Materials and Methods
The mycological techniques outlined in plant pathologists pocket book (1968) were followed for the preparation of media, culturing the fungus etc. Acid washed, corning brand glassware was used. Flasks containing media were autoclaved at 120°C (1.05 kg/cm²) for 30 min. Petridishes were heat sterilized at 160°C for 2.5h. Glass distilled water was used for the preparation of media and buffers.

**Culture media:**

**Potato Dextrose Agar (PDA):** Potato 200g was chopped into pieces and boiled for 15 min. in water. The extract was filtered through cheese cloth, made upto 1 litre with distilled water. Dextrose (20g) and agar (20g) were added to the potato extract before autoclaving.

**Czapek-dox Agar Medium** (Raper and Thom. 1949)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Nitrate</td>
<td>2.0g</td>
</tr>
<tr>
<td>DiPotassium Hydrogen Phosphate (K₂HPO₄)</td>
<td>1.0g</td>
</tr>
<tr>
<td>Magnesium Sulphate (MgSO₄)</td>
<td>0.5g</td>
</tr>
<tr>
<td>Potassium Chloride (KCl)</td>
<td>0.5g</td>
</tr>
<tr>
<td>Ferrous Sulphate (FeSO₄)</td>
<td>0.1g</td>
</tr>
<tr>
<td>Sucrose</td>
<td>30.0g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000ml</td>
</tr>
<tr>
<td>Agar Agar</td>
<td>20.0g</td>
</tr>
<tr>
<td>pH</td>
<td>5.5</td>
</tr>
</tbody>
</table>
**Nutrient Agar Medium** (Gerhardt et al., 1981)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto Peptone</td>
<td>5.0g</td>
</tr>
<tr>
<td>Beef extract</td>
<td>3.0g</td>
</tr>
<tr>
<td>Agar Agar</td>
<td>15.0g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000ml</td>
</tr>
<tr>
<td>pH</td>
<td>7.2</td>
</tr>
</tbody>
</table>

Yeast extract 0.1% was added to the media and the pH was adjusted to 7.0 with 0.1N NaOH before sterilization.

**Rose-bengal agar medium** (Martin, 1950):

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>1 gm.</td>
</tr>
<tr>
<td>Glucose</td>
<td>10 gm.</td>
</tr>
<tr>
<td>Peptone</td>
<td>5 gm.</td>
</tr>
<tr>
<td>Magnesium Sulphate</td>
<td>0.5 gm.</td>
</tr>
<tr>
<td>Rose-bengal</td>
<td>30 mg.</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>30 mg.</td>
</tr>
<tr>
<td>Agar-Agar</td>
<td>20 gm.</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml.</td>
</tr>
<tr>
<td>pH</td>
<td>4.6</td>
</tr>
</tbody>
</table>

**Sabouraud’s agar medium (SA medium)** (Sabouraud, 1952)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>10 g</td>
</tr>
<tr>
<td>Dextose</td>
<td>40 g</td>
</tr>
<tr>
<td>Agar-agar</td>
<td>30 g</td>
</tr>
<tr>
<td>20 % lactic acid</td>
<td>10 ml</td>
</tr>
</tbody>
</table>
Luria agar medium (LA Medium) (Gerhardt et al, 1981):

Luria agar medium was prepared to determine the effect of isolated toxins on different bacterial cultures.

**Ingredients:**
- Tryptone: 10 g.
- Yeast extract: 5 g.
- Sodium Chloride: 10 g.
- Agar-agar: 20 g.

**pH:** 7.2 – 7.4

Luria Broth:

Luria broth was prepared without adding agar to determine the antibacterial effect of the toxin in liquid media.

Malt extract agar:

Malt extract agar supplemented with 0.5 g yeast extract was prepared to study the effect of Dothistromin on different fungal strains.

**Ingredients:**
- Malt extract: 30.0 g
- Mycological peptone: 5.0 g
- Agar: 20.0 g
- Distilled water: 1000 ml.

**pH:** 5.4
Yeast Extract – Sucrose medium (YES medium) (Dawis N.D., et al., 1966)

YES medium was prepared to grow the isolated fungal cultures for toxin production with the following composition

Yeast extract 20 g.
Sucrose 150 g.
Distilled Water 1000 ml.
$p^H$ 5.5

Animal Cell culture Media RPMI 1640:

RPMI 1640 medium was prepared by using re-hydrating RPMI 1640 powder (Sigma USA) in double distilled water and supplemented with 27.3 ml. of 7.5% w/v Sodium bicarbonate solution, 10 units/ml and 100 µg/µl penstrep (pencillin streptomycin) (Sigma, USA). The volume was made upto 1 litre and $p^H$ was adjusted to 7.2, with Carbon dioxide (CO₂). Medium was sterilized by filtration through 0.45 µm Millipore membrane filters and tested for sterility before use.

