Materials and Methods
4. MATERIALS AND METHODS

The hypothesis is that the uses of culinary plants during oil frying have the toxigenic mold growth inhibitory, toxin detoxification, oil shelf life extension activity along with nutraceutical benefits. This section summarizes the materials and the standard methods used during this systematic study to test the proposed hypothesis.

4.1 SAMPLE COLLECTION

4.1.1 Selection of Oil Samples

The groundnut oils of twenty different popular marketed and local brands were chosen and used in the experiment. They were bought from local oil markets and shops. Three pockets of each brand having different production and expiry date were collected by random sampling, transferred to laboratory and were stored at room temperature till further analysis.

4.1.2 Period of Sampling

Oil samples were collected during the twelve months period from May 2006 to April 2007 from the local oil markets at Thiruvannamalai, Tamilnadu, India.

4.1.3 Glasswares, Chemicals and Reagents

All the glasswares used in this study were procured from Borosil Glass Works Ltd, India unless and otherwise specified. The chemicals and reagents used in this study are of analytical grade and were obtained from Central Drug House (CDH) (P) Ltd, New Delhi, India. The specialty chemicals were procured from E.Merck, Mumbai, India. All the readymade culture media and media ingredients were the products of HiMedia Pvt. Ltd, Mumbai, India.

4.2 DETERMINATION OF QUALITY OF THE OIL

The quality of the sampled groundnut oil is determined by following the widely and internationally used Association of Official Analytical Chemists (AOAC) methods as per the guidelines of The Directorate of Vanaspati, Vegetable Oils and Fats (DVVO&F) of the Ministry of Consumer Affairs, Food and Public Distribution, Govt. of India, the specialized organization in the field of vegetable oils and fats and
vanaspati at the Government level. Each sample was analyzed independently for the following important characters

4.2.1 Acid Value (AOAC 969.17)

The acid value is the number of milligrams of potassium hydroxide necessary to neutralize the free acids in 1 gram of test sample.

4.2.1 i) Principle

Acid value of fat is equal to milligram of KOH required to neutralize 1g fat, separated by melting butter, is dissolved in alcohol-ether mixture, and titrated with standard alkali.

4.2.1 ii) Apparatus

i) Erlenmeyer flasks (250 ml)
ii) Magnetic stirring device
iii) Burette (graduated 10 ml)
iv) Analytical balance

4.2.1 iii) Reagents

i) Alcoholic potassium hydroxide (KOH) 0.1M solution
ii) Alcohol-ether mixture
iii) Phenolphthalein indicator solution (1.0%)

4.2.1 iv) Procedure

10g of well mixed portion of oil was weighed into 250 ml Erlenmeyer flask. 100 ml of alcohol-ether mixture and 0.1 ml of phenolphthalein solution was added. The mixture is titrated with 0.1M alcoholic KOH until faint pink color appears and persists for 10 s.

The following formula is used for acid value calculation

\[
\text{Acid Value} = \text{Titration end point value} \times \frac{5.6}{\text{weight of sample}}
\]
4.2.2 Saponification Value (AOAC 920.160)

The saponification value is the amount of alkali necessary to saponify a definite quantity of the test sample. Saponification value is expressed as the number of milligrams of potassium hydroxide required to saponify 1 gram of oil.

4.2.2 i) Apparatus

i) Erlenmeyer flask (250 ml)
ii) Draining pipette
iii) Air condenser

4.2.2 ii) Reagents

i) Hydrochloric acid (HCl)-0.5 M
ii) Alcoholic potassium hydroxide (KOH)
iii) Phenolphthalein indicator solution

4.2.2 iii) Procedure

Five gram of filtered oil was taken accurately into 250 ml Erlenmeyer flask. About 50 ml of alcoholic KOH solution was pipetted and added into the flask. The flask is connected with air condenser and boiled until oil is completely saponified (30 minutes). Then cooled and titrated with 0.5 M HCl solution using phenolphthalein. Same procedure was repeated for blank using same pipette for measuring and draining same time.

Saponification value is calculated by
Saponification Value = 28.05 (B-S) / weight of oil.
B – Ml 0.5 M HCl required by blank
S – Ml 0.5 M KCl required for test sample.

4.2.3 Iodine Value (AOAC 993.20)

The iodine value is a measure of the unsaturation of oils and is expressed in terms of the number of centigrams of iodine absorbed per gram of sample.
4.2.3  i) Principle

When fat and oil samples are mixed with iodine monochloride solution halogenations of their double bonds takes place. Excess iodine monochloride is reduced to free iodine in presence of potassium iodide, and free iodine liberated can be measured by titration with sodium thiosulfate using starch as an indicator.

Iodine value is calculated as centigrams iodine absorbed per gram of sample (% iodine absorbed), is a measure of unsaturation of fats and oils.

4.2.3  ii) Apparatus

i) Glass stoppered iodine flasks (500 ml)
ii) Glass stoppered volumetric flasks (1000 ml)
iii) Volumetric dispensers
iv) Repeater pipette
v) Analytical balance
vi) Filters
vi) Hot air oven

4.2.3  iii) Reagents

i) Wij’s iodine solution
ii) Potassium iodide (KI) solution 15%
iii) Soluble starch solution
iv) Potassium dichromate (K₂Cr₂O₇)
v) Sodium thiosulfate solution (Na₂S₂O₃)
vii) Cyclohexane-acetic acid (1:1 v/v) solvent

4.2.3  iv) Standardization of Sodium Thiosulfate

Accurately 0.16 grams of dried and finely ground potassium dichromate was weighed in 500 ml flask, dissolved in 25 ml water. HCl 5 ml and 20 ml KI Solution was added. The contents were mixed by rotating and allowed to stand for 5 minutes. Hundred milliliters of water is added. This solution is titrated with sodium
thiosulphate solution by shaking continuously until yellow color has almost disappeared. One milliliter of starch indicator solution was added and titrated slowly with sodium thiosulfate until blue color disappears.

Sodium thiosulphate normality = [20.394 x K₂Cr₂O₇]/ml sodium thiosulphate.

### 4.2.3 v) Procedure

Oil sample is filtered through double layer of filter paper to remove any solid impurities and traces of water. Two grams of oil sample was weighed accurately into dry glass stoppered 500 ml bottle. Fifteen milliliter of cyclohexane – acetic acid solvent was added and swirled to dissolve completely. Twenty five milliliters of Wij’s solution was dispensed into the flask containing test portion, swirled to mix and kept in dark at 25°C for 1 hour & dissolved in chloroform in a flask containing iodine. The flask is removed from dark and 20 ml of KI solution was added and mixed. The mixture was added with 150 ml of water and gradually titrated with 0.1 M standard Na₂S₂O₃ solution with constant and vigorous shaking. Titration was continued till the yellow color disappears. About 1-2 ml of starch indicator solution was added to flask and continuously titrated till blue color disappears. Iodine value was calculated from the formula;

\[
\text{Iodine Value (IV)} = (B-S) \times M \times 12.69 / \text{weight of oil}
\]

Where B- Titration of blank
S- Titration of test solution
M- Molarity of Na₂S₂O₃ solution

### 4.2.4 Peroxide Value: (965.33)

Peroxide value was calculated according to AOAC 965.33 method.

### 4.2.4 i) Reagents

i) Acetic acid (CH₃COOH) – chloroform CHCl₃ (3:2 v/v) solvent solution
ii) Potassium iodide solution
iii) Sodium thiosulfate standard solution
4.2.4 ii) Procedure

Five grams of oil sample was weighed into a 250 ml glass stoppered Erlenmeyer flask. Thirty milliliters of acetic acid – chloroform solution was added and swirled to dissolve. About 0.5 ml of saturated KI solution was added and allowed for 1 minute with occasional shaking and 30 ml of water was added. The above mixture is titrated slowly with 0.1 M Na$_2$S$_2$O$_3$ with vigorous shaking till yellow color disappears. 0.5 ml of 1% starch solution was added and titration continued shaking vigorously to release all I$_2$ from CHCl$_3$ layer till blue color disappears. Peroxide value was determined by using the following formula

\[
\text{Peroxide value} = \frac{S \times M \times 1000}{\text{g test portion}}
\]

Where S- Ml of used Na$_2$S$_2$O$_3$

M- Molarity of Na$_2$S$_2$O$_3$ solution

4.3 EXAMINATION OF GROUNDNUT OIL FOR FUNGAL CONTAMINATION

The collected oil samples were subjected to initial mycotoxigenic fungal analysis. The fungal contaminants of marketed groundnut oils were examined and isolated as described (Okpokwasili and Molokwu, 1996). The moulds present in the oil samples were isolated using Sabouraud’s dextrose agar medium into which sterile streptomycin (50 µl) was incorporated to suppress bacterial growth.

4.3.1 Preparation of Sabouraud’s Dextrose Agar

Sabouraud’s dextrose agar was prepared by adding the following ingredients

i) Peptone - 1.0 g
ii) Dextrose - 4.0 g
iii) Agar - 1.5 g
iv) Distilled water - 100 ml
v) Streptomycin - 50 µl

pH was adjusted to 5.6
4.3.2 Preparation of 0.1% Peptone Water

0.1% of peptone water was prepared by dissolving 0.1 g of peptone in 100 ml of distilled water.

