28ºC. Control well was maintained by adding only methanol. Antifungal efficiency was calculated by measuring the zone of inhibition as earlier and evaluated its ameliorative effect on test organism. Further procedure was carried out according to Padmini et al., (2011).

3.2.7 Invitro Toxicity study of Aflatoxin and natural compound.

3.2.7.1 Cell culture :

HeLa cells provided by NCCS Pune, India. These cells were free from any kind of bacterial and fungal contamination. HeLa cells were cultivated in Eagles minimum essential medium (EMEM) at 37ºC in an incubator containing 90 % humidity 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 survival. Eagles 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, 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, 1987).

HeLa cells were cultivated in Eagles minimum essential medium (EMEM) at 37°C in an incubator. These cells were revived in tissue culture flask containing 20 % FBS, 10 % DMSO and 70 % EMEM. For further experiment cell inoculum seeded in 96 well plates incubated overnight with 10 fold dilution of aflatoxin B₁. After 48 hrs incubation the neutral red (NR) assay procedure for cell survival/viability was used to evaluate cytotoxicity by determination of the IC50 (50 % inhibiting concentration). 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, following the procedure of Van de Loosdrecht et al., (1994) with some modification.

Statistical analysis:

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

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

3.2.7.2 Neutral red assay procedure:

1. Cultures were removed from incubator into laminar flowhood.

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 solubilized 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. Spectrophotometrically measure absorbance at a wavelength of 570 nm.