Lactophenol mounting fluid (Cappuccino and Sherman, 1996).

Phenol crystals 20 g
Lactic acid 20 ml
Glycerol 40 ml
Distilled water 20 ml
1% cotton blue 5 ml
Lactic acid, glycerol and water were mixed, added to warmed phenol crystals. Cotton blue was added after cooling. This stain was used to prepare permanent slides of fungal cultures.

0.1 M Phosphate Buffer Saline (PBS)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium chloride (Nacl)</td>
<td>8 g</td>
</tr>
<tr>
<td>Potassium Chloride (Kcl)</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Di Sodium Hydrogen Phosphate (Na₂HPO₄)</td>
<td>1.15 g</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate (KH₂PO₄)</td>
<td>0.2 g</td>
</tr>
</tbody>
</table>

The above salts were dissolved in small quantity of distilled water and volume was made upto 1000 ml. pH was adjusted to 7.2 and autoclaved.

Trypan blue solution

400 mg. of Trypan blue was dissolved in 100 ml PBS buffer and was stored in a brown bottle at room temperature.

Reagents for Gram’s staining:

1. Crystal violet

   Solution A

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crystal violet (90% dye)</td>
<td>3 g</td>
</tr>
<tr>
<td>Ethanol (95%)</td>
<td>20 ml</td>
</tr>
</tbody>
</table>

   Solution B

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium Oxalate</td>
<td>0.8 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>80 ml</td>
</tr>
</tbody>
</table>
Solutions A and B are prepared separately and mixed and then kept in a brown colour bottle.

2. Gram’s Iodine

- Iodine 1 g
- Potassium iodide 2 g
- Distilled water 300 ml

3. Ethanol (95%)

- Ethanol (100%) 95 ml
- Distilled water 5 ml

4. Safranine

- Safranine (25% solution prepared in 95% ethanol) 10 ml
- Distilled water 100 ml

Microorganisms used in the present study:

- Escherichia coli
- Bacillus subtilis
- Staphylococcus aureus
- Micrococcus luteus
- Pseudomonas fluorescence
- Pseudomonas aeruginosa
- Streptococcus faecalis
- Staphylococcus albus
- Candida albicans
- Aspergillus niger
Trichoderma viride
Alternaria solani
Rhizopus nigricans
Aspergillus flavus
Aspergillus terreus
Cercospora personata
**Methods:**

**Isolation of fungal culture from infected rice husk:**

Sterile Czapek-dox, Rose Bengal agar and Sabouraud’s agar (500 ml) were prepared and poured into sterile petridishes. 5 gm of rice husk was added to 100 ml of sterile distilled water and used as inoculum. 0.1 ml of the inoculum from $10^{-1}$ to $10^{-5}$ dilutions was plated onto all the three media and plates were incubated at room temperature (28 $\pm$ 2°C) for 1 week.

Of the different fungal species grown on the media, two predominant species were selected and sub-cultured repeatedly on Czapek dox agar medium plates to get pure cultures.

**II. Identification of fungal cultures:**

Fungal species were identified by observing the conidial morphology at 24 hrs, 48 hrs, 72 hrs, using lactophenol cotton blue dye. Ocular and stage micrometers were used to determine the conidial morphology.

**III. Maintenance of Fungal Cultures:**

The fungal cultures isolated from the source were maintained in liquid medium i.e., Czapek-dox broth for further experimental work.

**Preparation of sporulating culture of C. personata and inoculation of media:**

The mycelium from PDA grown for 15 days was transferred to 250 ml Erlenmeyer’s flask containing glass beads and 15 ml sterile glass distilled water. The flask was agitated in a wrist action shaker for about 10 min. to break
mycelial clusters. One ml. of this macerated suspension was transferred aseptically to each flask containing medium. After inoculation, the flasks were incubated at 27 ± 1°C for 3 days in total darkness and placed under light provided by black fluorescent light tubes 300 lux for 7 days. The culture sporulated within 7 days.