4.3.3 Inoculation of Oil Sample

One milliliter of oil was transferred into 9 ml sterile 0.1% peptone water diluent. Oil and peptone water was mixed thoroughly by agitation to give 10⁻¹ dilution. Further decimal dilutions were carried out up to the 10⁻⁵ dilution. One milliliter portions of the oil dilutions were pipette into sterile empty petridishes. Twenty milliliters of sterile molten SDA (cooled to 45°C) was poured aseptically and mixed thoroughly by gently swirling the plates. The plates were incubated at room temperature (25°C±2°C) for 7 days. A portion of each mould colony which developed was picked with a sterile inoculating needle and aseptically cultured into fresh SDA plates for purification. Stock cultures prepared on SDA slants were stored in the refrigerator (4°C) until further use (Okpokwasili and Molokwu, 1996).

4.4 IDENTIFICATION OF FUNGAL ISOLATES

The isolated pure culture filamentous fungal isolates were presumptively identified by morphological examination including the colony morphology and microscopic features as described (Alexopoulos et al., 1996; Sampson et al., 2007).

4.4.1 Determination of Cultural Characteristics of Fungal Isolates

The nonselective, general-purpose media like potato dextrose agar (PDA), Rose Bengal Chloramphenicol Agar (RBA), Czepak-Dox Agar and Sabouraud’s Dextrose Agar (SDA) were used for determining the cultural characteristics of the isolates. Known quantity of nutrient agar was weighted, transferred using a clean spatula into 250 ml flask and it was dissolved in 100 ml of distilled water. It was stirred until the powder completed dissolved. The medium was sterilized at 121°C for 15 minutes. The medium was cooled at 45°C in a water bath and it was poured into the sterile Petri plate and kept it in the table until solidification. The spores of the fungal isolates were inoculated into the centre of the plates using inoculation loop and
the plates were incubated at room temperature. The Cultural characteristics like colony color, shape, texture, spore-bearing structures etc are noted.

4.4.1 i) Composition of PDA (Abbas et al., 2004)

i) Potato extract - 20 g
ii) Dextrose - 2.0 g
iii) Agar - 1.5 g
iv) Distilled water - 100 ml
pH adjusted to 6.5

4.4.1 ii) Composition of RBA

i) Soya peptone - 5 g
ii) Dextrose - 10 g
iii) Monopotassium phosphate - 1 g
iv) Magnesium sulphate - 0.5 g
v) Rose Bengal - 0.05 g
vi) Agar - 15 g
vii) Distilled water - 1000 ml
pH was adjusted to 7.2 ± 0.2

4.4.1 iii) Composition of SDA

i) Peptone - 1.0 g
ii) Dextrose - 4.0 g
iii) Agar - 1.5 g
iv) Distilled water - 100 ml
v) Streptomycin - 50 µl
pH was adjusted to 5.6

4.4.1 iv) Composition of Czepak-Dox Agar (Ghitakou et al., 2006)

i) NaNO3 - 0.2 g
ii) K2HPO4 - 0.1 g
iii) KCl - 0.05 g
iv) MgSO4·7H2O - 0.05 g
4.4.2 Microscopic Observation

The shape, size and arrangement of spores and hyphae were determined using the following

4.4.2 i) Lactophenol Cotton Blue Mount Preparation (Aneja, 2005)

Wet mount of isolated fungi was prepared for the morphological study using lactophenol cotton blue (LCB).

LCB stain contains

i) Lactic acid - 20.0 ml
ii) Phenol crystals - 20.0 g
iii) Glycerol - 40.0 ml
iv) Distilled water - 20.0 ml
v) Cotton blue (1% aqueous) - 2.0 ml

Glycerol was added to distilled water and mixed thoroughly. Phenol crystals was added and heated in hot water with frequent agitation until the crystals completely dissolved. 1% aqueous cotton blue was added and mixed thoroughly and used for wet mount preparation.

4.4.2 ii) Scotch Tape Method (Aneja, 2005)

Scotch tape method is a rapid and simple method for preparing a temporary microscopic mount of a fungus without disturbing the arrangement of conidia and conidia bearing hyphae, the conidiophores.

4.4.2 iia) Requirements

i) Strip of clear cellotape
ii) Lactophenol cotton blue
iii) Microscopic slide (Blue star, India)
iv) Fungal culture
4.4.2 iiib) Procedure

In this method, the sticky side of a cello tape was pushed over a fungal colony in a plate and stick it over the glass slide having a few drops of LCB. The preparation was allowed to stand for some time allowing the stain to penetrate the fungal tissues and then observed under high power of the microscope.

4.4.2 iii) Tease Mount Method

4.4.2 iiiia) Requirements

i) Microscopic slide
ii) Coverslip (Blue star, India)
iii) Dissecting needle
iv) Lactophenol cotton blue stain
v) Forceps

4.4.2 iiib) Procedure

In this method, a drop of lactophenol cotton blue was placed on a slide. Then using two flamed sterile stiff needles, a small amount of hyphal mass was removed by picking it up at the tip of the needle. The hyphal materials were transferred to the slide containing LCB and the hyphal mass was teased apart carefully with the help of the needle. Followed this, a cover slip was placed over the material without any air bubble formation and examined.

4.4.3 Slide Culture Technique (Aneja, 2005)

Slide culture technique is performed for the observation of fungi intact under microscope a vital one for their identification, since any disturbance during their transfer may disturb the shape of the spore bearing structure, arrangement of spores.

4.4.3 i) Requirements

i) Sterile Petri dish – Borosil make
ii) Glass microscopic slide – Blue star make
iii) Cover slip (22x30 mm) – Blue star
iv) Applicator sticks (7 mm)
v) Filter paper  
vi) Forceps  
vii) Dissecting needle  
viii) Transfer needle  
ix) SDA  

4.4.3 ii) Procedure  

A ‘U’ shaped bent glass rod, a microscopic glass slide and two cover slips was air oven sterilized inside a Petri dish by wrapping them in brown paper. A small agar block in square shape was cut from sterile SDA plate using a clean and sterilize scalpel and it was placed on a glass slide. An amount of growth or spores from the fungal plate transferred to a glass slides and the mycelial structure was teased with the help of inoculating needle in to small bits and it was transferred to the corners and edges of the agar block. The cover slip was positioned over the agar. 10 ml of sterile distilled water was poured into the Petridish, covered and it was incubated at room temperature (25-30°C). The preparation was checked every 48hrs for the occurrence of growth over the cover slip and slide, the same was examined under microscope when sufficient growth has occurred. Earlier the cover slip was removed with a sterile forceps and transferred to a drop of LCB put on a glass microscopic slide was observed.  

4.4.3 iia) Observation of Cover Slip  

The cover slip was lifted from the surface of the agar with sterile forceps. A drop of 95% alcohol was poured on fungus side of cover slip and drained off the alcohol. A drop of lactophenol cotton blue was placed on a slide and the cover slip is placed over it with fungus facing side down and observed under high power objective.  

4.4.3 iib) Observation of Culture Slide  

An agar block was removed from the slide using a sterile blade and was placed in the disinfectant solution. A drop of 95% alcohol was placed on fungal growth on the slide. Excess alcohol was drained off and a drop of lactophenol cotton blue was placed.
4.4.4 Biochemical Characterization (Aneja, 2005)

4.4.4 i) Urease Test

The urease is a hydrolytic enzyme which attacks the carbon and nitrogen bond amide compounds with the liberation of ammonia. Some fungi have the ability to produce the enzyme urease.

4.4.4 ia) Requirements

i) Urea agar base ingredients

ii) Urea

iii) Distilled water

iv) Test tubes

Composition of Urea agar base is

i) Peptone - 0.1 g

ii) Glucose - 0.1 g

iii) Sodium chloride - 0.5 g

iv) Potassium Monohydrogen phosphate - 0.2 g

v) Phenol red (1.2%) - 1.0 ml

vi) Agar - 2.0 g

vii) Dis.H₂O - 100 ml

pH is adjusted to 6.8

4.4.4 ii) Preparation of Urea Solution

Twenty grams of urea was dissolved in 100 ml of aqueous solution and sterilized by filtration.

4.4.4 iii) Procedure

The ingredients were dissolved by heating, pH is adjusted to 6.8 and sterilized at 121°C for 15 minutes and cooled to 50°C. Glucose – 0.1 g and phenol red (0.2%) – 0.6 ml was added to molten base, steamed for 1 hour and cooled to 50°C. Ten milliliter of 20% urea solution was added and mixed well. After solidification of medium as slant, fungal culture was inoculated into the slant and incubated at 25°C for 3 days.
4.5 DETERMINATION OF AFLATOXIGENIC POTENTIAL OF THE ISOLATES

4.5.1 Primary Screening of Aflatoxigenic Fungi

The presumptively identified *Aspergillus* isolates were screened for aflatoxin production using the screening media viz. Coconut Agar Medium, *A. flavus/ A. parasiticus* agar (AFPA), and Hara *et al.* (1974) medium.

4.5.1 i) Coconut Agar Medium (Scherm *et al.*, 2005)

Coconut agar medium was prepared by shredding 100 g of coconut and homogenized it for 5 minutes with 300 ml of hot distilled water. The homogenate was then filtered through four layers of cheese cloth and the pH of the filtrate was adjusted to pH 7.0 using 2N NaOH. 100 ml of filtrate was added with 2.0 g of agar. The medium was sterilized at 121°C for 20 minutes under 15 lbs. The *Aspergillus* sp colonies were inoculated into the center of the agar plates. The colonies showing blue fluorescence under long-wave UV light (365 nm) on the reverse side after 2 to 5 days of growth are detected as aflatoxin producing ones and selected for further study.

4.5.1 ii) *Aspergillus flavus/A. parasiticus* Agar (Ghitakou *et al.*, 2006)

The selected isolate’s aflatoxin production was further confirmed using *Aspergillus flavus/A. parasiticus* agar (AFPA) having the following composition

i) Yeast Extract - 2.0 g
ii) Peptone - 1.0 g
iii) Ferric Ammonium Citrate - 0.05 g
iv) Dichloran 0.2% in ethanol - 0.1 ml
v) Chloramphenicol - 0.01 g
vi) Agar - 1.5 g
vii) Distilled water - 100 ml

pH was adjusted to PH 6.0-6.5

Colonies showing bright orange yellow on the reverse, an indicative of aflatoxin production were selected for further study.
4.5.1 iii) Hara \textit{et al.} (1974) medium

Hara \textit{et al.} (1974) medium, another screening media normally used for the assessing the aflatoxigenic potential is also used for confirming the selected isolates aflatoxin production. This yeast extract agar containing yeast extract 2 g; sucrose 20 g was prepared and the selected isolates were inoculated. After incubation at 25\(^{\circ}\)C for 3 days, the colonies were exposed to UV (365 nm) and observed for fluorescence (Murugan \textit{et al.}, 2005).

4.5.2 Screening for Aflatoxin Producing Xerophilic \textit{Aspergillus} (Al-Shohaibani \textit{et al.}, 2011)

All the selected colonies were grown on Malt Extract Yeast Extract 50\% Glucose Agar (MY50G) for determining their xerophilic nature.

4.5.2 i) Composition of MY50G

i) Malt extract \hspace{1cm} -10.0 g;

ii) Yeast extract \hspace{1cm} -2.5 g;

iii) Agar \hspace{1cm} -10.0 g

iv) Glucose \hspace{1cm} -500.0 g

All the inoculated plates were incubated at 25\(^{\circ}\)C for 3 weeks and routinely examined for fungal growth.

4.5.3 Quantitative Screening of Aflatoxin Production (Leontopoulos \textit{et al.}, 2003)

Isolated fungus was inoculated in Yeast Extract Sucrose (YES) medium that induce aflatoxin production for quantitative screening.

4.5.3 i) Preparation of YES Medium

Yeast extract sucrose medium was prepared by adding the following ingredients

i) Yeast extract \hspace{1cm} - 2 g

ii) Sucrose \hspace{1cm} - 15 g

iii) Distilled water \hspace{1cm} - 100 ml

YES broth was sterilized at 121\(^{\circ}\)C for 20 minutes under 15 lbs.
4.5.3 ii) Inoculation of Fungal Spores in YES Broth

Ten milliliters of YES broth was inoculated with $10^2$ conidia flask$^{-1}$ of *A. flavus* MTCC 10680. Inoculated broth was incubated at 30°C for three days. After incubation broth was autoclaved at 115°C for 30 minutes. Immediately after 30 minutes of autoclaving the mycelial growth was removed.

4.5.3 iii) AFB$_1$ Determination

YES medium was mixed with 30 ml of methanol and shaken well for 10 minutes. An aliquot of 1 ml from broth was used for AFB$_1$ analysis by HPLC (AOAC 991.31) method.

4.6 MOLECULAR IDENTIFICATION OF FUNGI

4.6.1 Growth and Harvest of Fungi (Jin et al., 2004)

Stock cultures were grown on potato dextrose agar plates at 30°C. Potato dextrose broth (40 ml) was prepared in 125 ml flask and sterilized at 121oC for 15 minutes and inoculated with spores from stock cultures. Incubated at 30°C for 7 days and harvested by filtering through Whatman filter paper washed and blotted dry.

4.6.2 DNA Extraction

Two hundred milligrams of washed mycelia was added to a 1.7 ml microcentrifuge tube. 450 µl if cell lysis solution and 1 µl of 50 µg/ ml concentration of proteinase K were added to the tube. The tube was vortexed for 10 seconds, incubated in a 65°C heating block for 1 hour and chilled on ice for 5 minutes. About 225 µl of protein precipitation reagent was added and the tube was vortexed for 5 seconds. The suspension was then centrifuged at 20,800 x g for 10 minutes to pellet cell debris the supernatant was transferred to a new tube, spun again to remove residual cellular material and then transferred to new tube. An equal amount of phenol – chloroform isoamyl alocohol was added and the tubes were gently inverted several times to precipitate the DNA, which was then pelleted by centrifugation at 20,800xg for 10 minutes. Pellet was washed with 70% ice cold ethanol, centrifuged and vacuum dried. DNA was resuspended in 50 µl of Tris EDTA and treated with 2µl of a
5 mg/ml concentration of RNAase A at 65°C for 1 hour. This genomic DNA is used for amplification.

4.6.3 PCR Amplification (Rigo et al., 2002)

Amplification of fungal internal spacer gene was carried out following the procedure of Rigo et al. (2002). Fragments containing the ITS regions were amplified using ITS1 (forward) and ITS4 (reverse) primers. The 100 µl reaction mixture contained 200 µM of each dNTP, 1 µM of ITS1 (forward primer) (TCCGTAGGTGAACCTGCGG) and 1 µM of ITS4 (reverse primer) (TCCTCCGCTTATTGATATGC), 2.5 units of Taq DNA polymerase enzyme and 200 ng genomic DNA in an amplification buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X-100) was overlaid with a drop of mineral oil. In a programmable thermal cycler a programme was feed for an initial denaturation 35°C for 5 minutes, next 35 steps consisting of 94°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 2 minutes and final extension of 75°C for 5 minutes. Amplified DNA was mixed with MgCl₂ to get a concentration of 1.5 mM. The presence of and size of the amplicons were confirmed by electrophoresis on 1.5% agarose gel. Further nucleotide sequence of the amplicon was determined by sequencing which was carried at Ocimum Biosolution, MCV Chennai, India using the instrument ABI 3130, Applied Biosystems, CA, USA. The DNA sequences obtained for the part of the ITS region was deposited into the GenBank database.

4.7 DETERMINATION OF PHYLOGENETIC RELATIONSHIP

The phylogenetic relationship between the isolate and other oil contaminants were determined as described previously (Al-Shohaibani et al., 2011). The genetic similarity between acquired Aspergillus DNA sequences were determined using BLAST analysis (Tatusova and Madden, 1999). The evolutionary relationships among the isolates were determined by the analysis and comparison of DNA sequences comparing the 28S rRNA sequences of fungal isolates. The DNA sequences were aligned first using CLUSTAL W that calculate a crude similarity measure between all pairs of sequences by using a fast and approximate alignment algorithm described by Wilbur and Lipman (1983) and then determined the order of sequences to be aligned in the final multiple alignment. The resulting distances were
used to calculate a phylogenetic guide tree which uses pair wise sequence distance calculation to perform multiple sequence alignment. The guide tree is calculated with the MEGA 4 method (Saitou and Nei, 1987; Tamura et al., 2007).

4.8 AUTHENTICATION AND CULTURE COLLECTION DEPOSITION OF FUNGAL ISOLATE

To ensure the taxonomic reliability of the aflatoxigenic fungi isolated from groundnut oil, properly prepared voucher specimen and culture was deposited in the Indian national facility, Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. The identification of the cultures were authenticated by the mycological division head Dr. D. Ananthapadmanaban, Scientist F, IMTECH, and preserved. The MTCC reference number assigned to the fungal culture *A. flavus* MTCC 10680 is also recorded and the same was selected for further study.

4.9 EXPERIMENTAL GROUNDNUT INFECTION BY AFLATOXIGENIC ISOLATE

The aflatoxigenic nature of selected isolate *A. flavus* MTCC 10680 and its toxin production *in vitro* were confirmed by inoculating them artificially into selected good quality groundnut and allowing them to grow and toxin production. The groundnut oil was extracted from artificially infected groundnut by expeller pressing and the obtained oil used for further research work.

4.9.1 Selection of Groundnut for Inoculation

Groundnut oil seeds were purchased from the groundnut seed market located at Thiruvannamalai where the most of oil expellers purchase groundnut for expelling oil. Groundnut seeds free of dirt and good quality seeds were selected by hand picking and were collected in a sterile container.