**Preparation of spore suspension**

Sporulating cultures were flooded with 5 ml sterile distilled water and the conidia were dislodged by gently brushing the mycelium. The spore suspension was filtered through two layers of cheese cloth to remove hyphal fragments. The spore suspension was centrifuged at 3000 rpm for 5 min and resuspended in distilled water to get a concentration of 2×10⁵ spores /µl.

**Plants:**

Varieties of groundnut TMV2 and TMV3 was employed. The seeds were obtained from crop specialist ICRISAT, HYDERABAD, India. Plants were grown in a glass house in earthenware pots (15cm dia) filled with clay: red soil mixture 2:1 and watered on alternative days. The temperature in the green house ranged between 28° - 30°C and relative humidity fluctuated from 50 – 80 %/µl.

**Inoculation of Plants:**

A drop of tween 20 was added to 25 ml of spore suspension. The aboxial surface of third leaf (from below) of 30 day old plants grown in pots was sprayed with spore suspension using a glass sprayer, till it was completely wet. The pots were placed in a plastic chamber of size 75 × 75 × 30 cm. The set up
ensured high humid condition (90-100%) conducive for infection. After 36h the plants were transferred to the green house.

**Extraction and identification of toxins from field infected ground nut plant:**

Five grams of infected leaf spots punched out with cork - borer(3mm) were extracted with 50 ml ethyl acetate for 3 days and reduced to 5 ml. About 500μl was loaded onto a preparative thin-layer (silica gel) plate (20×20 cm).

**Pigments produced by C.personata:**

The virulent single spore isolate was used to study pigment production. The culture was grown on PDA in Petridishes overlaid with cellophane disc. After 4 weeks of growth under black light, they were harvested, extracted twice with ethylacetate and the pigments were purified. The pigments were separated into light yellow and Dothistromin as orange red band. Crystallization was achieved by dissolving the residue of orange red band and evaporated in vacuum to dryness.
Flow Chart for the isolation of pigments from C. personata mycelium

Fungal mycelium 75g

↓

Extracted twice with 50 ml. ethyl acetate

↓

Evaporated to dryness

↓

Residue dissolved in 20 ml of chloroform

↓

Dry silica agar column (20×20 cm)

↓

Pale yellow fraction

↓

Orange red fraction (Dothistromin)

↓

Deep red fraction

Isolation of Aflatoxin (Davis ND et al 1966):

Cultures were grown on 100 ml of YES Medium in 250 ml conical flasks. These flasks were incubated at 28° ± 1°C on the mechanical shaker for 3 weeks. After 3 weeks cultures were sterilized with methanol and culture filtrate was obtained by filtering through whatman’s filter paper no.1. 25 ml of culture filtrate and 10 ml of chloroform were transferred to a 125 ml separating funnel and shaken for 5 minutes and then allowed for the separation of chloroform layer. Chloroform layer was collected and filtered through anhydrous. Sodium Sulphate and finally washed with n-hexane to remove lipids. The chloroform extract thus obtained was evaporated to dryness at 39°C on a waterbath. 1 ml of
chloroform was added to this, which was later used for Thin Layer Chromatography (TLC).

**Thin Layer Chromatography (TLC)**

Separation of pigments from the mycelium buds carried out on thin layer silica gel plates of 20× 20 cm. size. TLC was carried out in subdued light. Thin layers (0.3 mm) absorbent silica gel were activated at 110°C for 2 hours. 5 μl. amounts dothistrin in an appropriate organic solvent was spotted and developed for a distance of 15 cm. in the following solvent systems with normal saturation.

- Benzene: Acetic acid: water (2:1:1 V/V)
- Ethyl acetate: Methanol (4:1 V/V)
- Ethyl acetate: Benzene (2:3 V/V)

Toxin from C. personata were visualized under ultra-violet light.

The extracted Aflatoxins from contaminated rice husk were spotted along with crude culture filtrate on 0.25 mm (300 μm) thick Silica gel – G plates (E. Morck A.G. Dormstedt, Germany). The plates were developed with Toluene: Ethyl acetate, Formic acid (6:3:1) and air dried. The developed plates were observed under UV light.