4.9.2 Sterilization of Groundnut

The collected groundnut seeds were crushed coarsely and distributed in a clean and presterilized tray. The tray was covered with an aluminium foil sheet and sterilized at 121°C for 15 minutes in an autoclave and cooled.
4.9.3 Preparation of Spore Suspension

SDA plates were incubated with the spores from the stock culture of *A. flavus* MTCC 10680 and incubated at 28°C for 5 days. Spore suspension was prepared by adding 10 ml of sterile distilled water to a SDA plate containing speculated colonies and adjusted to give a final spore concentration of approximately $10^6$ spores/ml with Neubauer haemocytometer.

4.9.4 Inoculation of Spore Suspension in Groundnut

Five hundred micro liters of spore suspension was added in the groundnut and mixed thoroughly for the even distribution of spores. The tray was incubated at room temperature (28 ± 2°C) for 7 days (Plate 1a & Plate 1b).

4.9.5 Extraction of Oil

The inoculated groundnut was subjected to heat treatment at 121°C for 30 minutes for complete destruction of spores and vegetative forms of fungi. Expeller crushing machine was cleaned thoroughly. Groundnut was crushed completely in expeller crushing to obtain oil and collected in a clean and sterile container. This oil was used for further research.

4.10 DETERMINATION OF AFLATOXIN LEVELS IN GROUNDNUT OIL

The strong fluorescence nature of aflatoxins under long-wave UV light makes their easy determination using thin layer chromatography (TLC). The aflatoxin elaboration of the fungi was determined as detailed below

4.10.1 TLC Analysis for Groundnut Oil Aflatoxin

The aqueous organic solvent extraction, their purification and analysis of groundnut oil aflatoxins were performed by following the standard method (Sandosskumar *et al.*, 2007) as detailed below

4.10.1 i) Principle

Thin layer chromatography is a type of planar chromatography. TLC is routinely used in the field of phyto-chemicals, to identify the components in a
compound mixture like alkaloids, phospholipids, amino acids. The separation depends on the relative affinity of compounds towards stationary and mobile phase. The compounds under the influence of mobile phase (driven by capillary action) travel over the surface of stationary phase. During this movement the compounds with higher affinity to stationary phase travel slowly while the others travel faster. Thus separation of components in the mixture is achieved. Once separation occurs individual components are visualized as spots at respective level of travel on the plate. The natures of the compounds are identified by suitable detection techniques.

4.10.1 ii) TLC Apparatus (AOAC 970.43)

i) Glass Plates (8x8 inches),
ii) Mounting Board,
iii) Spotting Template,
iv) Applicator,
v) Microsyringe (25 μl),
vi) Desiccating Storage Cabinate,
vii) Storage Rack,
viii) Developing Tank,
ix) Long wave 15 watts UV Lamp –Phillips make (long wave transilluminator),
x) Vortex Mixer,

4.10.1 iii) Reagents

i) Silica Gel
ii) Chloroform, acetone and water (88 ml+12 ml+1 ml) - developing Solvent.

4.10.1 iv) Preparation of Plates (AOAC 968.22Fa)

Thirty grams of silica gel was weighed into a 300 ml glass stoppered Erlenmeyer flask, 65 ml of water was added and vigorously shaken for 1 minute and poured into applicator. Amount of water is adjusted to obtain good consistency of slurry for spreading. The prepared slurry was immediately coated over 20 cm x 20 cm glass plate with 0.25 mm thickness of gel suspension and allowed to solidify without disturbance for 10 minutes. Coated plate was dried for 2 hours at 80°C and stored in
desiccating cabinet with active silica gel desiccant until before use. A line was scribed 16 cm from bottom edge of the silica gel as solvent stop. Line was scribed 0.5 cm in each side.

4.10.1 v) Standard Preparation (AOAC 971.22Bb)

The aflatoxin (Sigma Aldrich, USA) was prepared as per AOAC procedure. The prepared aflatoxin solution was transferred in a glass stoppered flask and diluted with 9 µl of benzene and acetonitrile (98 + 2) that gives 10 µg/µl.

4.10.1 vi) Extraction (Sandosskumar et al., 2007)

Five grams of peanut oil sample was mixed with 25 ml of methanol: Water (55+45), 10 ml of hexane and 0.2 g of sodium chloride and shaken vigorously using vortex mixer to ensure complete dissolution. The methanolic layer was separated and then extracted with 2.5 ml of chloroform for 3 times. The chloroform was concentrated to 1 ml under vacuum.

4.10.1 vii) Preparation of Solvent

Chloroform, acetone and water were mixed in the proportion of 88 ml + 12 ml + 1 ml respectively. Fifty milliliters of this solvent mixture was added to the developing tank.

4.10.1 viii) Chromatogram Development and Analysis

Twenty five microlitres of this concentrated aflatoxin extract was spotted four centimeters from bottom on to the 0.25 mm silica gel TLC plate. 25 µl of standards was placed on the same plate. The sample applied plate was placed in the tank dipping 2 cm gel in the solvent. The plate was developed for 40 minutes at 25°C. Plate is removed from tank and the solvent was removed by evaporation at room temperature. The plate was placed coated side up on UV (365 nm) lamp in dark room for illumination and the concentration of the aflatoxins were determined by visual comparisons.
4.10.2 HPLC Determination of Aflatoxins (AOAC 991.31)

Since the post-column derivatization of aflatoxins can increase detectability and/or selectivity of responses for the HPLC detector, the AOAC Official Method SM 991.31 is also used for the determination of groundnut oil aflatoxins.

The test portion was extracted with methanol water mixture CH₃OH-H₂O (7+3). The obtained extract was filtered, diluted with water, and applied to affinity column containing monoclonal antibody specific for aflatoxins B₁, B₂, G₁, G₂. Aflatoxins are isolated, purified and concentrated on column and removed from antibodies with CH₃OH. Total aflatoxins are quantitated by fluorescence measurement after reaction with bromine solution. Individual aflatoxins are quantitated by LC with fluorescence detection and post column iodine derivatization.

4.10.2 i) Apparatus

i) Blender – high speed. With 500 ml blender jar and cover.
ii) Filter paper – 24 cm, prefolded
iii) Glass microfibre filter paper
iv) Affinity column – aflatest P column
v) Syringe – 20 ml
vi) Hand pump – 20 ml
vii) Fluorometer cuvet – 12 x 75 mm
viii) Fluorometer – 360 nm excitation filter and 450nm emission filter
ix) Automatic dispenser – amber 2 oz bottle with 1.0 ml automatic dispenser
x) Volumetric flask – 2 ml
xi) LC pump – with flow rate at 1 ± 0.005 ml/min
xii) Injection system – Syringe injection valve with 50 μl loop
xiii) LC column - 4.6 mm x 25 cm, 5 μm, C₁₈
xiv) Post column derivatization system – second LC column pulseless pump, zero-dead volume piece, 610 cm x 0.05 mm id Teflon tubing and heating bath or post column reactor
xv) Fluorescence detector – 360 nm excitation filter and 420 nm cutoff emission filter
4.10.2 ii) Reagents

i) Solvents – LC grade methanol, acetonitrile, water and methanol
ii) Extraction solvent – CH$_3$OH – H$_2$O (7+3)
iii) Bromine developer solution – 0.002 %
iv) Fluorometer calibration standards – used 0.05 M H$_2$SO$_4$ as blank.
v) LC mobile phase – H$_2$O + CH$_3$CN – CH$_3$OH (3+1+1) degassed
vi) Postcolumn reagent – 100 mg iodine is dissolved in 2 ml CH$_3$OH and 200 ml of H$_2$O, stirred for 1 hour and filtered through 0.45µm filter
vii) Aflatoxin standard solutions for LC.

4.10.2 iii) Cleaning of Glass Wares

All the glass wares were soaked in 10% solution of house hold bleach which generally contains 5.25% NaOCl before using and discarding.

4.10.2 iv) Preparation and Extraction of Samples

Weighed 25 g of test portion added into blender jar. Five grams of NaCl and 125 ml extraction solvent was added and blended for 2 minutes at high speed. Then filtered through a prefolded filter paper. Fifteen milliliters filtrate was pipetted into 125 ml glass-stoppered Erlenmeyer flask. Thirty milliliters of water was added, stoppered and mixed. Diluted extract was filtered through glass microfibre paper for less than 30 minutes before affinity column chromatography.

4.10.2 v) Affinity Column Chromatography

The cap of the column was removed. Tip was cut off and used cap as connector between column and reservoir. Fifty milliliter of second filtrate was pipetted into reservoir. Reservoir was connected to air- filled hand pump. The end cap was removed from the column. Extract was pushed through column at flow rate of 2 drops/second. Hand pump was disconnected from the reservoir and pump was filled with air. Hand pump was reconnected to reservoir and 3 ml of air was passed through the column. Hand pump was again disconnected and 10 ml of water was added to the reservoir. Hand pump was filled with air and reconnected. Water was pushed through column at flow rate of 6 ml/minute. Repeated with another 10 ml of water and
discarded water washings. Hand pump was disconnected from the column and filled with air. Hand pump was reconnected and passed 3 ml air through column. Hand pump was again disconnected and 1.0 ml of LC grade CH₃OH was added to reservoir. Elute was collected in a container. Hand pump was filled with air, connected and passed CH₃OH through column. Three milliliters of air was passed additionally through column.

Two milliliters of CH₃OH elute was collected in 2 ml volumetric flask, diluted to volume with LC grade water, mixed and proceeded to quantitate individual aflatoxin by LC with fluorescence detection.

4.10.2 vi) LC Determination with Fluorescence Detection and Post Column Derivatization

Liquid chromatography column was connected to one arm of stainless steel, low dead volume T, using 0.01 inch id tubing. Outlet of second LC pump was connected, which delivers the post column reagents to second arm of T. One end of 610 cm x 0.5 mm id coil of Teflon tubing was connected to third arm of T and connected other end to detector.

The reaction coil temperature was maintained at 70°C using oven. Flow rate of mobile phase was set at 1.0 ml/minute, post column reagent flow rate at 0.3 ml/minute and total flow rate in reaction coil at 1.3 ml/minute.

The entire system was allowed to run for 20 minutes to stabilize. Sensitivity control of fluorescence detector was adjusted to give reasonable response for 0.125 ng aflatoxin G₂.

Fifty microliters of working standard mixture was injected into injector. Excess of 30 microliters of working standard mixture was injected into the injector to ensure complete filling. Aflatoxins eluted in order G₂, G₁, B₂ and B₁ with retention times of 6, 8, 9 and 11 minutes respectively.

Fifty microliters of CH₃OH eluted test solution was injected into injector. Aflatoxin B₁, B₂, G₁ and G₂ peaks in chromatogram was identified from analysis of test solution by comparing retention times with reference standard curves.
Concentration of aflatoxin B₁, B₂, G₁ and G₂ was calculated using
\[ W = 25 \text{ g} \times (15 \text{ ml} \times 125 \text{ ml}) \times (15 \text{ ml} / 45 \text{ ml}) = 1 \text{ g} \]
Where, \( W \) – Weight of sample represented by elute
Aflatoxin (ng/g) = \( A \times (T_v/I_v) \times (I \times W) = A \times 40 \)
\( A \) – ng aflatoxin in elute injected
\( T_v \) – final elute volume (2000\( \mu \)l)
\( I_v \) – elute injected (50 \( \mu \)l)

4.11 SELECTION AND DETERMINATION OF ANTIAFLATOXIGENIC ACTIVITY CULINARY PLANTS

Among the available wide range of species, parts, and secondary metabolites contributing to the significant variety and complexity of human diet, the following culinary plants widely used in south Indian diet especially in Tamilnadu, India were selected to understand their role in health benefits, aflatoxin detoxification and extension of shelf life of oil.

4.11.1 Vellai Puntu (Allium sativum)

4.11.1 i) Classification

Kingdom – Plantae

Phylum – Magnoliophyta
Class – Lilopsida
Order – Liliales
Family – Liliaceae
Genus – Allium
Species – Allium sativum

4.11.1 ii) English Names: Garlic

4.11.1 iii) Indian Names

Tamil : Vellai Puntu ; Hindi : Lasun, Lahasun ; Kannada : Bellulli ; Malayalam : Vellui; Sanskrit : Lasunah Rasonah; Telugu : Vellulli Tellagadda
4.11.1 iii) Morphology

It is a perennial plant which rises to a height of 1-2 ft (0.3-0.6 m) tall at maturity. The foliage comprises a central stem 25 - 100 cm tall, with flat or keeled leaves 30 - 60 cm long and 2 - 3 cm broad.

The leaves are long, narrow and flat like grass, with a crease down the middle and are held erect in two opposite ranks.

The flowers are placed at the end of a stalk rising direct from the bulb and are whitish, grouped together in a globular head and are surrounded by a papery basal spathe; each flower is white, pink or purple, with six tepals 3 - 5 millimetres long. The flowers are commonly abortive and rarely produce any seeds.

A garlic head is generally four to eight centimeters in diameter, white to pinkish or purple, and is composed of numerous (8 - 25) discrete bulbs. The bulb is of a compound nature, consisting of numerous bulblets, known technically as 'cloves,' grouped together between the membranous scales and enclosed within a whitish skin, which holds them as in a sac (Plate 2a).

4.11.1 iv) Culinary Uses

The bulb is the only edible part. When a cell of a garlic clove is broken by chopping, chewing, or crushing, enzymes stored in cell vacuoles trigger the breakdown of several sulfur-containing compounds stored in the cell fluids. The resultant compounds are responsible for the sharp or hot taste and strong smell of garlic. Garlic is most often used as a seasoning or a condiment. Garlic is widely used in many forms of cooking for its strong flavor, which is considered to enhance many other flavors. It is the most widely used component of the Lebanese cuisine. The shoots are often pickled in Russia and states of the Caucasus and eaten as an appetizer. In parts of Austria, salads are prepared with vinegar, oil and minced garlic. Raw garlic appears in quite a multitude of Mediterranean sauces.
4.11.2 Ginger (Zingiber officinale)

4.11.2 i) Classification

Kingdom – Plantae
Subkingdom: Trachaeobionta
Super division: Spermatophyta
Division: Magnoliophyta
Class: Liliopsida
Subclass: Zingiberidae
Order: Zingiberales
Family: Zingiberaceae
Genus: Zingiber Mill
Species – Zingiber officinale Roscoe

4.11.2 ii) Indian Names

Hindi: Adrak; Manipuri: Shing; Marathi: Alha, Aale; Tamil: Ingee, Inji;
Gujarati: Sunth; Sanskrit: Adraka.

4.11.2 iii) Morphology

Ginger has a perennial rhizome or stems which creeps and increases in size underground. Roots grow from the bottom of the rhizome and shoots from the upper surface. In the spring it sends up from its rhizome a green reed-like stalk about 2 feet high, with narrow lanceolate leaves. These leaves die back after the growing season. The flowering stalk rises directly from the rhizome with the leaves and consists of an oblong spike with scalloped green bracts. From each bract one or more white or yellowish-green flowers is produced, blooming for several days. The underground rhizome is the source of commercial "ginger root" (Plate 2b).
4.11.2 iv) Culinary Uses

“Ginger produces a hot, fragrant kitchen spice. Young ginger rhizomes are juicy and fleshy with a very mild taste. They are often pickled in vinegar or sherry as a snack or just cooked as an ingredient in many dishes. They can also be steeped in boiling water to make ginger tea, to which honey is often added; sliced orange or lemon fruit may also be added. Ginger can also be made into candy. Mature ginger roots are fibrous and nearly dry. The juice from old ginger roots is extremely potent and is often used as a spice in Indian recipes, and is a quintessential ingredient of Chinese, Japanese and many South Asian cuisines for flavoring dishes such as seafood or goat meat and vegetarian cuisine. Ginger acts as a useful food preservative. Fresh ginger can be substituted for ground ginger at a ratio of 6 to 1, although the flavors of fresh and dried ginger are somewhat different. Powdered dry ginger root is typically used as a flavoring for recipes such as gingerbread, cookies, crackers and cakes, ginger ale, and ginger beer. Candied ginger is the root cooked in sugar until soft, and is a type of confectionery. Fresh ginger may be peeled before eating. For longer-term storage, the ginger can be placed in a plastic bag and refrigerated or frozen” (wikipedia).

4.11.3 *Chinna vengayam (Allium cepa L.)*

4.11.3 i) Classification Kingdom –

Plantae Subkingdom:

Trachaeobionta Super
division:Spermatophyta
Division: Magnoliophyta
Class: Liliopsida
Subclass: Lilidae
Order: Liliales
Family: Liliaceae
Genus: *Allium* L.
Species – *Allium cepa* L.
4.11.3 ii) English Name: Onion

4.11.3 iii) Indian Names

Hindi : Chota piyaz, Choti piyaz.

4.11.3 iv) Morphology

*A. cepa* L. is an evergreen bulb growing to 0.6 meter (2 feet). It forms a bulb, this is, a cluster of subterranean leaves designed to store energy to allow for a rapid growth in spring. These edible bulbs are composed of food-storage leaves that are rich in sugar and pungent oil, the source of its strong taste. The above-ground green leaves, typically long and tubular, are also eaten. It is hardy to zone 5 and is not frost tender. It is in flower from Jun to July. The flowers are hermaphrodite (have both male and female organs) and are pollinated by Bees, insects. Besides the bulb, the superterranean green leaves can also put to culinary use, most often in the form of young onion plants (green onion or spring onion). Their flavour is more similar to chives (Plate 2c).

4.11.3 v) Culinary Uses

In contemporary Indian cooking, onion is the basis of most sauces and gravies. Onions are often chopped and used an ingredient in various hearty warm dishes, although onions can also be a more prominent ingredient, for example in French onion soup, or be used raw in cold salads. Onions pickled in vinegar are eaten as a snack. These are often served as a side serving in fish and chip shops throughout the United Kingdom and Australia, often served with cheese in the United Kingdom, and are referred to simply as "pickled onions" in Eastern Europe.