The silica gel of the aflatoxin samples were scraped off carefully dissolved in 2 ml ethyl acetate and allowed to stand for two hours. Supernatant is
collected to which 0.5ml of PBS is added. Later this was used to carry out toxicity assays.

**Absorption Spectrum of Dothistromin:**

As the pigments were insoluble in water, a stock solution of 10mg in 10 ml methanol was prepared and stored at -4°C whenever necessary, 1 ml of this concentrated solution was withdrawn and diluted with 3 ml methanol to give the required concentration. Absorption spectra of orange red fraction was determined in UV – visible recording spectrophotometer, in the range of 200-600nm.

**Antibiotic activity of Dothistromin:**

Microorganisms were from cultures routinely maintained at the Mycology culture room. They were tested by nutrient agar diffusion method using analytical filter paper discs at the center of agar plates freshly seeded with the microorganisms. Fungi were cultured on plates containing malt extract agar medium supplemented with Yeast extract (0.5 g) and were maintained at room temperature (25°C) throughout the test experiments.

**Antimicrobial activity of culture filtrates of A.flavus and A.terreus:**

Culture filtrates were assayed for antimicrobial activity by adding concentrated dried ethanolic solutions. Final concentration is 50 µg/ml x 10 ml volumes of appropriate medium (luria broth) which had been autoclaved and cooled. Control experiments contained quantities of ethanol equivalent to that in the test experiments. Growth was assessed nephelometrically against broth
blanks after 24 hours, 48 hours and 72 hours of incubation. E.coli, B.subtilis, S.aureus and M.luteus were used.

The same organisms were also tested for sensitivity by luria agar diffusion method using analytical filter paper discs at the center of agar plates freshly seeded with Micro organisms.

**Haemolysis:**

This was assayed by the method of Mew et al., (1963). For experiments in an inert atmosphere the red blood cell suspension (400 ml) was placed in a test tube (15 ml) which was closed with a rubber septum and then purged with oxygen free N\textsubscript{2} admitted and voided by means of hypodermic needles for 45 minutes. The needles were removed and dothistrin solution (10 ml) was injected through the septum in a dark room. Experiments in the presence of air were done similarly in open tubes not purified with N\textsubscript{2}. The blood was taken from healthy staff from our laboratory. It was stabilized by the addition of heparin and stored at 4°C in the dark until used.

**Isolation of Peripheral Blood Lymphocytes (PBL) (Boyum 1968):**

Peripheral Blood Lymphocytes were isolated from fresh heparinised venous blood from healthy persons. 10 ml of venous blood was drawn from the donor after obtaining his consent using 10 ml vacuette tubes, coated with heparin. Blood was then diluted with equal volume of RPMI 1640 medium and carefully layered over Ficoll-Conray (2 parts of diluted blood 1 part of Ficol Conray). After centrifugation at 400 xg for 30 min., at 25°C the whole lymphocyte
cell layer seen at the interface was carefully transferred to a tube containing 5 ml of RPMI 1640 medium. The cells were thoroughly mixed with medium and washed by centrifugation at 300 xg for 10 minutes. The procedure of washing with RPMI 1640 medium was repeated thrice and the final suspension was made in 1 to 5 ml of RPMI 1640 medium.

Peripheral blood lymphocytes are used for cycotoxicity of dothistromin PBL (1x10^6) in RPMI 1640 medium were incubated for two hours at 37°C under air with dothistromin as log concentrations, 50 µl, 150 µl and 200 µl. Control subjects were tested as lymphocytes pre incubated with RPMI 1640 medium alone.

Cytoxicity of aflatoxins of A.flavus and A.terreus is similarly tested using PBMC (1x10^6) in RPMI 1640 medium were incubated for 2 hours at 37°C in CO₂ incubator as log concentrations, 25 µl, 50 µl and 100 µl control subjects were tested as lymphocytes pre incubated with RPMI 1640 medium alone. Cytoxicity was assessed by trypan blue dye exclusion following the 2 hours incubation Riley et al., (1988).

The plasma membrane of a viable cell does not permit the entry of non-electrolyte dye substance. This phenomenon is used to distinguish dead from living lymphocytes. Percentage of viability was calculated as follows:

\[
\text{% of viable cells} = \frac{\text{No. of viable cells}}{\text{No. of viable cells} + \text{No. of dead cells}} \times 100
\]