4.11.4 Shallot (*Allium cepa* var. *aggregatum*)

4.11.4 i) Classification

Kingdom – Plantae
clade: Angiosperms
clade: Monocots
Order – Asparagales
Family – Amaryllidaceae
Genus – *Allium*
Species – *Allium cepa var. aggregatum*

4.11.4 ii) English Name: Shallot

4.11.4 iii) Indian Names

Tamil : *pallari vengayam*; Hindi : *kanda* or *gandana*; Marathi: *kanda*; Marwari *gandana*; Punjabi : *kanda*; Bengali : *gundhun*; Malayalam :*cheriya ulli* or *chuvanna ulli.*

4.11.4 iv) Morphology

This group contains shallots and potato onions, also referred to as multiplier onions. It is a small bulbous plant. Bulbs clustered, narrowly ovoid or cylindric-ovoid. Scape not developed. They are propagated almost exclusively from daughter bulbs, although reproduction from seed is possible. Shallots are the most important subgroup within this group and comprise the only cultivars cultivated commercially. They form aggregate clusters of small, narrowly ovoid to pear-shaped bulbs. Potato onions differ from shallots in forming larger bulbs with fewer bulbs per cluster, and having a more flattened (onion-like) shape. However, intermediate forms exist (Plate 2d).

4.11.4 v) Culinary Uses

The distinction between onion and shallot is weak in Indian cuisine; larger varieties of shallot are often confused with small red onions and used interchangeably. Eaten raw, it can be sliced up and used in salads, sandwich fillings etc, it can be baked or boiled as a vegetable in its own right and is also commonly used as a flavoring in soups, stews and many other cooked dishes. The southern regions of India distinguish shallots from onions in recipes more often, especially the much loved tiny varieties (about the width of a finger); these are widely used in salads, curries and different types of sambar. Shallots pickled in red vinegar are common in many Indian restaurants, served along with the sauces and papad on the condiments tray. Indians also use it as a home remedy for sore throats, mixed with jaggery or sugar. It is widely used in Southeast Asian cuisines. Crispy shallot chips are also used in southern Chinese cuisine. In Indonesia, sometimes it is made into pickle which is usually added
in several traditional foods. Its sourness is thought to increase one's appetite. It is a common one in Arabian foods.

4.11.5 Karuveppilei (*Murraya koenigii*) (L.) Spreng.

4.11.5 i) Classification

- Kingdom: Plantae
- Division: Angiosperms (unranked): Rosids
- Class: Eudicots
- Order: Sapindales
- Family: Rutaceae
- Genus: *Murraya*
- Species: *Murraya koenigii*

4.11.5 ii) English name: Curry leaf-tree

4.11.5 iii) Indian names: karivempu, karuveppilei (Tamilnadu) karepaku (Andhra Pradesh); narasingha, bishahari (Assam); barsanga, kartaphulli (Bengal); gorenimb, kadhilimbdo (Gujrat); mitha neem, gandhla, gandhela, gandhelu (Himachal Pradesh); kathnim, mitha neem, kurry patta gandhela, barsanga (Hindi); karibeva (Karnataka); kariveppilei (Kerala); gandhela, gandla, gani (Kumaon); bassan, basango, bhursanga (Orissa); surabhinimba, kalasaka, mahanimb (Sanskrit).

4.11.5 iv) Morphology

A small spreading shrub, about 2.5 metres high; the main stem, dark green to brownish, with numerous dots on it; its bark can be peeled off longitudinally, exposing the white wood underneath; the girth of the main stem is 16 cm.

Leaves, exstipulate, bipinnately compound, 30 cm long, each bearing 24 leaflets, having reticulate venation; leaflets, lanceolate, 4.9 cm long, 1.8 cm broad, having 0.5-cm-long petiole (Plate 2e).

Flowers, bisexual, white, funnel-shaped, sweetly scented, stalked, complete, ebracteate, regular, actinomorphic, pentamorous, hypogynous, the average diameter of a fully opened flower being 1.12 cm; inflorescence, a terminal cyme, each bearing
60 to 90 flowers; calyx, 5-lobed, persistent, inferior, green; corolla, white, polypetalous, inferior, with 5 petals, lanceolate; length, 5 mm; androecium, polyandrous, inferior, with 10 stamens, dorsifixed, arranged into circles of five each; smaller stamens, 4 mm. long whereas the longer ones, 5 to 6 mm; gynoecium, 5 to 6 mm long; stigma, bright, sticky; style, short; ovary, superior.

Fruits, round to oblong, 1.4 to 1.6 cm long, 1 to 1.2 cm in diameter; weight, 880 mg; volume, 895 microlitres; fully ripe fruits, black with a very shining surface; pulp, Wistaria blue 640/2; the number of fruits per cluster varying from 32 to 80.

Seed, one in each fruit, 11 mm long, 8 mm in diameter, color spinach green 0960/3; weight, 445 mg; volume, 460 microlitres.

4.11.5 v) Culinary Uses

Almost every part of this plant has a strong characteristic odour. The people of the plains, particularly of southern India, use the leaves of this plant as a spice in different curry preparations. The leaves are used as a spice in different curries and impart a very good flavour to the preparations. This plant is quite ornamental due to its compound leaves. It can, therefore, be used as a hedge and as an ornamental shrub. The shiny-black fruits are liked both by children and adults. As revealed by the chemical composition of the fruits, they are very nutritious. These fruits have also many medicinal properties. The branches of M. koenigii (L.) Spreng. are very popular for cleaning the teeth as datun and are said to strengthen the gums and the teeth.

4.12 EXTRACTION OF BIOACTIVE COMPOUND FROM SPICES (Irkin and Korukluoglu, 2007)

4.12.1 Collection of Culinary Plants

A. sativum, Z. officinale, A. cepa L. and A. cepa var. aggregatum were collected from the local markets in Thiruvannamalai, Tamilnadu. These spices were collected in clean bags and transported to the lab and processed.
4.12.2 Extraction of Bioactive Compounds From *A. sativum*

4.12.2 i) Materials Used

i) Domestic blender
ii) Membrane filter apparatus (Borosil) with Cellulose acetate membrane filter (0.45 μm pore size)
iii) Refrigerator
iv) Sterile distilled water
v) Glass beaker (500 ml)

4.12.2 ii) Preparation of *A. sativum* for Extraction

An extract of *A. sativum* was prepared from fresh garlic bulb commercially available in the market. Cloves were separated from the bulb of *A. sativum* and were hulled. Hullled cloves were used for the extraction of bioactive compounds.

4.12.2 iii) Extraction: (Irkin and Korukluoglu, 2007)

Two hundred and seventy grams of fresh hulled *A. sativum* was homogenized in 300 ml of sterile distilled water in a domestic blender at an average speed for 2 minutes bursts for a total of 10 minutes. Pour the pulp in a glass beaker and macerated for 24 hours at 4°C in a refrigerator. The resulting extract was a thick fluid and filtered through a sterile 0.45 μm pore size cellulose acetate membrane filter under nitrogen gas pressure (Plate 3a). The extract was used directly for analysis of antifungal activity and antitoxigenic activity against *A. flavus* MTCC 10680 isolated from groundnut oil. The extract was preserved at 4°C till its use.

4.12.3 Extraction of Bioactive Compounds from *Z. officinale*

4.12.3 i) Materials Used

i) Domestic blender
ii) Membrane filter apparatus (Borosil) with Cellulose acetate membrane filter (0.45 μm pore size)
iii) Refrigerator
iv) Sterile distilled water
v) Glass beaker (500 ml)
4.12.3 ii) Preparation of Z. officinale for Extraction

Rootstalk or rhizome part of Z. officinale was used for extract of bioactive compounds. Fresh rhizomes of Z. officinale was collected from the market and washed with clean water, cut into small pieces and used for extraction.

4.12.3 iii) Extraction (Irkin and Korukluoglu, 2007)

Two hundred and seventy grams of pieces of fresh rootstalk of Z. officinale was blended at an average speed in 300 ml of sterile distilled water in a domestic blender for 10 minutes with 2 minutes of burst time. The pulp of Z. officinale was transferred to a 500 ml glass beaker covered with an aluminium foil sheet and placed in a refrigerator at 4°C for 24 hours. Refrigerated extract was filtered through a 0.45 µm pore size cellulose acetate membrane filter under nitrogen gas pressure (Plate 3b). The filtrate was stored at 4°C till further use.

4.12.4 Extraction of Bioactive Compounds from A. cepa L. and A. cepa var. aggregatum

4.12.4 i) Materials Used

i) Domestic blender
ii) Membrane filter apparatus (Borosil) with Cellulose acetate membrane filter (0.45 µm pore size)
iii) Refrigerator
iv) Sterile distilled water
v) Glass beaker (500 ml)

4.12.4 ii) Preparation of A. cepa L. and A. cepa var. aggregatum for Extraction

Bulp part of A. cepa L. and A. cepa var. aggregatum was used for extraction of bioactive compounds. Fresh A. cepa and A. cepa var. aggregatum bulb parts were collected from the market. Bulbs of A. cepa L. were separated from the cluster. The outer layer of bulb of A. cepa and A. cepa var. aggregatum was removed, cut into small pieces and used for extraction.
4.12.4 iii) Extraction: (Irkin and Korukluoglu, 2007)

Two hundred and seventy grams of pieces of fresh *A. cepa* and *A. cepa* var. *aggregatum* bulb was chopped separately in 300 ml of sterile distilled water in a domestic blender at an average speed till it becomes paste. The blended paste was transferred into a 500 ml beaker and covered with aluminium foil. The mixtures were macerated for 24 hours at 4°C. After 24 hours resulting extracts of material were filtered and sterilized using a 0.45 µm pore size cellulose acetate membrane filter under nitrogen gas pressure (Plate 3c & Plate 3d). The extracts were used directly for analysis of fungal growth inhibition and inhibition of aflatoxin. The extracts were preserved at 4°C.

4.12.5 Bioactive Compound Extraction from *M. koenigii*

4.12.5 i) Materials Used

i) Soxhlet extractor

ii) Heating mantle (250 ml capacity)

iii) Domestic blender

vi) Filter paper

v) Methanol

vi) Acetone

vii) Acetonitrile

viii) Chloroform

ix) Reagent bottle (500 ml)

4.12.5 ii) Collection of *M. koenigii* Leaves

Fresh disease free matured leaves of *M. koenigii* were collected in and around Thiruvannamalai. The leaf was examined morphologically for shape, apex, base and margin for identity confirmation. The leaves were thoroughly washed, shade dried for three days and powdered with the help of domestic blender at a medium speed for 10 minutes.
4.12.5 iii) Extraction (Satish \textit{et al.}, 2007)

Twenty five grams of \textit{M. koenigii} powder was weighed and packed in a filter paper and placed in the thimble of Soxhlet extractor. Methanol (125 ml) was taken in a conical flask. Inlet of condenser was connected to the tap and an outlet pipe was connected. Soxhlet extractor was assembled and placed in the heating mantle and extracted successively for 48 hours (Plate 3c). The same procedure was followed for extraction using acetone, acetonitrile and chloroform as the solvent. All the extracts were concentrated using rotary flash evaporator and preserved at 4°C in air tight brown bottle till further use.

4.13 DETERMINATION OF ANTIAFLATOXIGENIC POTENTIAL OF CULINARY PLANT EXTRACTS

4.13.1 Effect Extracts on Fungal Biomass (Dry Weight) and Aflatoxin Elaboration.

The Atanda \textit{et al.} (2007) method was followed for determining the effect of selected culinary plant extract on the growth; biomass production and toxin elaboration of the aflatoxigenic isolate \textit{A. flavus} MTCC 10680.

4.13.1 i) Preparation of Culture Medium

Sabouraud’s dextrose broth (SD broth) was used for the determination plant extract's effect on \textit{A. flavus} MTCC 10680 fungal growth. The SD broth was prepared using the following ingredients

\begin{itemize}
  \item i) Peptone - 1.0 g
  \item ii) Dextrose - 4.0 g
  \item iii) Distilled water - 100 ml
  \item iv) Streptomycin - 50 μl
\end{itemize}

All the ingredients were dissolved in distilled water and the pH was adjusted to 5.6. SD broth was dispensed in 50 ml quantities into 100 ml Erlenmeyer flasks and were sterilized by autoclaving it at 121°C for 15 minutes under 15 lbs.
4.13.1 ii) Preparation of Spore Suspension

SDA plates were inoculated with the spores from the stock culture of *A. flavus* previously maintained on Sabouraud’s dextrose agar (SDA) slant at 4°C and were incubated at 28°C for 5 days. After profuse growth, the inoculums spore suspension was prepared by adding 10 ml of sterile distilled water to the SDA plates and scraping it with loop. The numbers of spores were adjusted to give a final spore concentration of approximately $10^6$ spores/ml with Neubauer haemocytometer counting. 50 µl of these prepared spores suspension was inoculated into sterilized Saboraud’s dextrose broth and mixed well for even distribution of spores throughout the broth.

4.13.1 iii) Addition of Extracts

The different concentrations of culinary plant extracts were added to each flask to obtain a final concentration of 1-5% (v/v) (500 µl – 2500 µl) and were incubated at room temperature for 7 days. Sabouraud’s dextrose broth that is not added with spice extracts served as control. After the incubation period of 7 days, the flasks were given heat treatment at 121°C for 30 minutes in an autoclave to destroy spores and vegetative mycelia.

4.13.1 iv) Separation of Mycelial Mat

The mycelial mat and broth were separated by filtration on dry Whatman No.1 filter paper by applying vacuum. The mats were washed with sterile distilled water and completely transferred to dry preweighed aluminium foil and dried at 85°C for 24 hours in a hot air oven. The dry weight of mycelial mats was measured using digital weighing balance and noted. Dry weight was determined by subtracting the weight of the preweighed butter paper from the dry weight butter paper and mycelial mats.

4.13.1 v) Estimation of Aflatoxin in Broth

Aflatoxin released into the broth was estimated from mycelial mat removed filtered broth. The growth in the flask previously added with 5% (2500 µl) of spice extract was used for the estimation of aflatoxin by HPLC (AOAC 991.31).
4.13.2 FUNGAL MYCELIAL GROWTH INHIBITION ASSAY

Fungal mycelia growth inhibition assay was carried out using potato dextrose agar (PDA) as described (Sandoss kumar et al., 2007).

4.13.2 i) Preparation of Plant Extract Incorporated PDA Plates

PDA was prepared by adding the following ingredients

i) Potato extract - 20 g
ii) Dextrose - 2.0 g
iii) Agar - 1.5 g
iv) Distilled water - 100 ml

The ingredients were dissolved in distilled water in 250 ml Erlenmeyer flasks and pH was adjusted to 6.5 and the flasks were sterilized at 121°C for 15 minutes under 15 lbs in an autoclave. Different concentrations (1200 μl, 1400 μl, 1600 μl, 1800 μl and 2000 μl) filter sterilized culinary plant extracts were added to PDA medium previously maintained at 45°C, mixed uniformly and poured into the sterile petridishes. The plates were allowed to solidify.

4.13.2 ii) Measurement of Fungal Growth Inhibition

A 3 mm diameter agar block containing 5 day old A. flavus MTCC 10680 grown in PDA medium was transferred to the centre of the plate containing spice extracts and incubated at 28°C for 4 days. Plate without the spice extracts was used as control. After incubation fungal growth was measured.

4.13.3 Antifungal Activity Determination by Agar Cup Method

The antifungal activity of the plant extracts were determined also by agar cup diffusion method as described (Panda et al., 2010).

4.13.3 i) Medium Preparation and Inoculation

In this method the Sabouraud’s dextrose agar (SDA) was used for antifungal assay of culinary plant extracts on the isolate A. flavus MTCC 10680. SDA was prepared by using the following ingredients in the given proportion
i) Peptone - 1.0 g
ii) Dextrose - 4.0 g
iii) Agar - 1.5 g
iv) Distilled water - 100 ml
v) Streptomycin - 50 µl

$p^H$ was adjusted to 5.6 and sterilized at 121°C for 15 minutes under 15 lbs in an autoclave and poured in the sterile petridishes. After the medium solidification, (0.1%) of previously prepared spore suspension was spreaded on SDA surface and were allowed to dry for 30 minutes.

4.13.3 ii) Extract Addition and MIC Determination

Using 6 mm sterile cork borer wells were made in the medium. Different concentrations of spice extracts were added to the wells and incubated at room temperature (28 ± 2) for 4 days. All the experiments were carried out in triplicates and the zone of inhibition were measured and noted.

4.14 ANALYSIS OF CULINARY PLANT TREATMENT ON CONTAMINATED OIL

To determine the efficiency of the spices treatment on aflatoxin detoxification and oil quality, the regularly used parts of culinary plants *A. sativum*, *Z. officinale*, *A. cepa* L. and *A. cepa* var. *aggregatum*, *M. koenigii* were fried in the artificially contaminated oil and the changes in aflatoxin level and fatty acid were determined.

4.14.1 Preparation of Spices for Boiling in Oil

4.14.1 i) *A. sativum*

Cloves from the bulb of *A. sativum* was separated and hulled. Twenty five grams of hulled cloves of *A. sativum* were coarsely crushed in the mortar and pestle and used for oil heat treatment.

4.14.1 ii) *Z. officinale*

Rhizome or rootstalk of *Z. officinale* was washed cleanly and was cut into small pieces with a help of knife and used for heat treatment.
4.14.1 iii) *A. cepa* L. and *A. cepa* var. *aggregatum*

Bulb part of the *A. cepa* L. and *A. cepa* var. *aggregatum* were used for boiling with oil. They were cut into pieces after removing the outer layers of the bulb and were used.

4.14.1 iv) *M. koenigii*

Fresh leaves from the stems were removed and were washed with clean water, air dried and used for treatment.

4.14.2 Heat Treatment

Twenty five grams of each culinary plant was added with 50 ml oil separately and fried for 5-7 minutes at a medium flame. After characteristic change of the culinary plants, the heat treatment was stopped. The oil was filtered using a sterile filter paper and were subjected to chemical analyses like acid value (AOAC 969.17), saponification value (AOAC 920.160), iodine value (AOAC 993.20), HPLC aflatoxin (AOAC 991.31) analysis as previously described and fatty acid analysis by Gas chromatography- Mass spectrophotometry (GC-MS).

4.15 FATTY ACID ANALYSIS OF GROUNDNUT OIL BY GAS CHROMATOGRAPHY–FID

Gas chromatography–mass spectrometry (GC-FID) Agilent 6890N make is a method that combines the features of gas liquid chromatography and mass spectrometry to identify different substances within a test sample.

4.15.1 Apparatus

4.15.1 i) Gas Chromatography Column – Varian M1 33-10-1 Factor 4 Capillary Column,

- a) Length of the column -30.0 m,
- b) Diameter -250 µm,
- c) Film thickness in column -0.25 µm
4.15.1 ii) GC Conditions

a) Column oven temperature -40°C  
b) Injector temperature -250°C  
c) Injection mode –split  
d) Split ratio -25:1  
e) Flow control mode –linear velocity  
f) Column flow -1 ml/minute  
g) Carrier gas –Hydrogen  
h) Injection volume – 1 µl

4.15.1 iii) Flame Ionization Detector (FID)

a) Detector temperature : 250°C  
b) H₂ flow : 30 ml/minute  
c) Air flow : 300 ml/minute  
d) Makeup flow : 25 ml/minute

4.15.2 Injector.

4.15.3 Reagents

Hydrogen gas

4.15.4 Injection of Carrier Gas

Hydrogen gas was used as carrier gas. It was injected at the rate of 30 ml/min through the column.

4.15.5 Preparation of Oil Sample

About 0.25 g of oil was transferred into a 250 ml Erlenmeyer flask. Ten millilitre of 0.5 N sodium hydroxide in methanol was added. The solution is boiled with shaking in the reflux condenser till the solution become clear( for 10 minutes) and after reflux stopped 2 drops of phenolphthalein was added. One normality solution of sulphuric acid was added till solution becomes colourless and then added 1 ml of 1 N sulphuric acid in excess. The solution was extracted with petroleum ether and evaporate to dry. Twenty millilitre of 5% sulphuric acid in methanol was added.
Condenser is fixed and boiled for 20 minutes. Flask is cooled under running water. Extraction was repeated for 3 times and finally collected the ether layer into another separator and washed with water for 3 times. The ether layer is passed through anhydrous sodium sulphate and evaporated to dry. Resulting sample is reconstituted with petroleum ether and used for injection into GC.

4.15.6 Standard Preparation

Mixed standards of 37 compounds were diluted to 10 ml of dichloromethane to achieve a working solution concentration of 40 ppm.

4.15.7 Injection of Sample

One microlitre of oil sample was injected into the sample injector.

4.15.8 Injector Temperature and Oven Temperature

The source and inlet line temperature was maintained at 250°C. The injector temperature was kept at 250°C. The gas chromatography oven temperature was initially maintained at 40°C for 3 minutes and increased at 25°C/minute to 195°C and immediately raised 3°C/minute to 205°C for 2 minutes. Then the temperature was raised to 240°C for 5 minutes at the rate of 8°C/minute and hold for 1.09 minutes. Split ratio is maintained at 25:1.

4.15.9 Identification of Compounds

Peak identification was accomplished by comparison of the mass spectra with the corresponding ones that contained in the NIST 08s, WILEY 8 and Fame library.

4.16 GC-MS PHYTOCHEMICAL ANALYSIS

The bioactive components of the culinary plant extracts were identified by subjecting them to GC–MS phytochemical analyses. Compounds were separated by GC. The structures of the components were identified using a mass spectrophotometer. GC–MS analyses were carried out in a Shimadzu QP2010 (Shimadzu corporation, Japan) instrument as outlined below.
4.16.1 Apparatus

4.16.1 i) Gas Chromatography Column – Shimadzu QP2010,

a) Length of the column -30.0 m,
b) Diameter -0.25 mm,
c) Film thickness in column -0.25 µm

4.16.1 ii) GC Conditions

a) Column oven temperature -70°C
b) Injector temperature -200°C
c) Injection mode –split
d) Split ratio -40
e) Flow control mode –linear velocity
f) Column flow -1.51 ml/minute
g) Carrier gas –Helium (99.9995% purity)

4.16.1 iii) Mass Spectrometry

a) Iron source temperature - 200°C 
b) Interface temperature – 24°C 
c) Scan range – 40 – 1000 m/z 
d) Event time – 0.5 sec 
e) Solvent cut time – 5 minutes
f) MS start time – 5 minutes

g) MS end time – 35 minutes
h) Ionization – EI (-70ev)

4.16.1 iv) Injector

4.16.2 Reagents

a) Helium gas

4.16.3 Injection of Carrier Gas

Helium gas was used as carrier gas. It was injected at the rate of 1.51 ml/min through the column.
4.16.4 Injection of Sample

One milliliter of spice extract was injected into the sample injector.

4.16.5 Injector Temperature and Oven Temperature

The source and inlet line temperature was maintained at 200°C. The injector temperature was kept at 200°C. The gas chromatography oven temperature was initially maintained at 70°C and increased at 8°C /minute to 150°C for 2 minutes. Then the temperature was raised to 280°C for 5 minutes at the rate of 8°C/minute. Slit ratio is maintained at 1:10.

4.16.6 Identification of Compounds

Peak identification was accomplished by comparison of the mass spectra with the corresponding ones that contained in the NIST 08s, WILEY 8 and Fame library.

4.17 MOLECULAR INTERACTIONS

The molecular interaction between the bioactive compounds of culinary plants and protein ver1A involved in the conversion of versicolorin A to demethylsterigmatocystin, an important step during aflatoxin biosynthesis were determined using the LigandFit protocol available in Accelrys Discovery studio 2.5 as detailed below.

4.17.1 Databases

4.17.1 i) SWISSPROT

Swiss-Prot is a curated protein sequence database which strives to provide a high level of annotation such as the description of the function of a protein, its domains structure, post-translational modifications, variants, a minimal level of redundancy and high level of integration with other databases. It is a computer-annotated supplement of Swiss-Prot that contains all the translations of EMBL nucleotide sequence entries not yet integrated in Swiss-Prot.
4.17.1 ii) PDB

The PDB archive contains information about experimentally-determined structures of proteins, nucleic acids, and complex assemblies. As a member of the wwPDB, the RCSB PDB curates and annotates PDB data according to agreed upon standards.

The RCSB PDB also provides a variety of tools and resources. Users can perform simple and advanced searches based on annotations relating to sequence, structure and function.

4.17.1 iii) Pubchem

Pubchem is a database of chemical molecules and their activities against biological assays. The system is maintained by the National Center for Biotechnology Information (NCBI), a component of the National Library of Medicine, which is part of the United States National Institutes of Health (NIH). PubChem can be accessed for free through a web user interface. Millions of compound structures and descriptive datasets can be freely downloaded via FTP. PubChem contains substance descriptions and small molecules with fewer than 1000 atoms and 1000 bonds.

4.17.1 iv) Blast

Basic Local Alignment Search Tool, or BLAST, is an algorithm for comparing primary biological sequence information, such as the amino-acid sequences of different proteins or the nucleotides of DNA sequences. A BLAST search enables a researcher to compare a query sequence with a library or database of sequences, and identify library sequences that resemble the query sequence above a certain threshold.

4.17.2 SOFTWARES

4.17.2 i) Accelry’s Discovery Studio 2.5

It is a powerful client-server software system that implements a full set of all the necessary tools needed to predict the fundamental properties and processes of organic crystalline materials, inorganic materials, as well as polymeric and soft materials.
4.17.3 Methodology

i) The ver1 protein of *A. flavus* has been retrieved from swissprot database

ii) The template chosen by homology search using BLAST tool

iii) The 3D structure of the ver1 was modeled using Build Homology model available in Acclery’s D.S 2.5

iv) The modeled protein has been verified using Verify 3D profile.

v) The structure of the ligand molecules were retrieved from pubchem databases

vi) The active site of the target protein were predicted using Eraser and flood filling algorithm

vii) The ligands were docked with the protein using Ligand fit algorithm

viii) The hydrogen bond interaction between the docked ligand and protein were predicted with its distance
Plate 1a: Showing the contamination of groundnut kernel

Plate 1b: Showing growth of *A. flavus* MTCC 10680 in groundnut
Plate 2a: Showing *A. sativum*

Plate 2b: Showing *Z. officinale*

Plate 2c: Showing *A. cepa* L.

Plate 2d: Showing *A. cepa* var. *agregatum*

Plate 2e: Showing *M. koenigii*
Plate 3a: Showing the extract of *A. sativum*

Plate 3b: Showing the extract of *Z. officinale*

Plate 3c: Showing the extract of *A. cepa* L.

Plate 3d: Showing the extract of *A. cepa* var. *aggregatum*

Plate 3e: Showing the extract of *M. koenigii*